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DCS-120 Confocal and Multiphoton FLIM Systems

with

SPCImage NG FLIM Data Analysis

**User Handbook
10th Edition**



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Preface

The author of this handbook has designed and used TCSPC and TCSPC FLIM for more than 40 years, and he plans to continue do so for at least another decade. He is convinced and, probably, has good reasons to believe that TCSPC FLIM is a beautiful technique with a large potential of applications. Nevertheless, it is used by a relatively small community of researchers. Why do others, who certainly could benefit from using FLIM, not do so? The reason is, almost certainly, lack of knowledge about the technique and the benefits it offers for a wide range of applications.

What is the purpose of FLIM? You will probably get as many answers to this question as there are people you ask. A standard microscope user will tell you that it is a way to discriminate the signals of fluorophores which have similar fluorescence spectra. A physicist will tell you that FLIM delivers the effective decay rates of the fluorophores in your sample, and that these rates depend of the radiative and the non-radiative decay rates and (if you take it strictly) on the intersystem-crossing rate. The photochemist will add that the fluorophore molecules need to have a chain of conjugate double bonds, and that the structure has to be as rigid as possible to make them fluoresce brightly and with a long fluorescence lifetime. A biologist will possibly admit that he hasn't used FLIM yet because he considers it too difficult. But he believes that it helps obtain information about the proteins and enzymes in biological systems.

So, why and for what should you use FLIM? You should use it because it is the direct way to look at the molecular parameters of the objects you are investigating. The fluorescence decay function of a fluorophore depends on its molecular environment. It is influenced by interaction with ions, by exchanging energy with other fluorophores, by binding to proteins, by the conformation of the protein it is bound to, by local viscosity and refractive index, and a number of other more or less important molecular parameters. Because the decay function is independent of the fluorophore concentration the information you get is an absolute one, i.e. it does not depend of the variable and, usually, unknown concentration of the fluorophore.

Why should you use the DCS-120 FLIM system? The DCS-120 system uses bh's multi-dimensional TCSPC technique. By combining TCSPC with laser scanning it combines the most sensitive and most precise techniques to record in time and to record in space: TCSPC delivers precise decay functions in the individual pixels of the scan, and confocal or multiphoton scanning suppresses out-of-focus signals and lateral scattering.

But there is more than that. Multi-dimensional TCSPC builds up photon distributions. For basic FLIM, the distribution is built up over the scan coordinates and the times of the photons in the decay functions. The DCS system thus delivers FLIM data which contain a full fluorescence decay curve in each pixel, not just a 'lifetime'. Simultaneously, the bh TCSPC modules deliver by far the highest time resolution of all TCSPC products. Complex decay functions can thus efficiently be split in their components, revealing information that is not available by recording just the lifetime of a single-exponential approximation.

However, multi-dimensional TCSPC FLIM can do much more than simply record lifetime images. When it builds up a photon distribution it can include other parameters of the photons, such as their wavelength, the time from a stimulation of the sample, an electrical voltage, or the time within an the period of an additional modulation of the laser. By doing so, the DCS system can perform experiments which are beyond the capabilities of other FLIM systems: FLIM of fast physiological processes, excitation-multiplexed FLIM, multi-wavelength FLIM, FLIM from moving objects, or simultaneous FLIM / PLIM.

The aim of this handbook is to bridge the gap in knowledge between the biological and biochemical problems and the capabilities of state-of-the art FLIM systems. After an introduction

into the general capabilities of the system it describes the electronic and optical principles of FLIM data acquisition, basic and advanced techniques and procedures, and system configurations for different applications. A large chapter is dedicated to FLIM applications in biology. It covers the measurement of local environment parameters, protein interaction experiments by FRET, autofluorescence FLIM, metabolic FLIM, and metabolic FLIM in combination with pO₂ measurement. Analysis of FLIM data is described in a special chapter on bh's new SPCImage NG data analysis software. The book ends with a list of 650 references to publications related to the bh FLIM technique.

As supplementary literature we recommend the bh TCSPC Handbook, which provides basic information on multi-dimensional TCSPC, photon counting detectors, TCSPC devices, TCSPC operation modes, and TCSPC applications.

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Introduction

Principle of the DCS-120 FLIM Systems

The DCS-120 is a family of confocal or multiphoton laser scanning microscope systems for fluorescence lifetime imaging.

The DCS-120 systems use a combination of bh's multidimensional time-correlated single-photon counting (TCSPC) process [67, 427] with confocal or multiphoton laser scanning. In other words, the systems use a combination of the most accurate and sensitive principles to record in space and in time. Laser scanning yields depth resolution, optical sectioning capability, and suppression of longitudinally and laterally scattered light. The result is high-contrast recording of images from a clearly defined focal plane. Multi-dimensional TCSPC yields ultimate time-resolution and sensitivity, high dynamic range, recording of the full decay functions in a large number of time channels, and the capability to split multi-exponential fluorescence decay functions in their exponential components.

The basic principle of the DCS-120 systems is shown in Fig. 1. The sample is repetitively scanned by a high-repetition rate pulsed laser beam. Single photons of the fluorescence signal are detected, and each photon is characterised by its time in the laser pulse period and the coordinates of the laser spot in the scanning area in the moment its detection. The recording process builds up a photon distribution over these parameters.

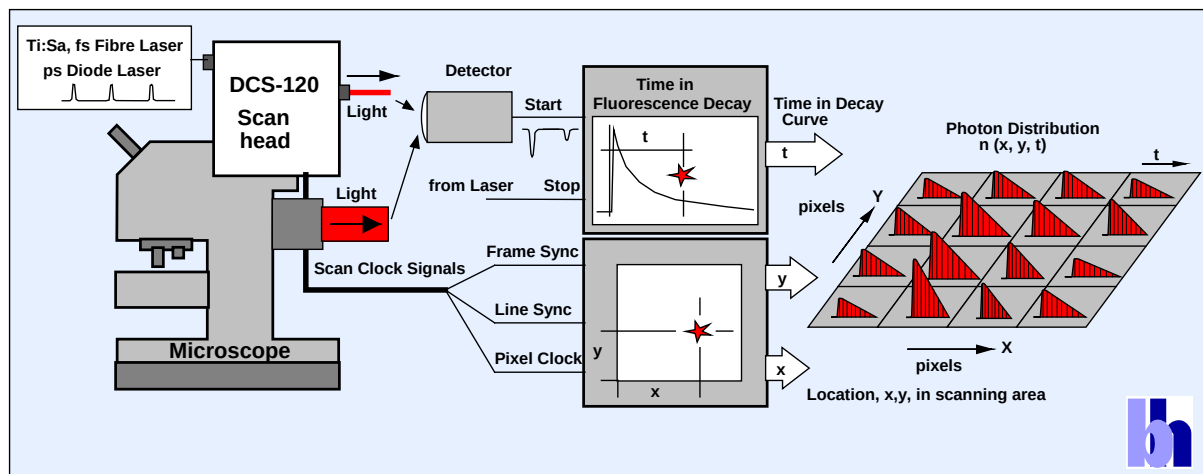


Fig. 1: Basic Principle of TCSPC FLIM

The photon distribution can be interpreted as an array of pixels, each containing a full fluorescence decay curve in a large number of time channels. In other words, the result is a perfect FLIM image, see Fig. 2.

The recording process is independent of the scan rate of the microscope. The signal-to-noise ratio depends only on the total acquisition time and the photon rate available from the sample. This gives the recording process additional flexibility. On the one hand, recording of FLIM within surprisingly short acquisition time can be performed. On the other hand, high accuracy FLIM can be recorded by simply increasing the acquisition time.

The technique can further be extended by including additional parameters in the photon distribution. These can be the depth of the focus in the sample, the wavelength of the photons, the time after a stimulation of the sample, or the time within the period of an additional modulation of the laser. These techniques are used to record Z stacks or lateral mosaics of FLIM

images, multi-wavelength FLIM images, images of physiological effects occurring in the sample, or to record simultaneously fluorescence and phosphorescence lifetime images.

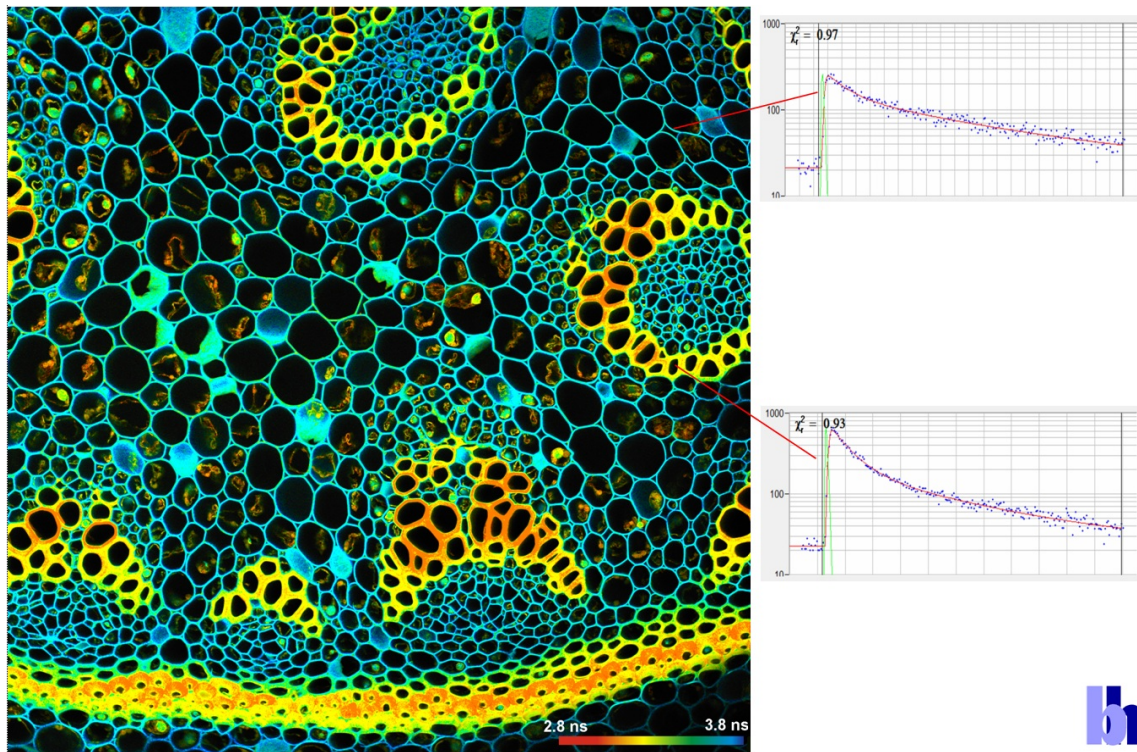


Fig. 2: FLIM Image recorded with the DCS-120. Fluorescence decay curves in selected pixels are shown on the right. Each pixel contains a full fluorescence decay curve in a large number of time channels, enough for multi-exponential decay analysis.

The DCS-120 system has been introduced in 2007. Since then, it has been upgraded constantly with improved scanner optics, new detectors, new lasers, new TCSPC / FLIM modules, and new data acquisition and data analysis software.

Two of the early DCS-120 confocal versions are shown in Fig. 3. A DCS-120 system with an inverted microscope is shown on the left, a system with an upright microscope on the right.



Fig. 3: DCS-120 Confocal Scanning FLIM system. Left: Inverted version with Zeiss Axio Observer. Right: Upright version with Zeiss Axio Examiner

DCS-120 Versions

DCS-120 and DCS-120 CMI Confocal Systems

Recent DCS-120 versions are shown in Fig. 4 through Fig. 14. Fig. 4 shows the basic confocal DCS-120 system. It has two BDS-SM ps diode lasers, two detection channels with HPM-100-40 or HPM-100-06 hybrid detectors, two SPC-180NX or one SPC-QC-104 TCSPC/FLIM module, and an upgraded optical scanner module. The new scanner has improved optical and mechanical stability, improved transmission, electronic pinhole alignment, and a super-stable main dichroic beamsplitter which can be replaced by the user.

An upscaled version of the basic DCS-120 system is the DCS-120 CMI Molecular Imaging System. It uses the bh LHB-104 'Laser Hub' to deliver the excitation light. The Laser Hub contains four lasers of different wavelength, and is able to multiplex them for simultaneous FLIM of fluorophores of different excitation spectra. The DCS-120 CMI is shown in Fig. 5.

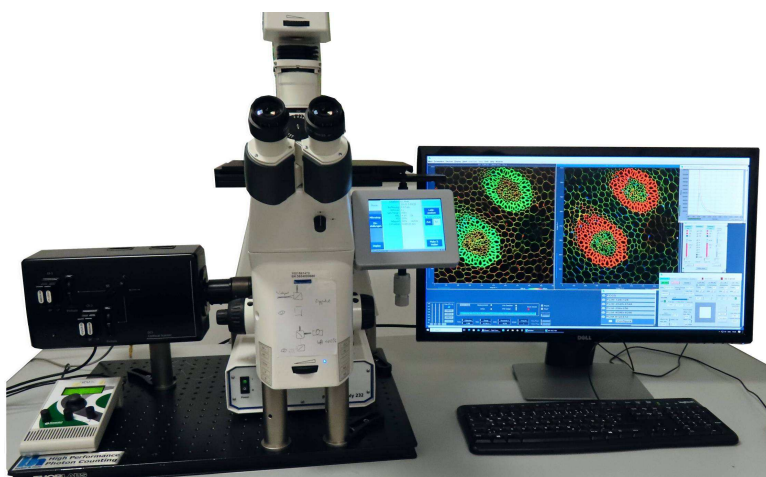


Fig. 4: DCS-120 Confocal Scanning FLIM systems. Left: Basic version with two lasers and two detection channels. Right: DCS-120 CMI Molecular Imaging Confocal FLIM System. Four lasers and up to four parallel detection channels

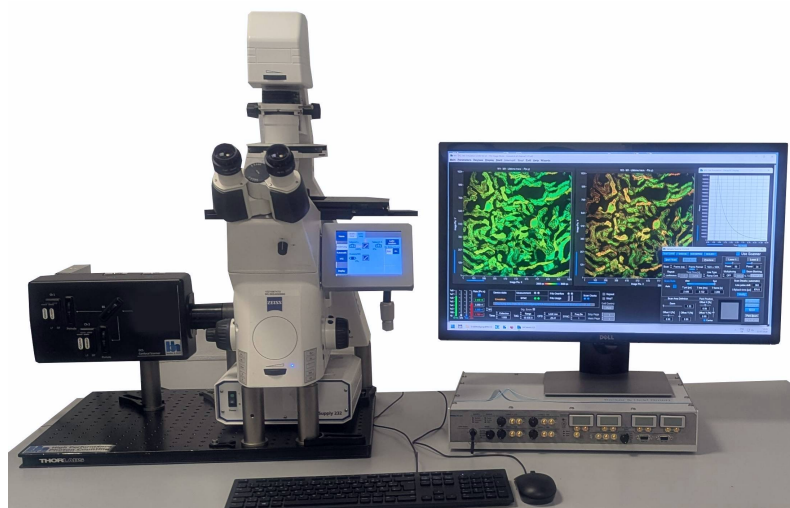


Fig. 5: DCS-120 CMI Molecular Imaging Confocal FLIM System. Four lasers and up to four parallel detection channels

The optical principles of the DCS-120 and the DCS-120 CMI are shown in Fig. 6 and Fig. 7. In the DCS-120 the beams of two lasers are delivered separately into the scan head. In the DCS-120 CMI the beams of the four lasers of the bh Laser Hub are delivered via a single optical fibre.

The beams pass the main dichroic beamsplitter, and are deflected by two fast-moving galvanometer mirrors. The scan lens projects the beams down the microscope light path into the back aperture of the microscope lens. As the system is scanning, the angle of the beams is changing, and the illuminated spot in the sample is raster-scanning over the image area.

The fluorescence light from the sample is collected by the microscope lens and again reflected by the galvanometer mirrors. This compensates the angular motion of the beam so that the reflected beam becomes stationary. It is separated from the excitation by the main dichroic beamsplitter and split in two wavelength intervals by the secondary beamsplitter. It then passes a pinhole in a plane conjugated with the image plane in the sample. Only light from this plane is focused in the pinholes, and only light from the image plane can pass the pinholes. The light passing the pinholes is cleaned up by filters and, finally, fed to the detectors.

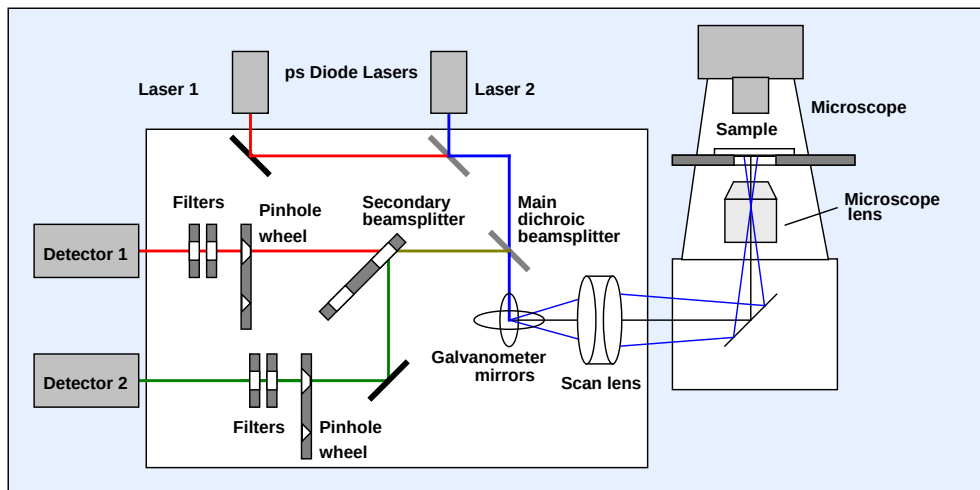


Fig. 6: DCS-120 Basic confocal System with two lasers and two detection channels

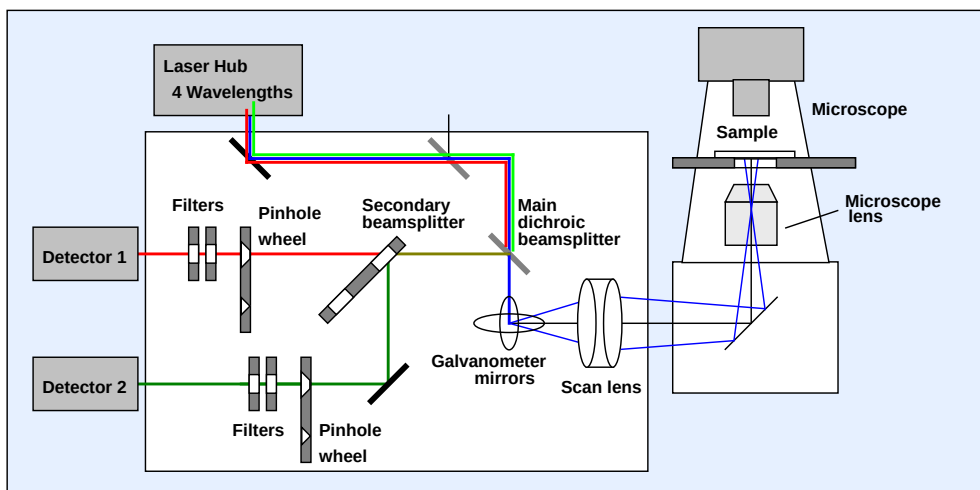


Fig. 7: Principle of the DCS-120 CMI Confocal Molecular Imaging System. The system has four laser wavelengths and two detection channels.

DCS-120 MP and DCS-120 DMP Multiphoton Versions

Multiphoton versions of the DCS-120 are shown in Fig. 8 and Fig. 9. Fig. 8 shows the basic DCS-120 MP multiphoton system. It uses a 785 nm Toptica femtosecond fibre laser (either a Femto Fibre Pro or a Femto Fibre Smart) for excitation. With these fibre lasers, the system is not only the least expensive multiphoton FLIM microscope in the world, it is also the one easiest to use. The system is ready for use immediately after turn-on, the sample is positioned visually through the eyepieces, and fine focusing is done in the Preview Mode of the SPCM software. The excitation wavelength is ideal for label-free imaging with NAD(P)H, FAD, carotenoids and melanin. It also works for metabolic FLIM with NADH, and for molecular imaging with most of the commonly used exogenous fluorescence probes. Another outstanding feature is time resolution. Because the laser pulse is much shorter than the detector response, the system reaches less than 20 ps fwhm IRF width with the fast HPM-100-06 hybrid detectors. With an IRF width this short, the DCS-120 MP is able to detect fluorescence lifetimes down to 10 ps.

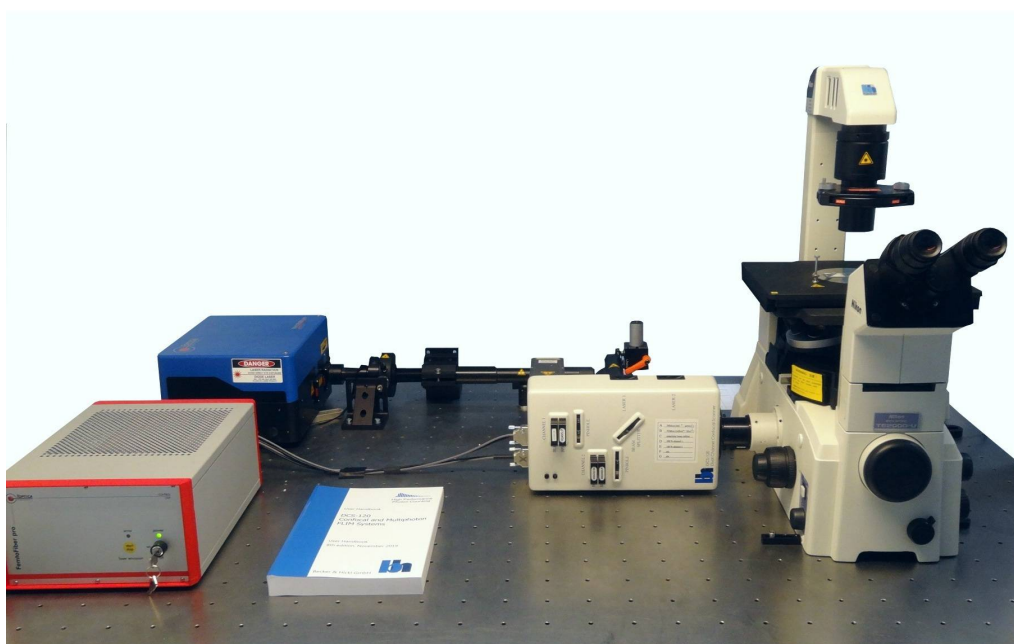


Fig. 8: DCS-120 MP system. Excitation by femtosecond fibre laser of 785 nm.

Fig. 9 shows the DCS-120 DMP Dual Multiphoton Excitation system. The DCS-120 DMP uses two synchronised femtosecond Toptica Femto Fibre Ultra lasers of 780 nm and 920 nm. In the DCS-120 DMP, the lasers can be multiplexed for simultaneous FLIM of NAD(P)H and FAD. With this capability, the DCS-120 DMP is the ideal multiphoton metabolic FLIM system. It can also be used for metabolic FLIM with NAD(P)H and FAD in combination with exogenous fluorescence probes or oxygen measurement by PLIM. Also here, the time resolution with the ultra-fast HPM-100 detectors is better than 20 ps fwhm. As the DCS-120 MP, it resolves fluorescence lifetimes down to 10 ps.

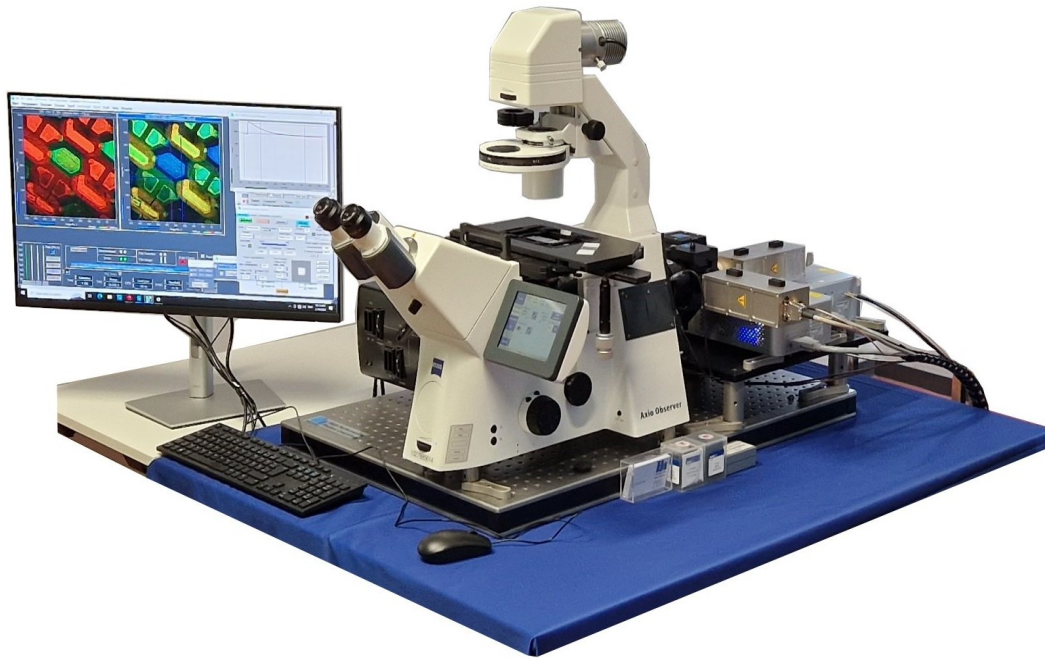


Fig. 9: DCS-120 DMP system. Two femtosecond fibre lasers of 785 and 880 nm.

The optical principle of the DCS-MP and the DCS-DMP is shown in Fig. 10 and Fig. 11. The systems use the same scanner optics as the confocal systems. However, there is an important difference in the way the fluorescence light is detected. Two-photon excitation occurs only in a thin layer around the focal plane of the microscope lens. Therefore, no pinhole is needed to suppress the detection of out-of focus fluorescence. Consequently, there is no need to send the fluorescence light all the way back through the scanner. Instead, the fluorescence is split from the excitation light directly behind the microscope lens, and directly send to the detectors. The result is that even photons scattered on the way out of the sample have a chance to reach the detectors. The fact that scattered photons are detected does not impair the image quality - the data acquisition system automatically assigns them to the x-y position of the laser focus, not to the position where they left the sample. The result is high image quality, high contrast, and high detection efficiency from deep sample layers.

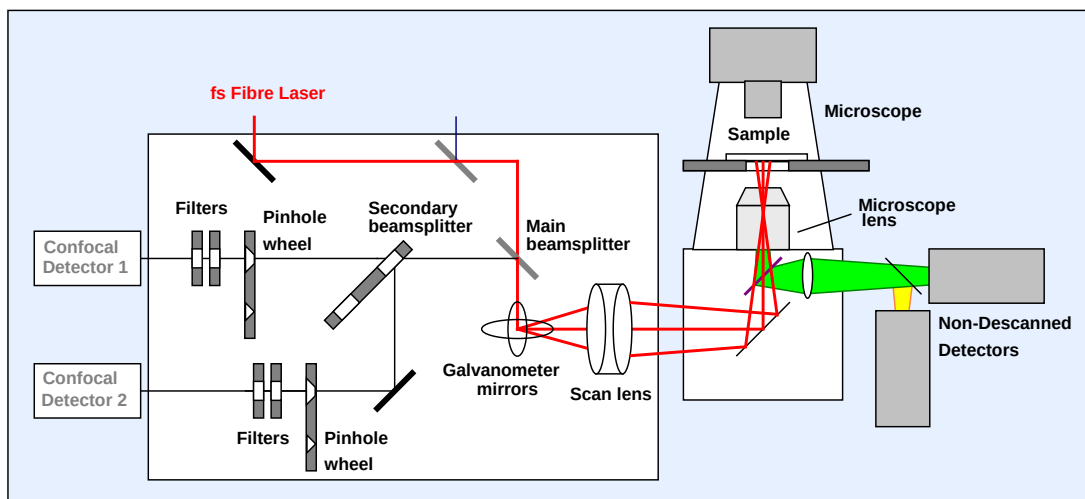


Fig. 10: Optical principle of the DCS-120 MP system

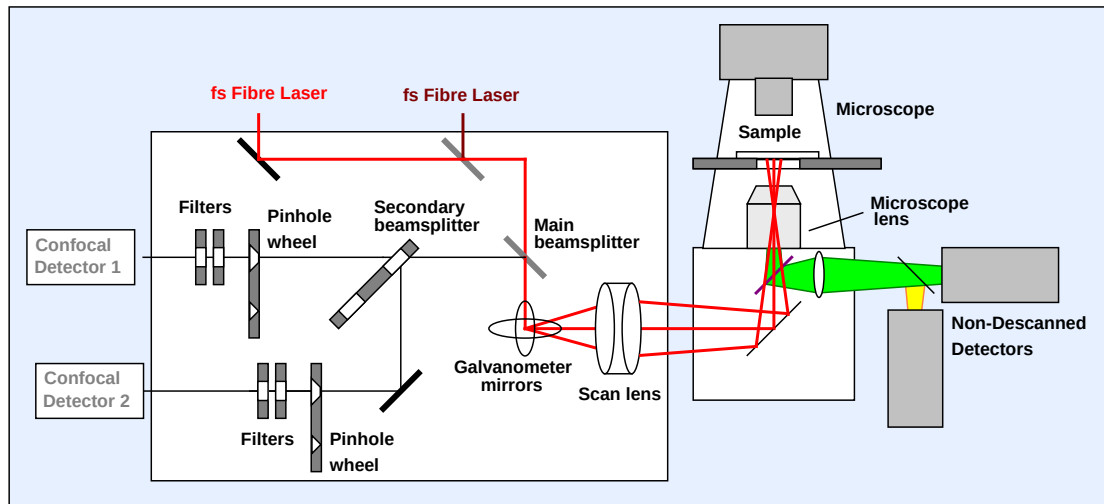


Fig. 11: Optical principle of the DCS-120 DMP system

DCS-120 Super MPC Combined Multiphoton / Confocal System

Fig. 12 shows another unique FLIM system of the DCS family, the DCS-120 Super MPC. The system uses a Ti:Sa laser or an OPO for tuneable multiphoton excitation. In addition, the SHG of the Ti:Sa laser is available for tuneable excitation in the visible range. Multiphoton FLIM is performed via a non-descanned beam path, one-photon excitation via a confocal beam path. The DCS-120 Super MPC can be used for deep-tissue FLIM and single-cell imaging as well. Because the excitation pulses are derived from the pulses of the Ti:Sa laser the time resolution of the DCS-120 Super MPC system is extremely high. The system is able to resolve lifetimes down to 10 ps even for excitation in the visible range. No other FLIM system has these capabilities. The principle of the DCS-Super-MPC system is shown in Fig. 13.



Fig. 12: DCS-120 Super MPC system. Multiphoton FLIM by Ti:Sa laser combined with tuneable confocal FLIM via SHG of Ti:Sa laser

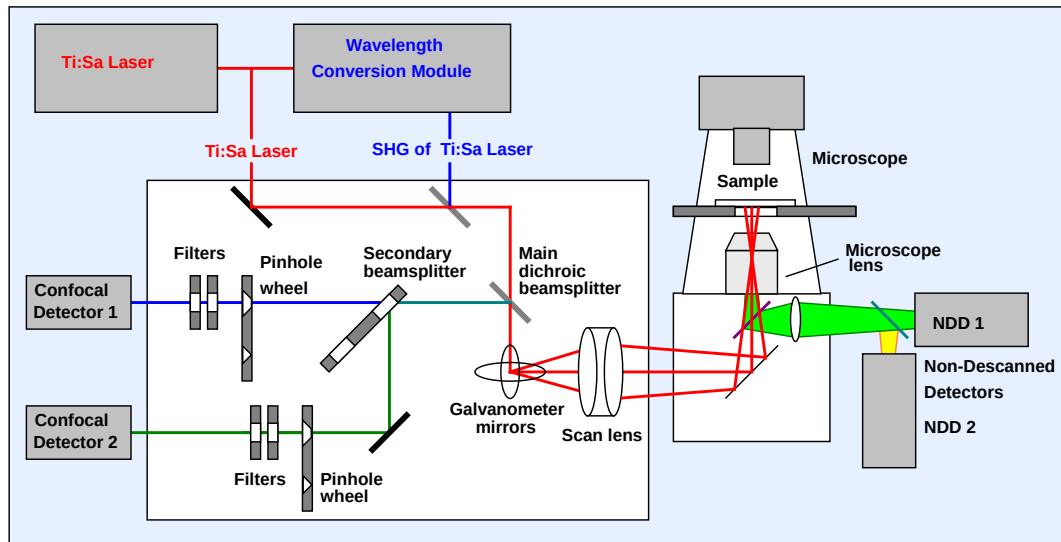


Fig. 13: Principle of DCS-Super-MPC Multiphoton / Confocal System

DCS-120 MACRO System

The DCS-120 MACRO system records images of macroscopic objects directly in the image plane of the scanner. FLIM images of objects as large as 15 mm can be scanned directly, larger images by mosaic FLIM in combination with a motorised sample stage. Like the basic DCS system, the DCS-120 MACRO uses two ps diode lasers for excitation and two HPM-100 hybrid detectors for detection. With lasers of 375 nm and 405 nm or 445 nm, the system is perfectly suitable for metabolic imaging by laser multiplexing. Main applications are inspection of excised tissue during tumor surgery, imaging of whole small organisms, and quality control of solar cells.

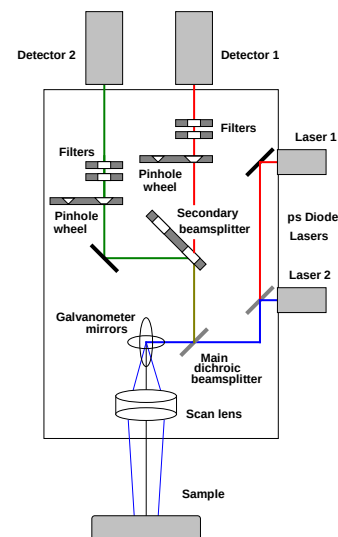
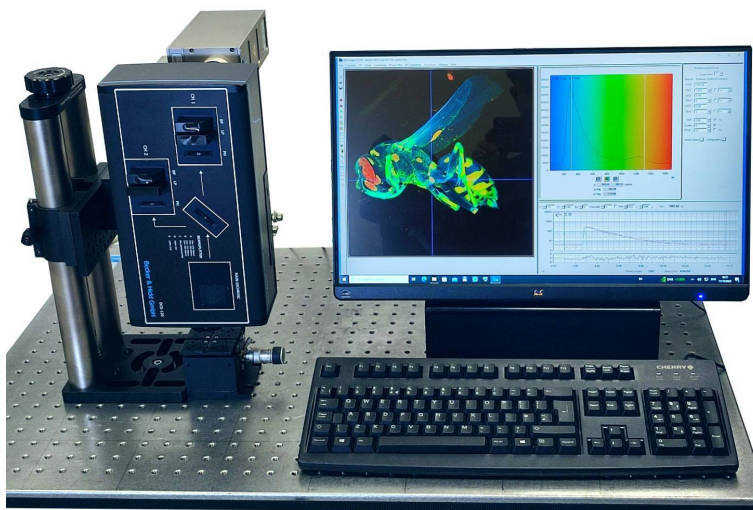


Fig. 14: DCS-120 MACRO System: Right: Optical principle

DCS-120 Systems with Incubator Box

DCS-120 systems can be delivered with an incubator box. The box helps maintain controlled environmental conditions during the measurement of live cells. The box also keeps off environment light from the detection beam path. A DCS-120 DMP system with an incubator box is shown in Fig. 15.

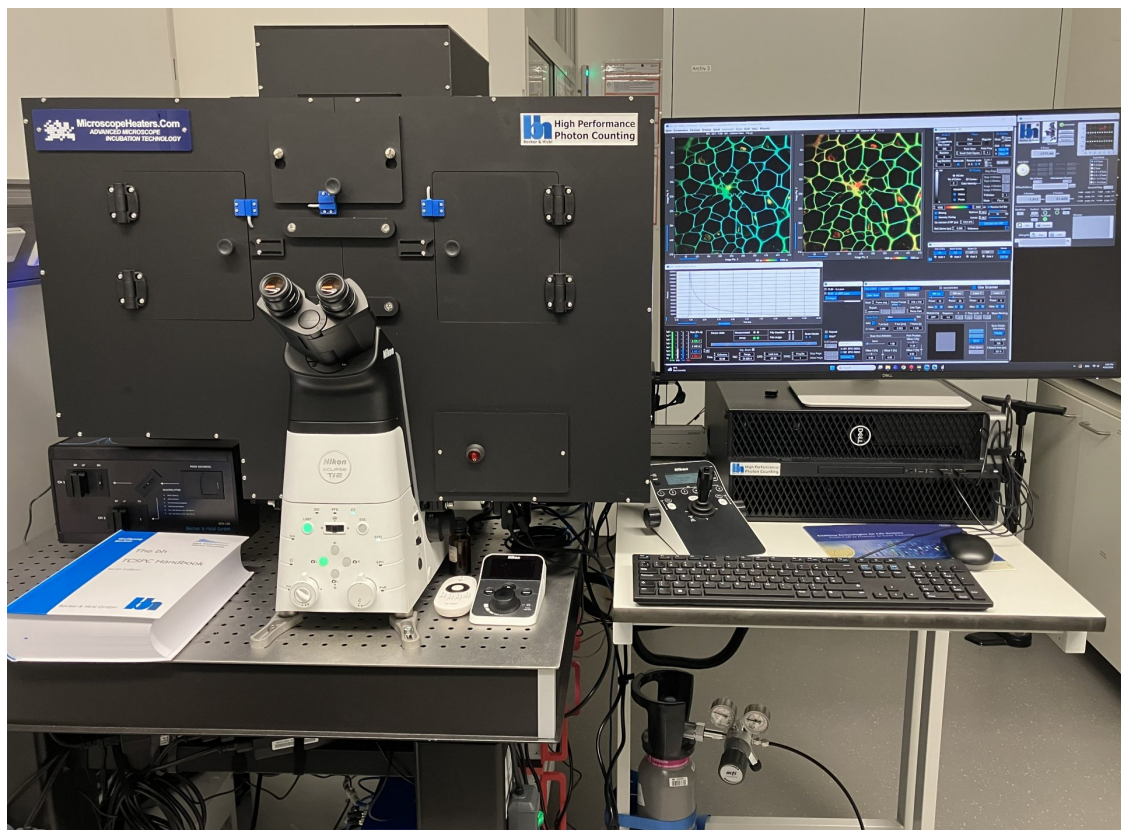


Fig. 15: DCS-120 system with an incubator box. DCS-120 DMP dual-excitation multiphoton system with a NIKON microscope.

Overview on DCS-120 FLIM Features

Technical Features

Fully Integrated Systems

The DCS-120 systems are 'fully integrated systems', meaning they are controlled by one piece of software running on one computer. Scanner control, laser control, detector control, and control of single measurements or measurement sequences are implemented in the SPCM data acquisition software. As an example, Fig. 16 shows the SPCM main panel with the scanner control panel. The configuration allows the user to select the image format, scan rate, and scan area, configure the multiplexing of the lasers, start the preview, and start and stop the measurement. Imaging parameters can be changed at any time, even during a measurement.

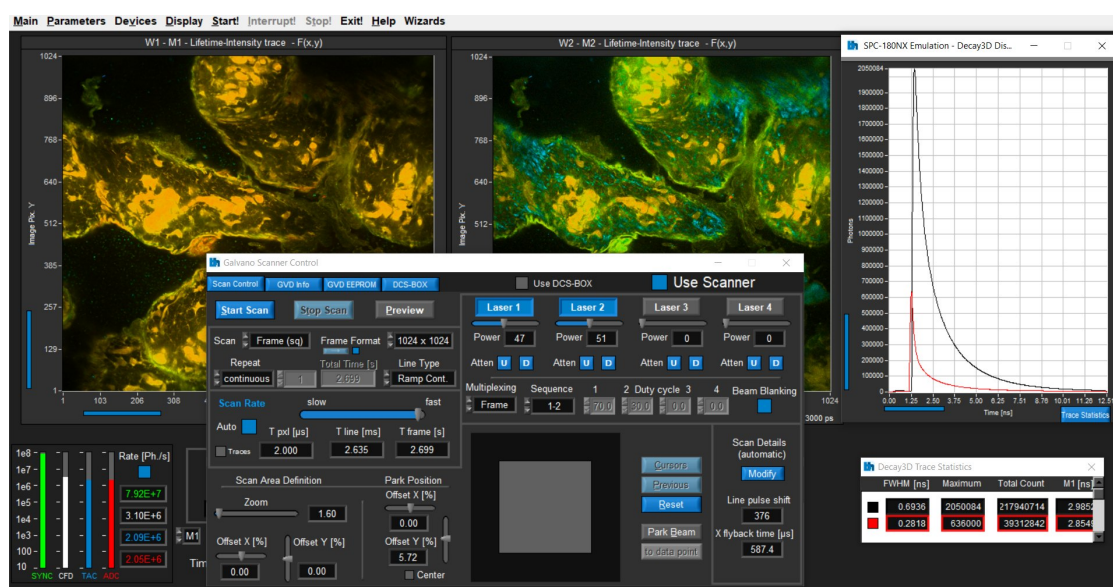


Fig. 16: SPCM Version 9.92 data acquisition software with integrated scanner control

Fast Preview Function

When FLIM is applied to live samples the time and the excitation dose needed for sample positioning, focusing, laser power adjustment, and region-of-interest selection has to be minimised. Therefore, the FLIM systems have a fast preview function. The preview function displays images in intervals on the order of 1 second and less, see Fig. 17. Scan parameters can be changed during the preview, without stopping the scan.

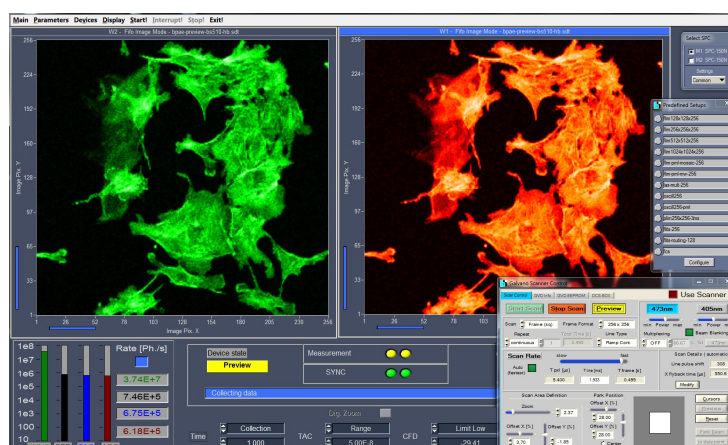


Fig. 17: SPCM software in fast preview mode, display rate one image per second.

Online Lifetime Display

Starting from Version 9.72 SPCM software the DCS-120 system is able to display lifetime images online, both during the accumulation of FLIM data and for the individual steps of a fast image sequence [49]. Lifetime images can be displayed at images rates as fast as 10 images per second. The calculation of the lifetime images is based on the first moment of the decay data in the pixels of the images. The first-moment technique combines short calculation times with near-ideal photon efficiency. Importantly, it does not require to reduce the time resolution (number of time channels per pixel) to obtain high calculation speed. Even if the fast online lifetime function is used during the FLIM acquisition the data can later be processed by precision SPCImage multi-exponential data analysis.

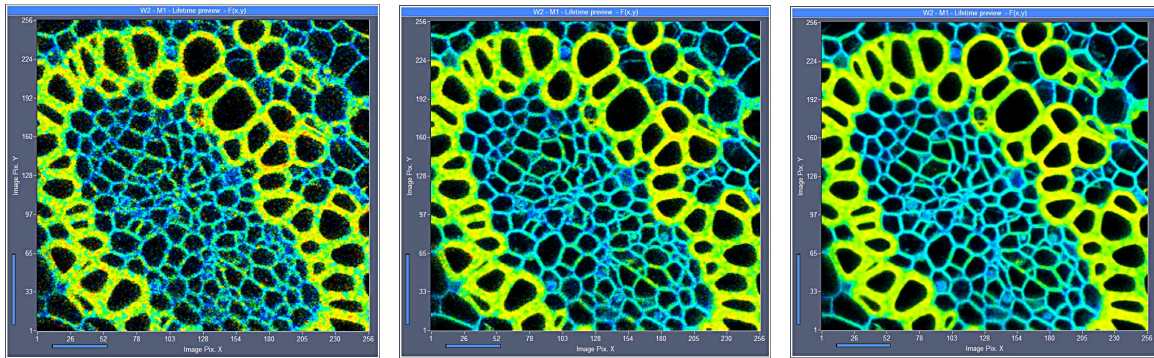


Fig. 18: 256x256-pixel images obtained by the online FLLIM display function. Acquisition time 0.2s, 0.5s, and 2s.

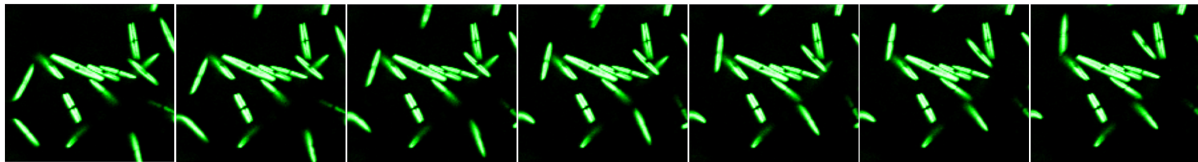


Fig. 19: Bacteria in motion. Autofluorescence, acquisition speed 2 images per second, scan speed 6 frames per second

Direct Interaction with SPCImage NG Data Analysis

FLIM data can be sent directly to SPCImage NG data analysis, without prior saving them into a file and importing this file into SPCImage.

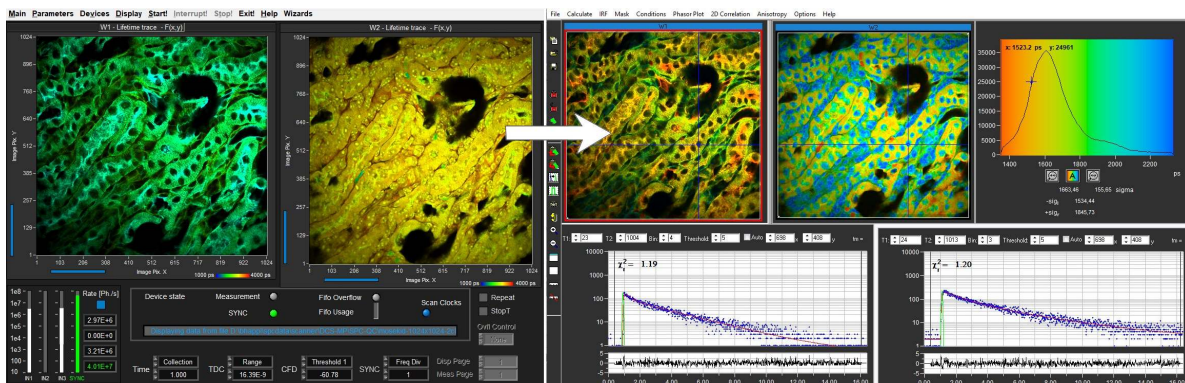


Fig. 20: Interaction with SPCImage NG Data analysis

Megapixel FLIM Images in Two Parallel Channels

The DCS-120 systems are able to simultaneously record two high-resolution images in different wavelength or polarisation channels, see Fig. 21 and Fig. 22. Pixel numbers can be up to 2024 x 2024 in both channels, with 256 time channels per pixel. Recording is performed in two fully parallel TCSPC channels, avoiding any electronic lifetime or intensity crosstalk. Even if one channel should saturate the other is still producing correct data.

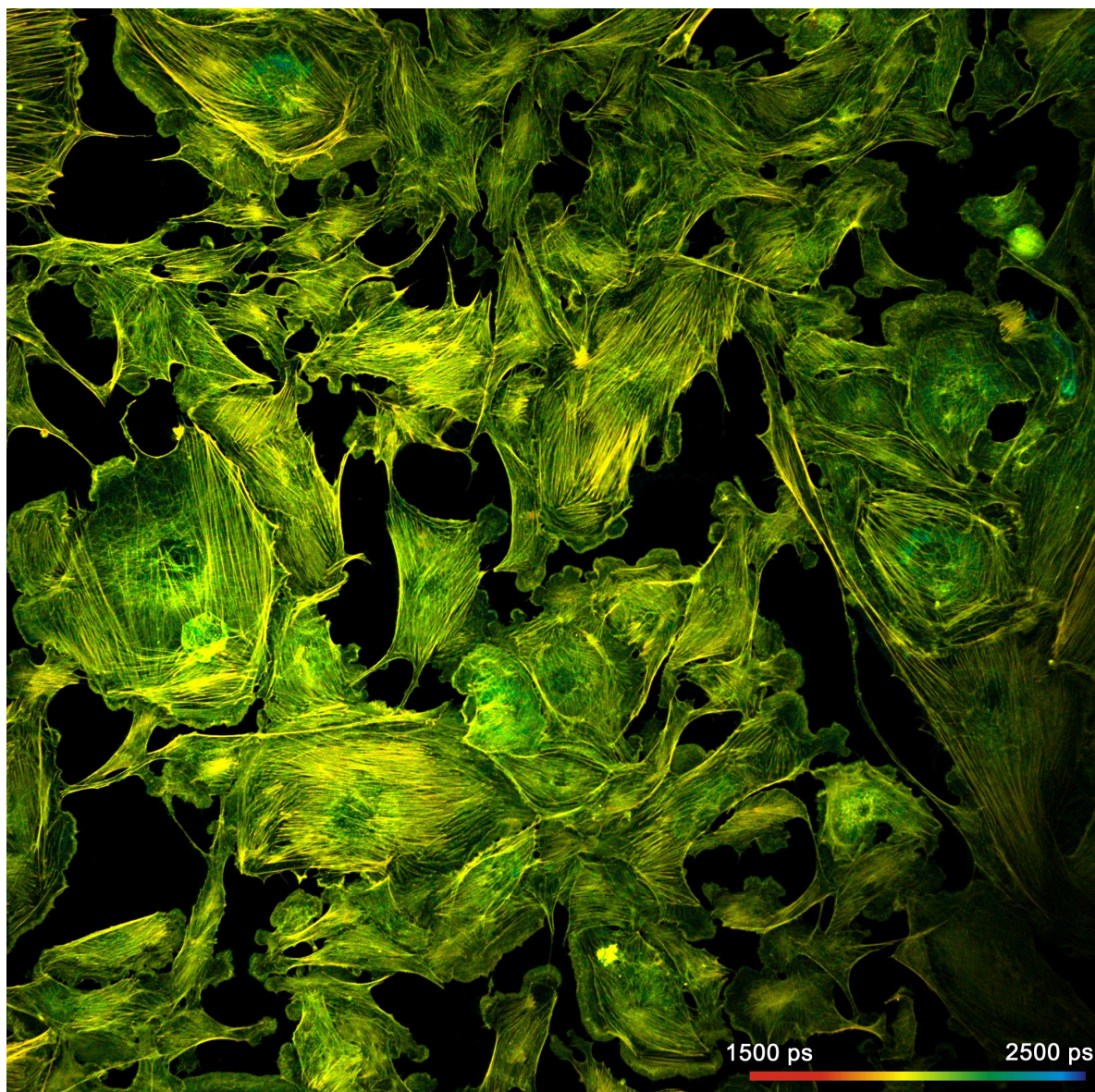


Fig. 21: BPAE sample (Invitrogen) scanned with 2048 x 2048 pixels. Green channel, 485 to 560 nm

The capability to record images of large pixel numbers is beneficial for a wide range of FLIM applications. One example is tissue imaging where the samples are large, and the images are containing a wealth of detail. It is also useful when a large number of cells have to be investigated and the FLIM results to be compared. Megapixel FLIM records images of many cells simultaneously, and under exactly identical environment conditions. Moreover, the data are analysed in a single analysis run, with identical IRFs and fit parameters. The results are therefore exactly comparable for all cells in the image area.

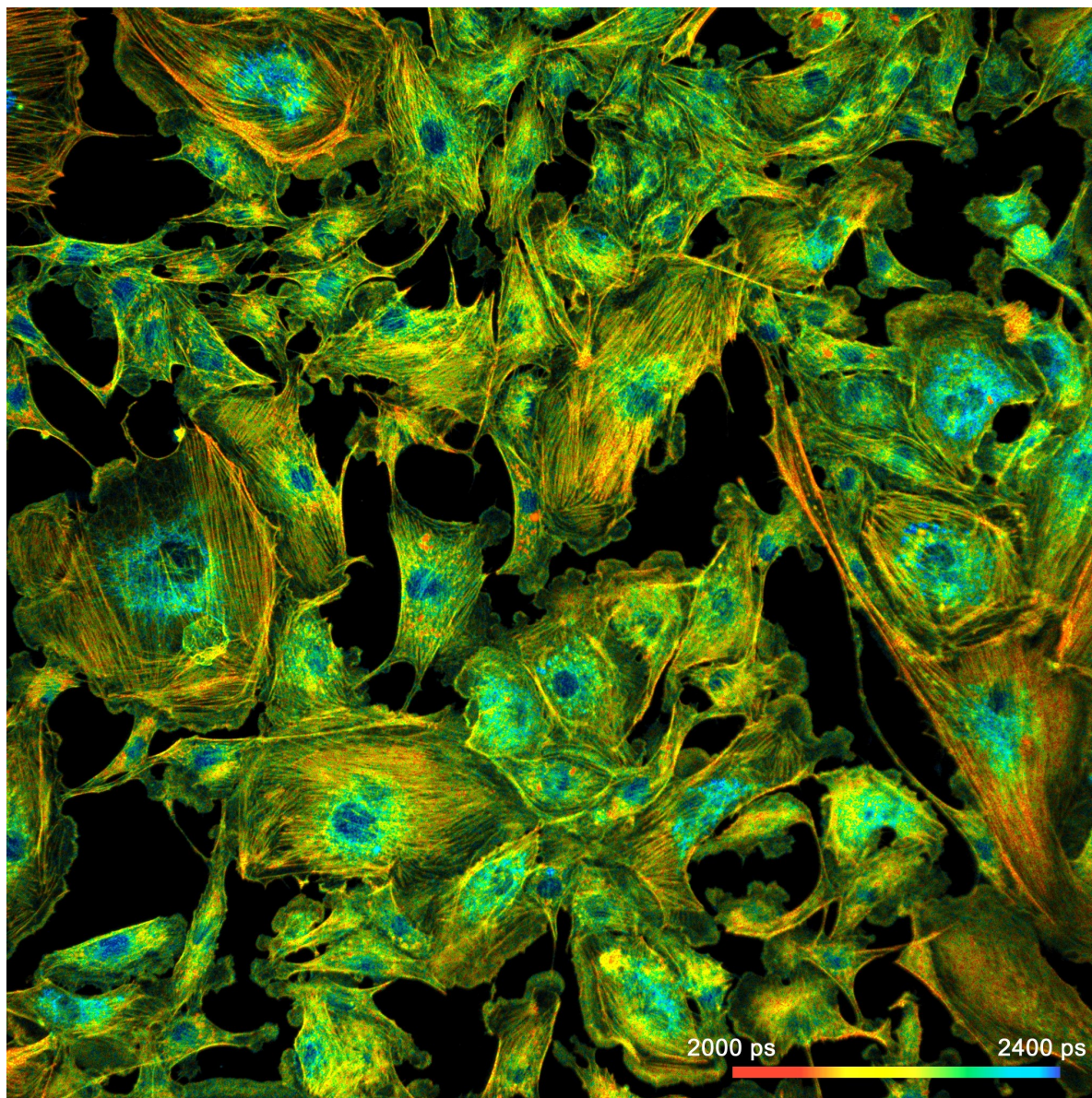


Fig. 22: BPAE sample (Invitrogen), scanned with 2048 x 2048 pixels. Red channel, 560 to 650 nm

Ultra-High Efficiency

The bh HPM-100-40 and -06 hybrid detectors of the DCS-120 combine ultra-high sensitivity with large active area, low background, and short IRF width. The combination of these features makes it easy to detect fluorescence from endogenous fluorophores in single cells. Advanced data analysis by MLE procedures can be used to split the decay curves in the pixels into several decay components and determine the lifetimes and amplitudes of the individual decay components, see Fig. 23. This capability is important for advanced applications like metabolic imaging or calibration-free FRET imaging.

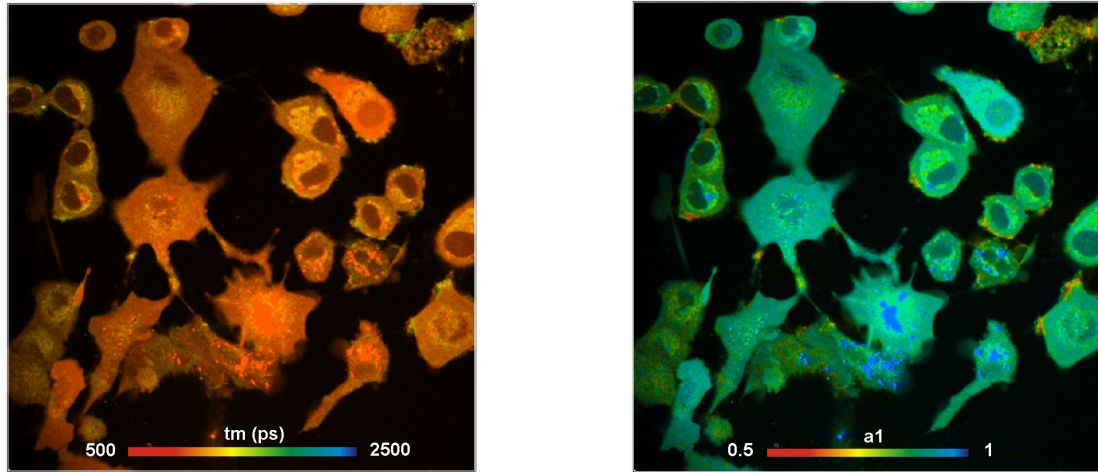


Fig. 23: Autofluorescence lifetime images of NADH in single cells. Lifetime image of mean lifetime of double exponential decay (left) and image of amplitude of fast decay component, a_1 (right). SPCImage NG data analysis, MLE fit.

Sub-20 ps Time Resolution

The DCS-120 multiphoton systems can be equipped with ultra-high speed HPM-100-06 and -07 hybrid detectors. The time resolution (IRF width) with these detectors is less than 20 ps, full width at half maximum [52]. Lifetimes in the range of 5 to 10 ps can be detected by these systems, even if they are part of a multi-exponential decay. Applications are FLIM of carotenoids, melanins, and other natural short-lifetime fluorophores. An example is shown in Fig. 24.

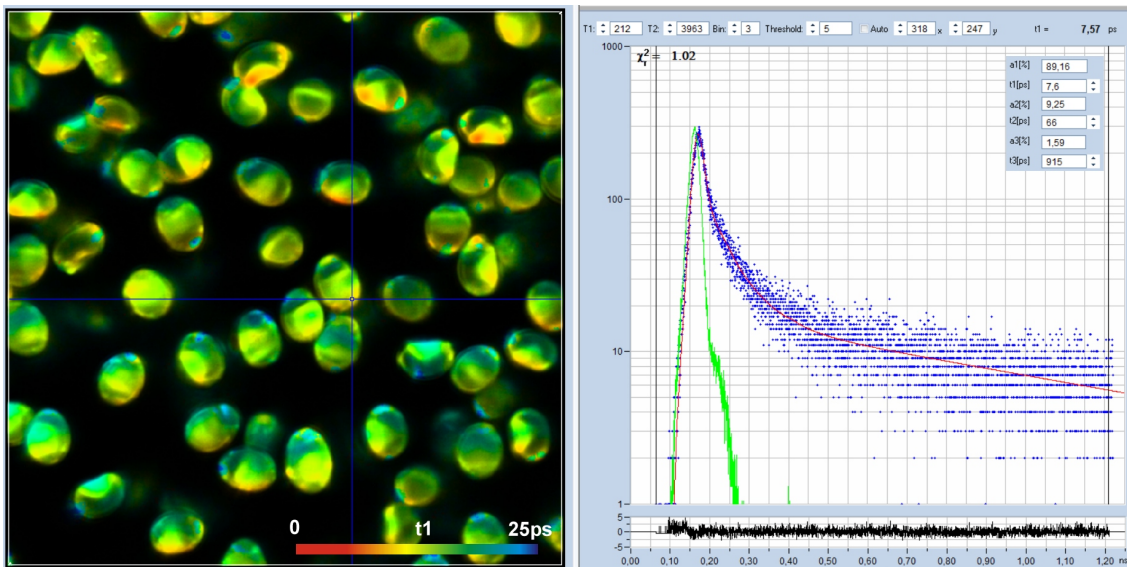


Fig. 24: Spores of *Paxillus involutus*, time-channel width 300 fs, 4096 time channels, observation-time interval 1.25 ns. Left: Image of the fast decay component, t_1 , colour scale 0 to 25 ps. Right: Decay curve (blue), IRF (green), and decay parameters at the cursor position. DCS-120 MP system with HPM-100-06 detector.

Other applications of high time resolution are in the field of metabolic FLIM, which requires separation of the decay components from bound and unbound NADH and/or FAD. High time resolution greatly improves the accuracy at which these parameters are obtained [53]. Fig. 25 shows NAD(P)H FLIM recorded with a DCS-120 MP multiphoton system. It shows an image of the mean lifetime, t_m , an image of the amplitude ratio, a_1/a_2 , the system IRF, and a decay curve from the cursor position in the images. Please see ‘NADH FLIM with Ultra-Fast Detectors’, page 217.

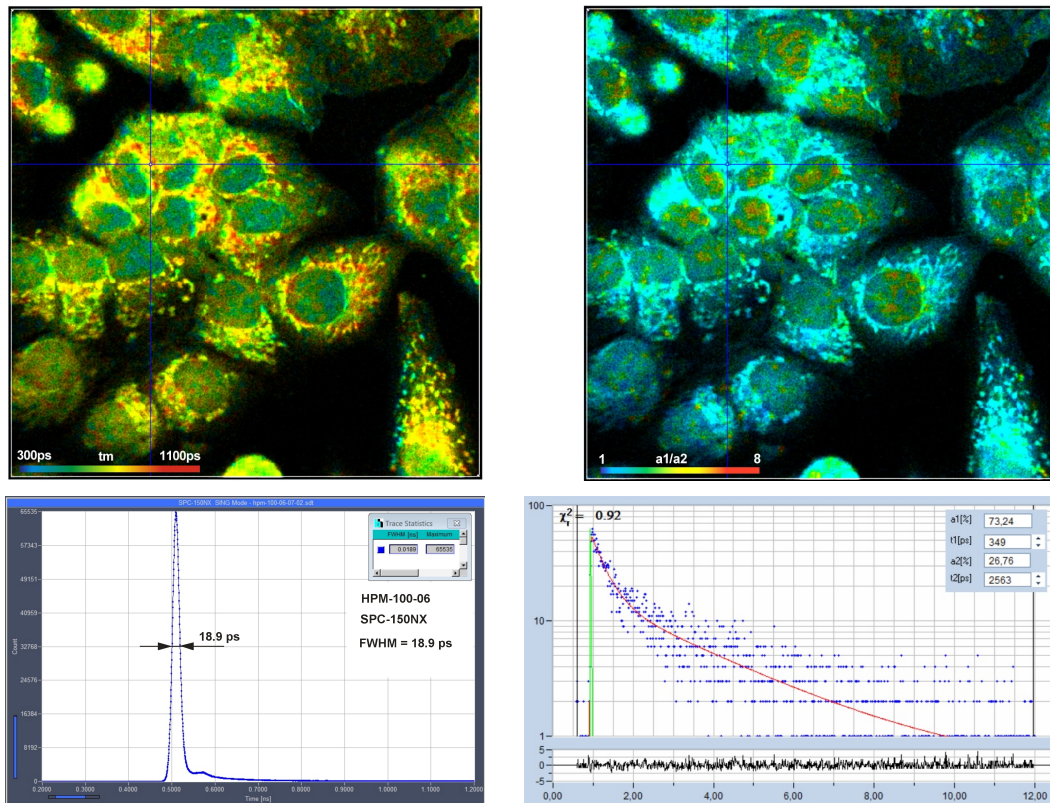


Fig. 25: Image of the mean lifetime, t_m , and image of the amplitude ratio, a_1/a_2 . Lower row: system IRF and decay curve at the cursor position. DCS-120 MPC system with HPM-100-06 detectors, two-photon excitation.

Fast Acquisition

The DCS-120 uses fast beam scanning by galvanometer mirrors. A complete frame is scanned within a time from 100 ms to a about one second, with pixel dwell times down to one micro-second. With its fast scanner and its multi-dimensional TCSPC process the DCS systems achieve surprisingly short acquisition times. In most cases the acquisition speed is not limited by the recording process but by the ability of the sample to withstand high excitation power. For highly fluorescent samples excellent FLIM images can therefore be obtained within a second or less. An example is shown in Fig. 26.

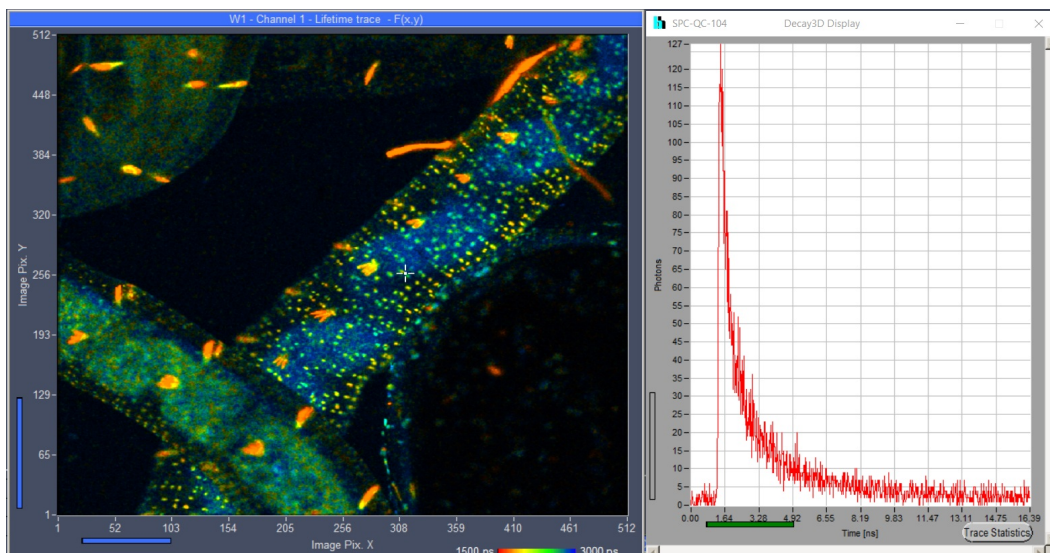


Fig. 26: Enchytraeus Albidus, Autofluorescence, Acquisition Time 1 second, Image Area 1mm x 1mm. Online-lifetime display of SPCM software. Right: decay curve in selected spot of the image.

Precision Confocal FLIM

In confocal systems with ps diode lasers and ultra-fast hybrid detectors the temporal instrument response function is essentially given by the laser pulse width. With the 375 nm and the 405 nm lasers the IRF width is less than 40 ps FWHM. A system IRF and a decay curve recorded with the HPM-100-06 and a diode laser are shown in Fig. 27. Please note the smooth residuals of the fit, an indication that the data are free of IRF afterpulses, laser afterpulses, or optical reflections.

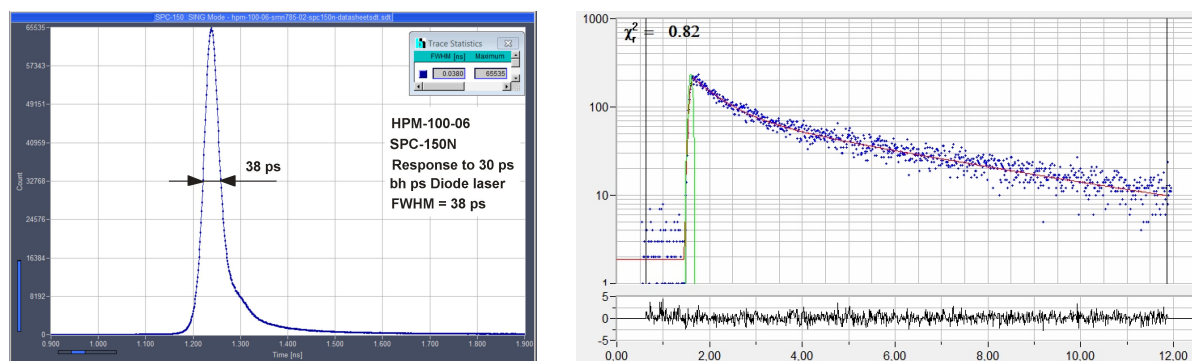


Fig. 27: Left: IRF with HPM-100-06 and bh 405 nm ps diode laser. Right: Fluorescence decay recorded with HPM-100-06 detector and bh 375 nm diode laser.

Wide Range of Excitation Wavelengths

The DCS-120 confocal system can be used with a wide range of excitation wavelengths. Available laser wavelengths range from 375 nm for excitation of NADH to 785 nm for excitation of NIR dyes. An NADH (autofluorescence) image is shown in Fig. 28, an image of a pig skin sample incubated with 3,3'-diethylthiatricarbocyanine in Fig. 29.

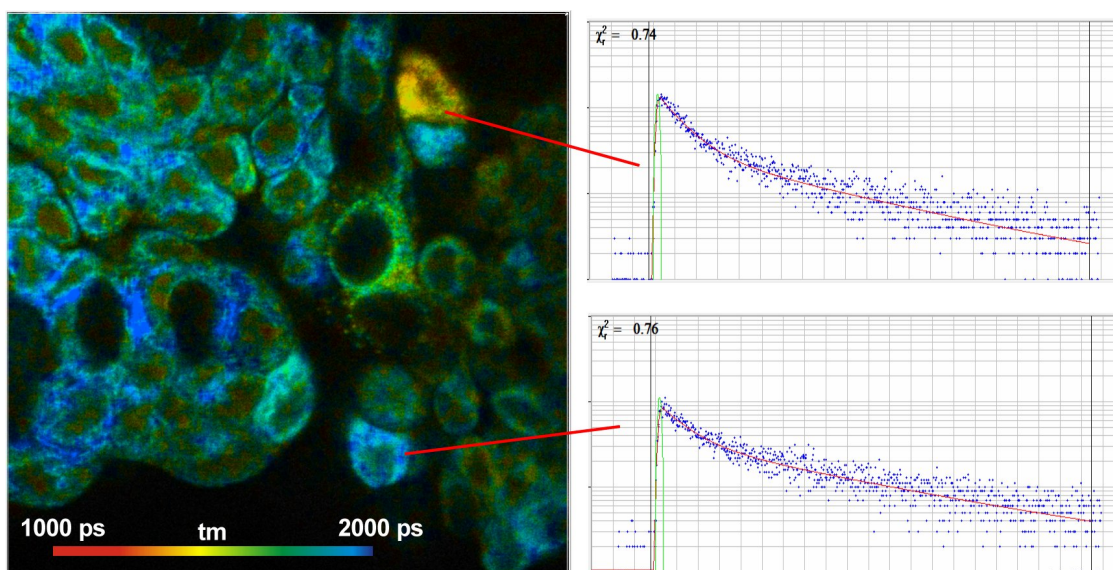


Fig. 28: UV-Excitation FLIM. NADH image of cells, excitation 370 nm, detection 420 to 475 nm.

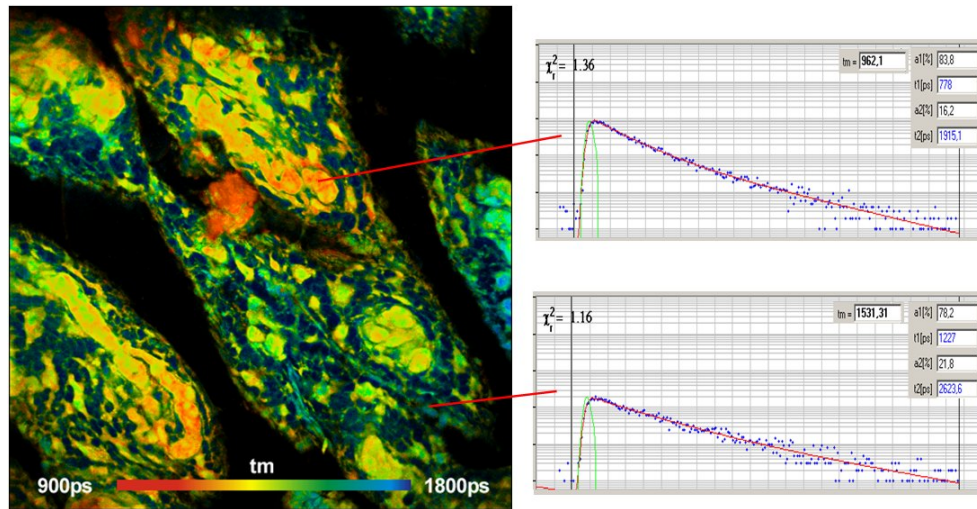


Fig. 29: Near-Infrared FLIM. Pig skin sample stained with 3,3'-diethylthiatricarbocyanine, detection wavelength, excitation 690 nm, detection wavelength from 780 nm to 900 nm.

Applications

Autofluorescence FLIM of Small Organisms

The wide range of excitation and detection wavelengths, the high sensitivity, and the ability to record images of large pixel number makes the DCS-120 an excellent system for autofluorescence FLIM of small organisms. Fig. 30 shows an autofluorescence image of *Artemia salinas*, a small shrimp living in briny water. The excitation wavelength was 405 nm, the detection wavelength from 420 nm to 560 nm.

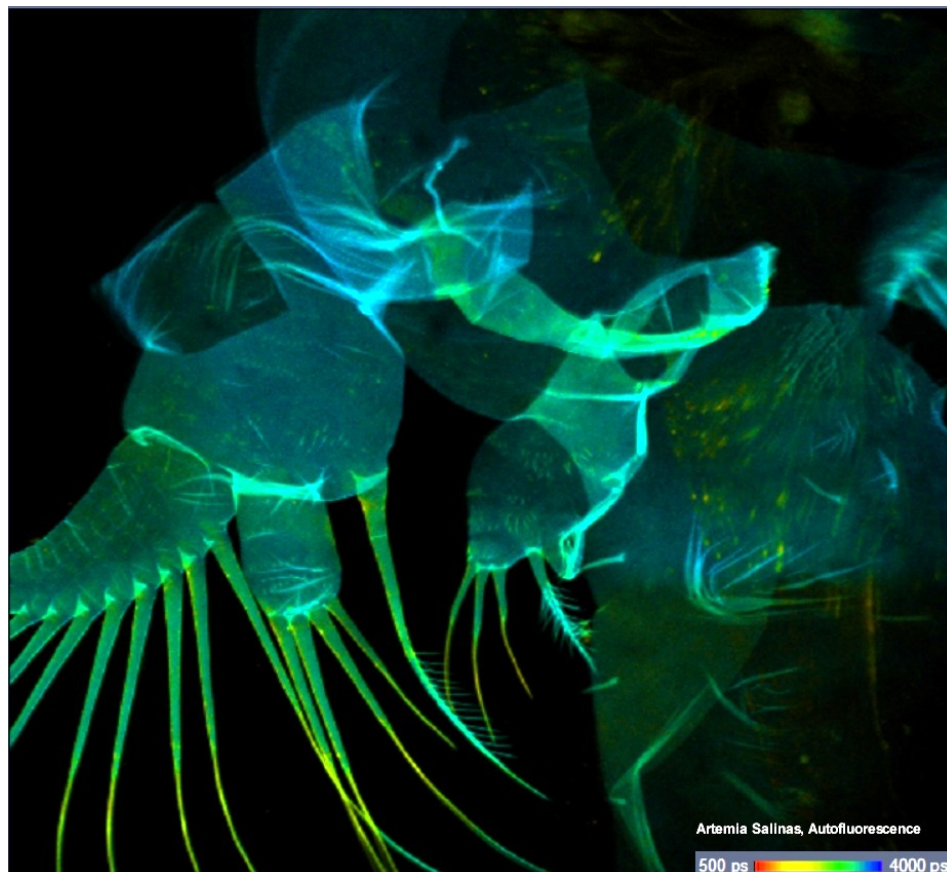


Fig. 30: Autofluorescence FLIM of *Artemia salinas*, basic DCS-120 confocal system

Laser-Wavelength Multiplexing

The two ps-diode lasers of the DCS-120 system can be multiplexed on a pixel-by-pixel, line-by-line, or frame-by-frame basis. With the two detection channels of the DCS system, images for three or four combinations of excitation and emission wavelength are obtained. An example is shown in Fig. 31.

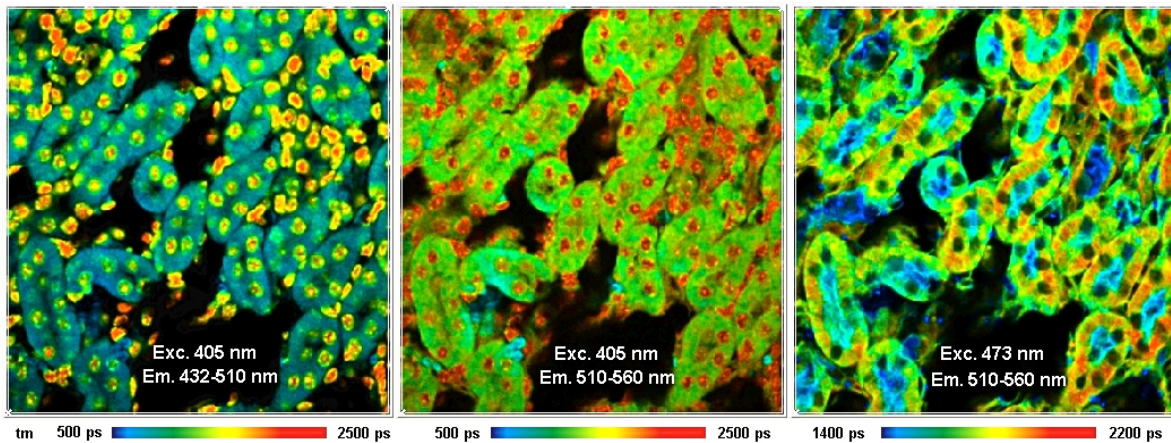


Fig. 31: Excitation-wavelength multiplexing, 405 nm and 473 nm. Detection wavelength 432 nm to 510 nm and 510 nm to 550 nm. Mouse kidney section, stained with Alexa 488 WGA, Alexa 568 phalloidin, and DAPI.

Unlike images recorded to sequentially, wavelength multiplexing records the images at the two excitation wavelengths virtually simultaneously. This reduces the influence of photochemical or physiological effects, changes in the laser power, or focus depth. Such changes influence the results for the two wavelengths in a similar way. Therefore the FLIM data for the two wavelengths remain comparable. With the DCS-120 CMI system and its four lasers FLIM at four different excitation wavelengths can be recorded, substantially increasing the amount of information obtained from samples with multiple fluorophores. An important application of excitation wavelength multiplexing is metabolic FLIM with simultaneous detection of NAD(P)H and FAD. Please see below.

Metabolic FLIM with NAD(P)H and FAD

Metabolic FLIM is based on simultaneous recording of lifetime images of NAD(P)H (nicotinamide adenine (pyridine) dinucleotide) and FAD (flavin adenine dinucleotide). The DCS-120 FLIM systems use laser wavelength multiplexing to simultaneously record lifetime images of the two fluorophores. By multi-exponential decay analysis of the NADH and FAD signals, the system delivers information on the metabolic state of the cells or the tissue investigated.

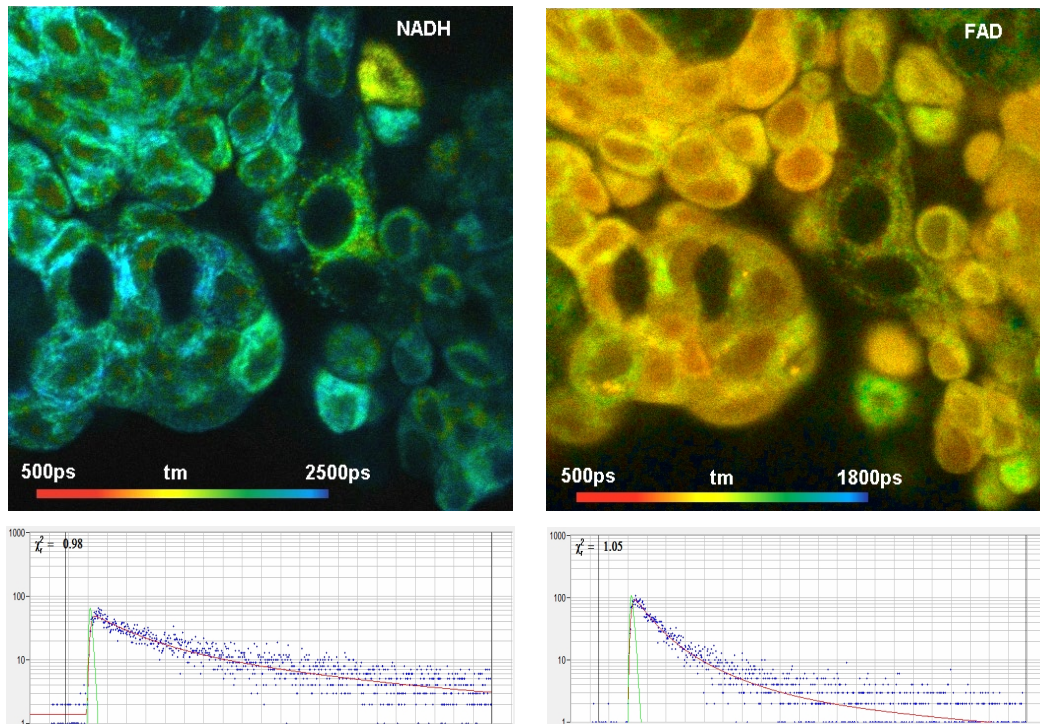


Fig. 32: NADH and FAD images, recorded simultaneously by the DCS-120 metabolic-FLIM system

In metabolic FLIM, the primary information is not in the mean lifetime, t_m , but in the amplitudes of the decay components, a_1 and a_2 . Due to its superior photon efficiency and time resolution the DCS-120 metabolic FLIM system provides such images at unprecedented accuracy. An example is shown in Fig. 33.

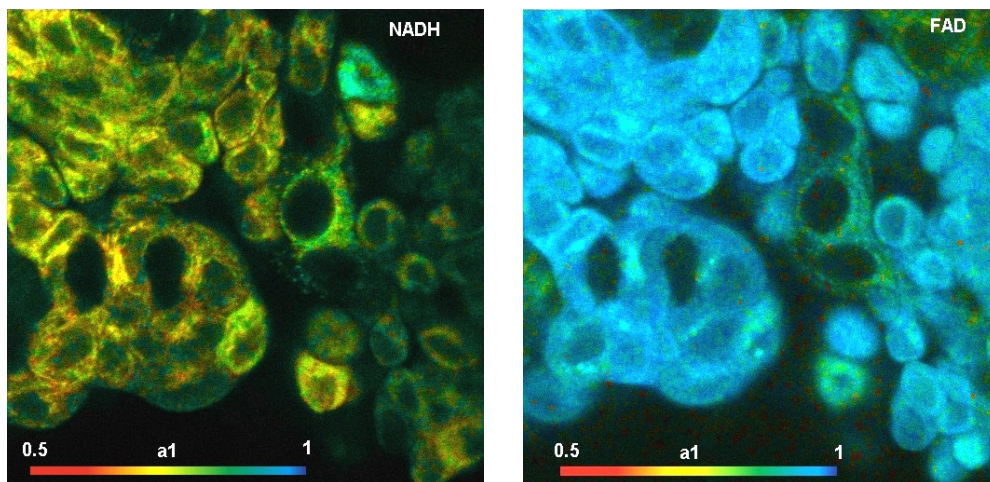


Fig. 33: NADH and FAD images, showing the amplitude of the fast decay component, a_1 .

DCS-120 multiphoton systems: Multiphoton FLIM of Small Organisms

Multiphoton FLIM is used when images from deep layers of biological tissue have to be recorded. A two-photon FLIM image of *Artemia salinas* (a small shrimp) recorded by the DCS-120 MP Multiphoton system is shown in Fig. 34. The image was recorded with the Femto-Fibre-Pro (fibre-laser) version of the DCS-120 MP [87].

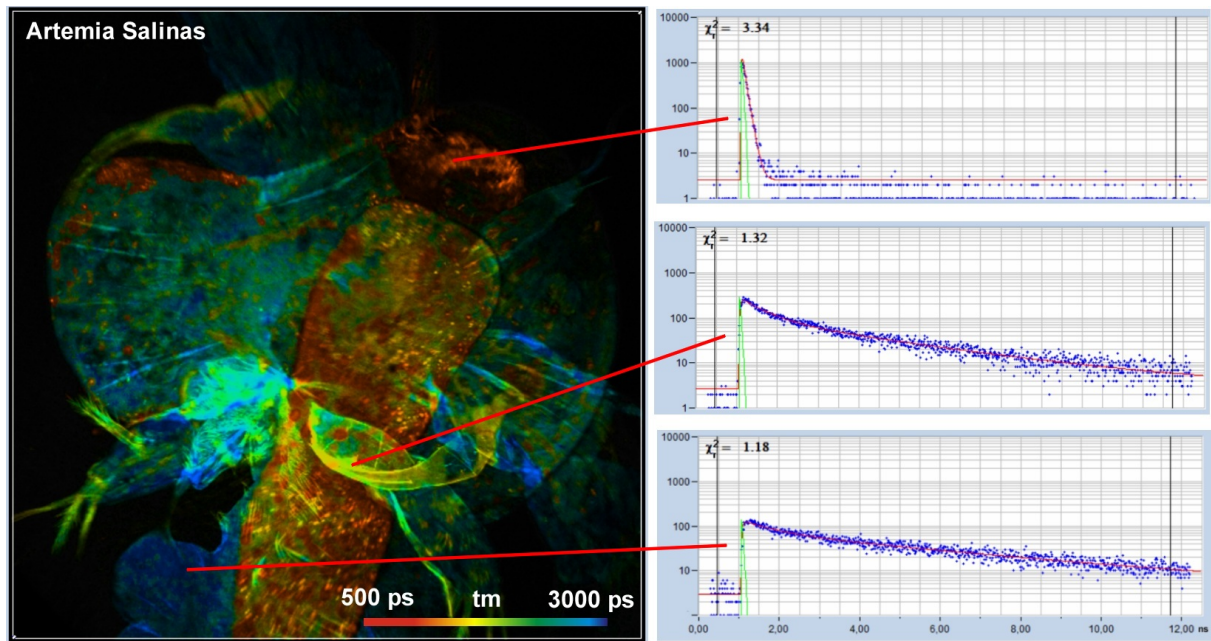


Fig. 34: Autofluorescence FLIM image of a brine shrimp, *artemia salinas*. Mean (amplitude-weighted) lifetime of double-exponential decay. Decay functions of selected areas shown on the right.

Multiphoton FLIM of Skin

Fig. 35 show a FLIM image of a pig skin sample, excited at 785 nm and detected from 510 to 600 nm. The image was recorded with 1024 x 1024 pixels and 1024 time channels.

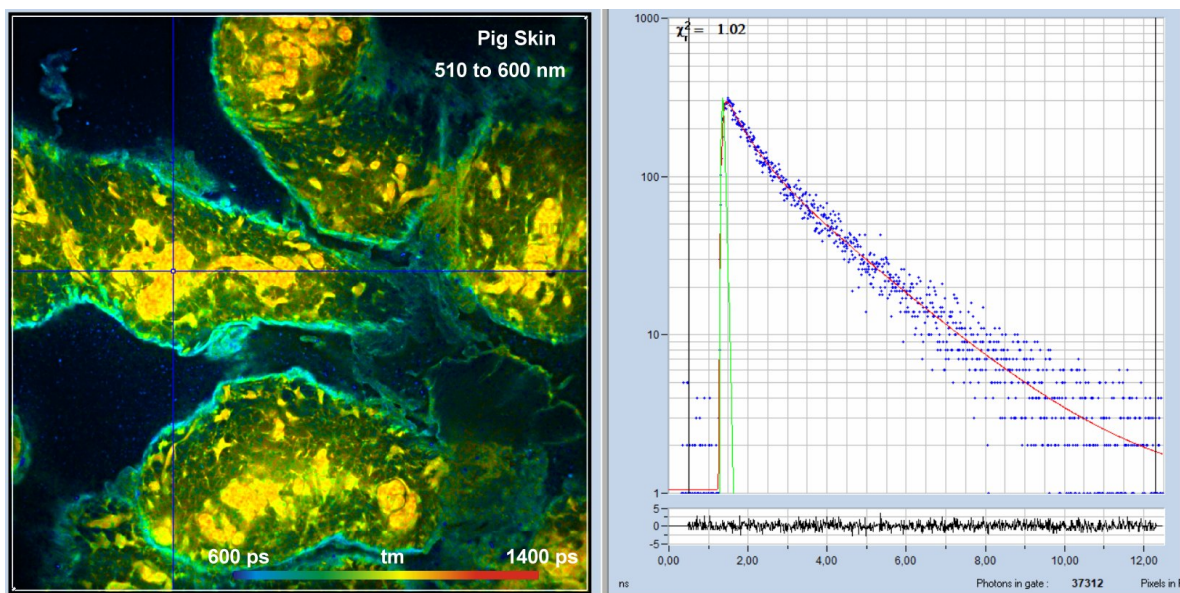


Fig. 35: Pig skin, autofluorescence, excitation 785 nm, detection 510 to 600 nm. 1024 x 1024 pixels, 1024 time channels. DCS-120 MP with Topica Femto Fibre Smart laser. Amplitude-weighted lifetime, tm. Decay curve in 5x5 pixel area around the cursor position shown on the right. SPCImage NG data analysis software.

Wavelength-Multiplexed Multiphoton FLIM

The DCS-120 DMP system is able to multiplex two Topica Femto Fibre Ultra lasers of 780 nm and 920 nm. The typical application is metabolic FLIM of tissues by simultaneous detection of NAD(P)H and FAD data. An example is shown in Fig. 36 and Fig. 37.

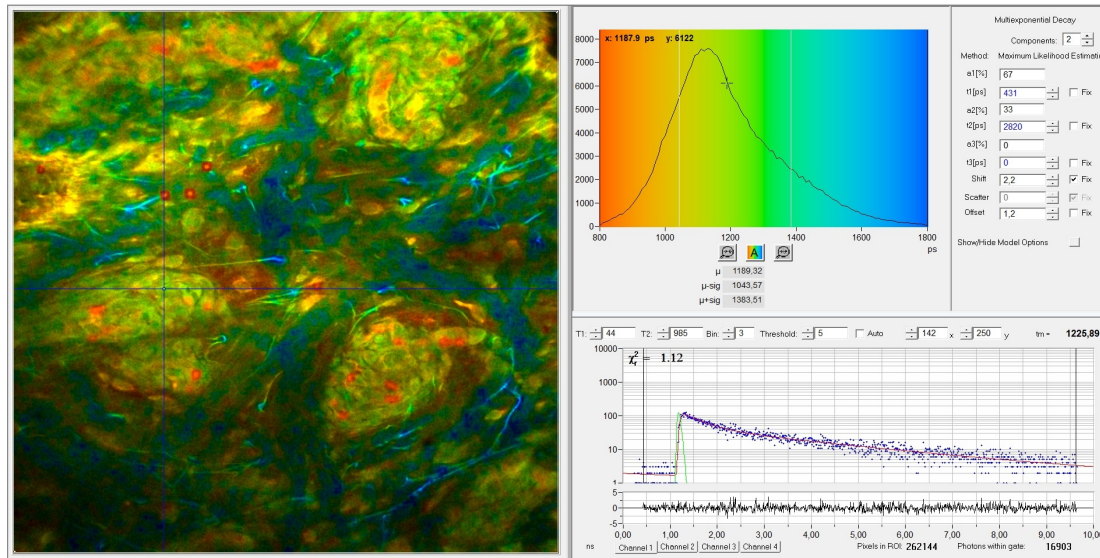


Fig. 36: 2p metabolic FLIM of Pig skin, t_m image in the NADH channel. DCS-120 DMP, SPCImage NG data analysis

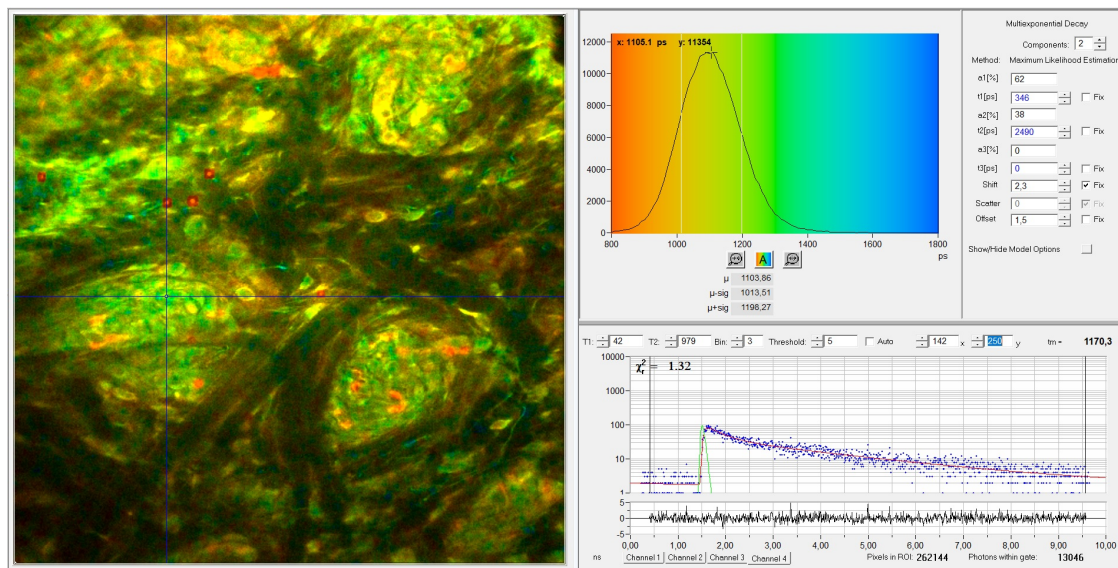


Fig. 37: 2p metabolic FLIM of Pig skin, t_m image in the FAD channel. DCS-120 DMP, SPCImage NG data analysis

Multiphoton Skin Imaging in Combination with pH Imaging

Biological imaging often requires that several biological parameters are recorded simultaneously and in their mutual dependence. Fig. 38 shows simultaneous imaging of the metabolic state via the decay function of NADH, and pH imaging via the fluorescence lifetime of BCECF.

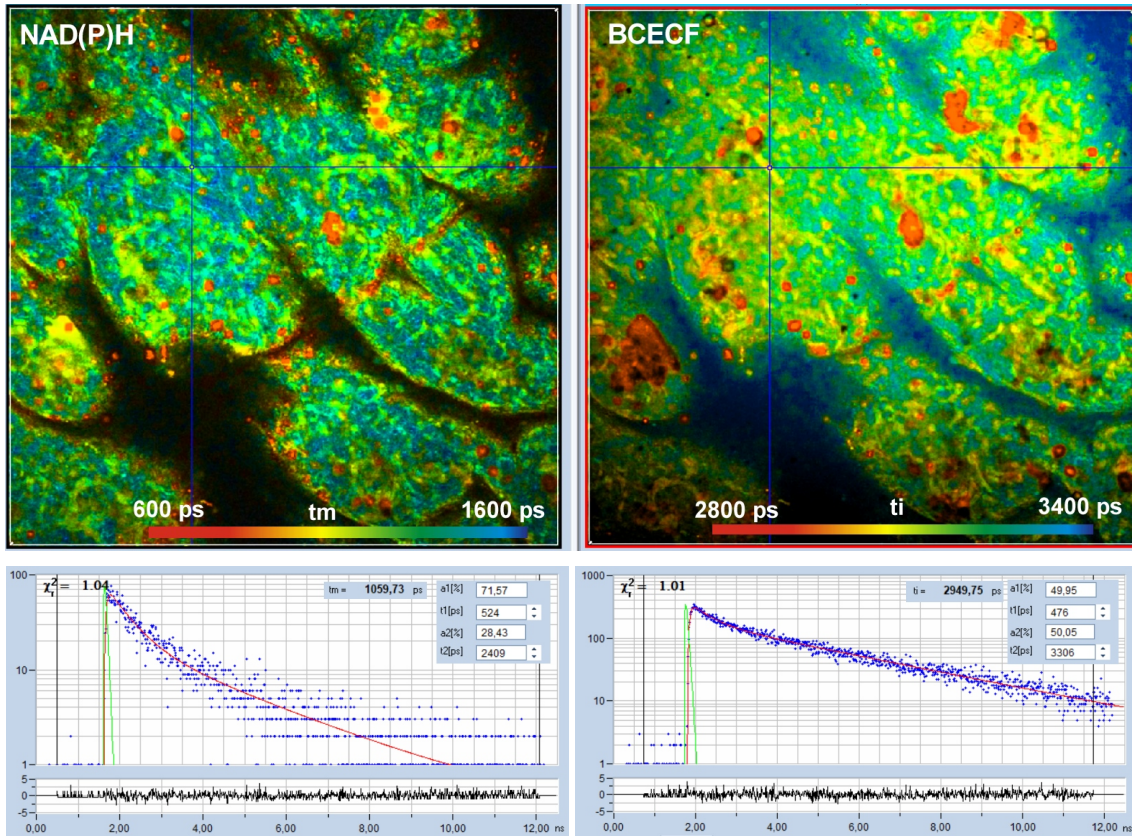


Fig. 38: Upper row: NAD(P)H image of the amplitude-weighted lifetime, t_m , and intensity-weighted lifetime image of BCECF. Lower row: Decay function of NAD(P)H and decay function of BCECF at cursor position. Both images 512 x 512 pixels, 1024 time channels. DCS-120 MP system, data analysis with bh SPCImage NG, MLE fit.

Multi-Wavelength FLIM

With the bh multispectral FLIM detectors the DCS-120 records FLIM simultaneously in 16 wavelength channels [61, 69, 77]. The images are recorded by a multi-dimensional TCSPC process which uses the wavelength of the photons as a coordinate of the photon distribution. There is no time gating, no wavelength scanning and, consequently, no loss of photons in this process. The system thus reaches near-ideal recording efficiency. Moreover, dynamic effects in the sample or photobleaching do not cause distortions in the spectra or decay functions. Multi-wavelength FLIM got an additional push from the new 64-bit SPCM software, and from the introduction of a highly efficient GaAsP multi-wavelength detector. 64-bit software works with enormously large photon distributions, and the GaAsP detector delivers the efficiency to fill them with photons. As a result, 16 images in 16 wavelength channels can be recorded at a resolution of 512x512 pixels and 256 time channels. An example is shown in Fig. 39.

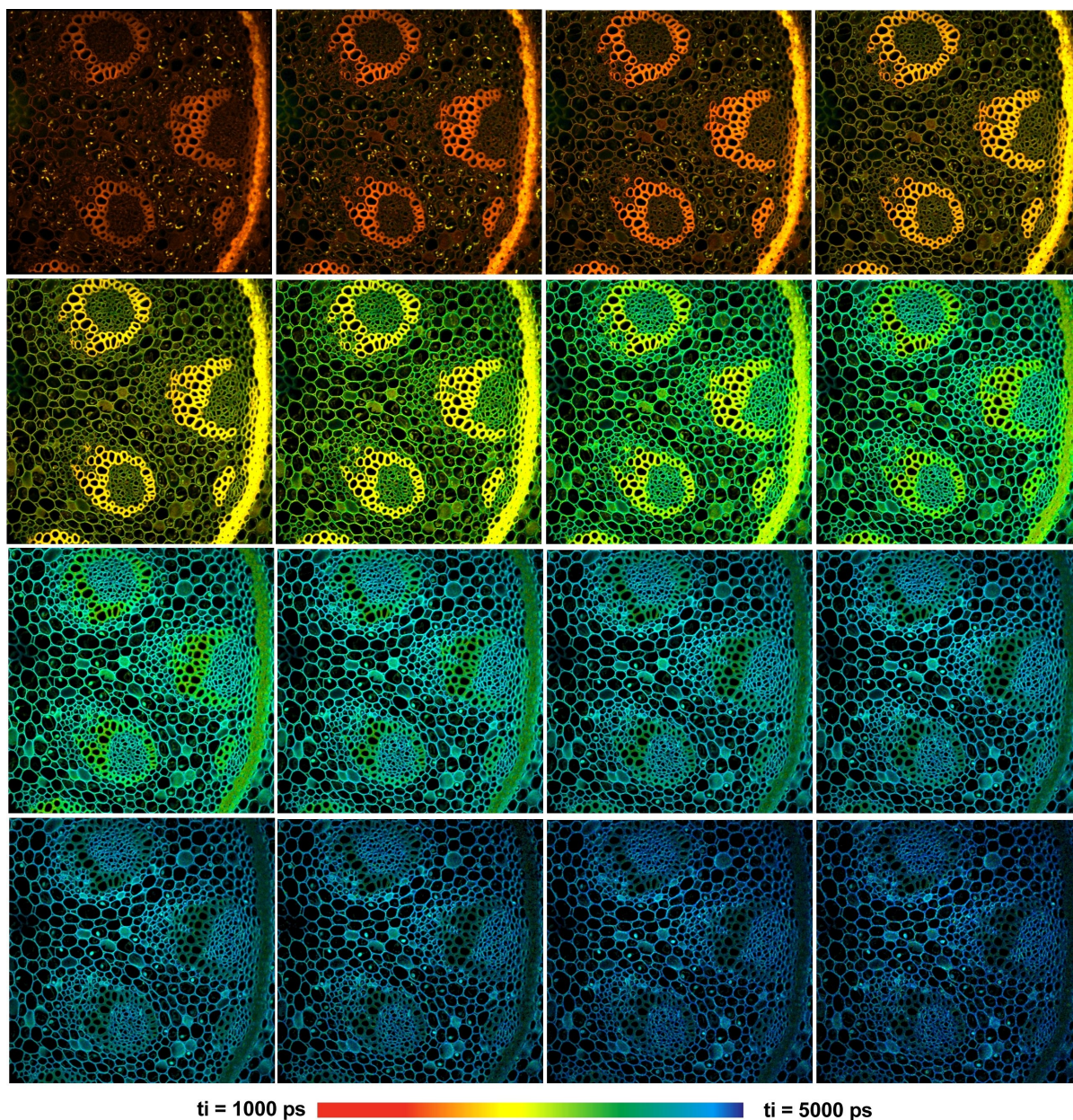


Fig. 39: Multi-wavelength FLIM with the bh MW-FLIM GaAsP 16-channel detector. 16 images with 512 x 512 pixels and 256 time channels were recorded simultaneously. Wavelength from upper left to lower right, 490 nm to 690 nm, 12.5 nm per image.

Fig. 40 demonstrates the true spatial resolution of the data. Images from two wavelength channels, 502 nm and 565 nm, were selected from the data shown Fig. 39, and displayed at larger scale and with individually adjusted lifetime ranges. With 512x512 pixels and 256 time channels, the spatial and temporal resolution of the individual images is comparable with what previously could be reached only for FLIM at a single wavelength. Decay curves for selected pixels of the images are shown in Fig. 41.

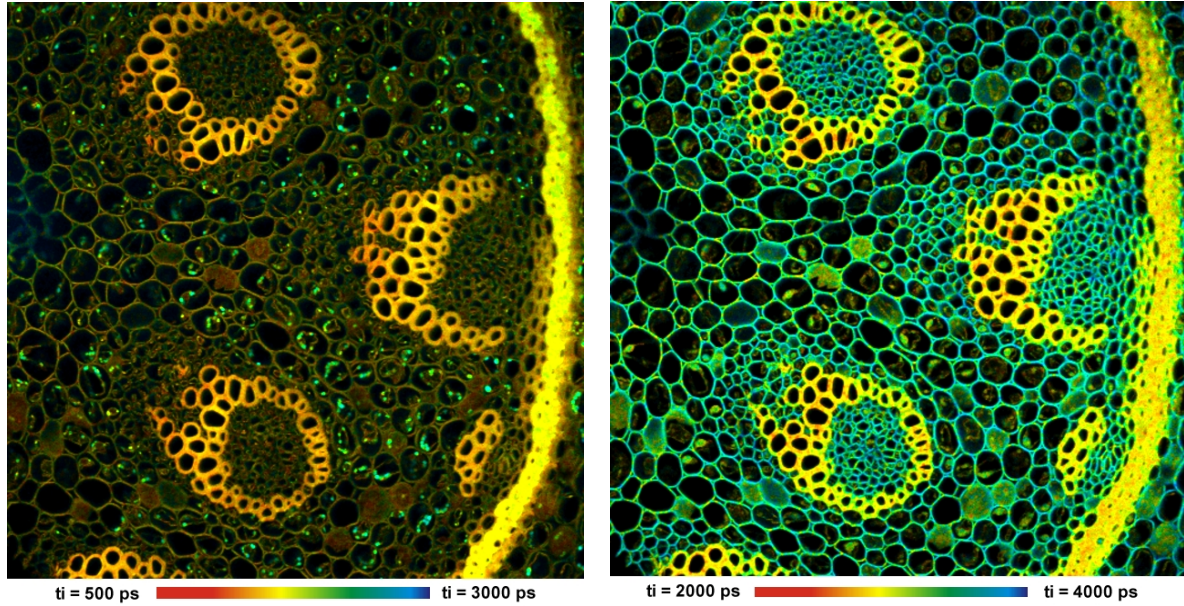


Fig. 40: Two images from the array shown in Fig. 39, displayed in larger scale and with individually adjusted lifetime range. Wavelength channels 502 nm (left) and 565 nm (right). The images have 512 x 512 pixels and 256 time channels.

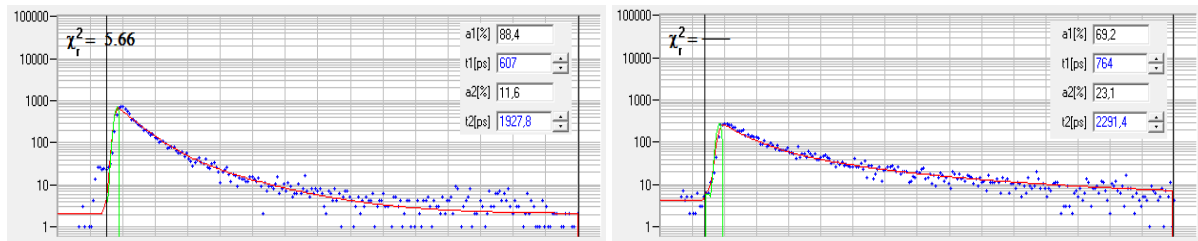


Fig. 41: Decay curves at selected pixel position in the images shown above. Blue dots: Photon numbers in the time channels. Red curve: Fit with a double-exponential model.

Lateral Mosaic FLIM

Mosaic FLIM records a large number of consecutive images into a single FLIM data array. The individual images within this array can represent the elements of a tile scan (x-y mosaic), images in different depth in the sample (z-stack mosaic), or images for different times after a stimulation of the sample (temporal mosaic). An example of an x-y mosaic is shown in Fig. 42. The complete data array has 2048 x 2048 pixels, and 256 time channels per pixel. Compared to a similar image taken through a low-magnification lens the advantage of mosaic FLIM is that a lens of high numerical aperture can be used, resulting in high detection efficiency and high spatial resolution.

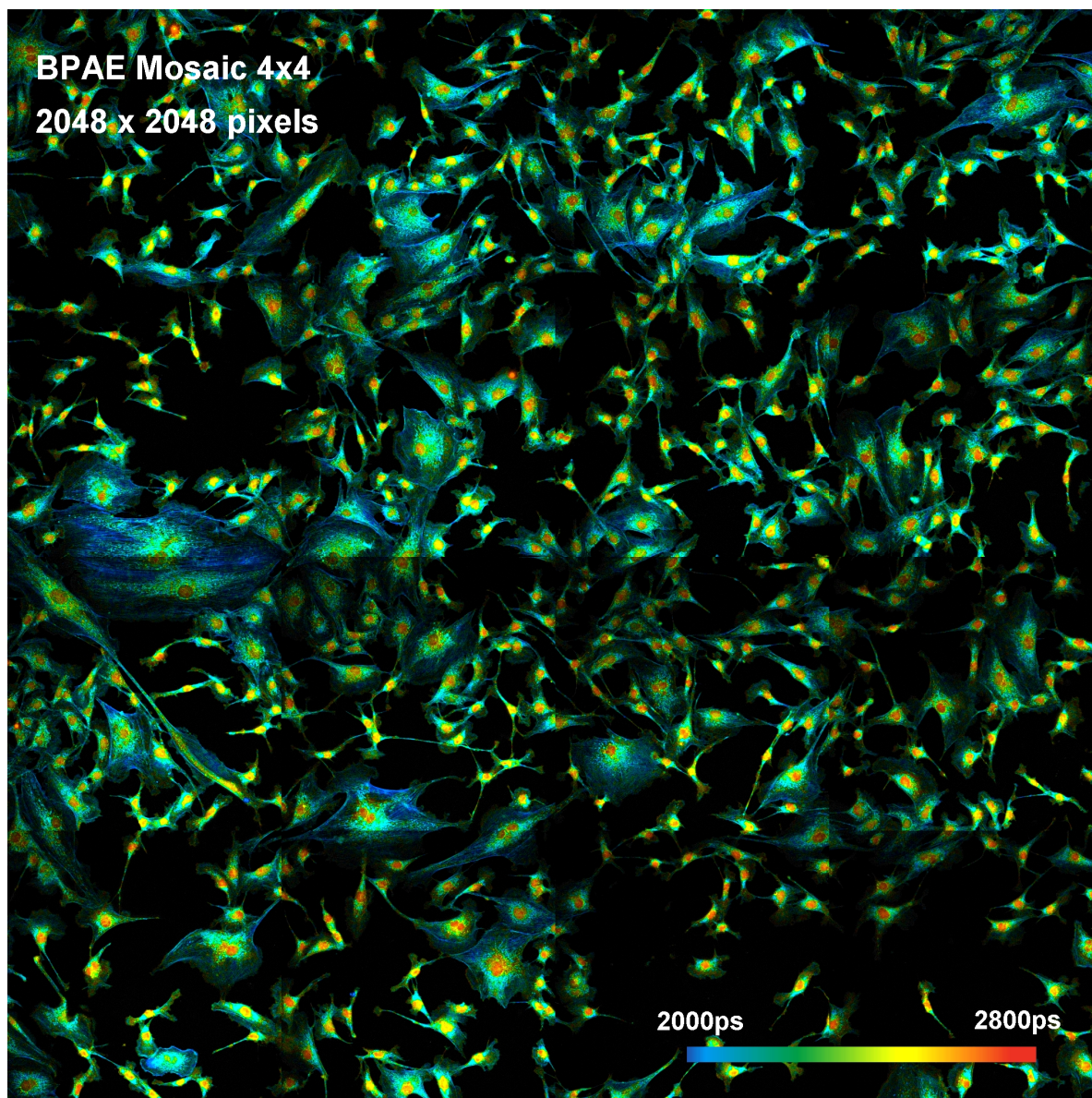


Fig. 42: Mosaic FLIM of a BPAE sample. The mosaic has 4x4 elements, each element has 512x512 pixels with 256 time channels. The entire mosaic has 2048 x 2048 pixels. DCS-120 MP multiphoton system with motorised sample stage, SPCImage NG data analysis.

Z-Stack recording by Mosaic FLIM

The Mosaic FLIM function of SPCM can be used to record Z Stacks of FLIM images. As the microscope scans consecutive images planes in the sample the FLIM system records the data into consecutive elements of a FLIM mosaic. The advantage over the traditional record-and-save procedure is that no time has to be reserved for save operations, and that the entire array can be analysed in a single analysis run. An example is shown in Fig. 43.

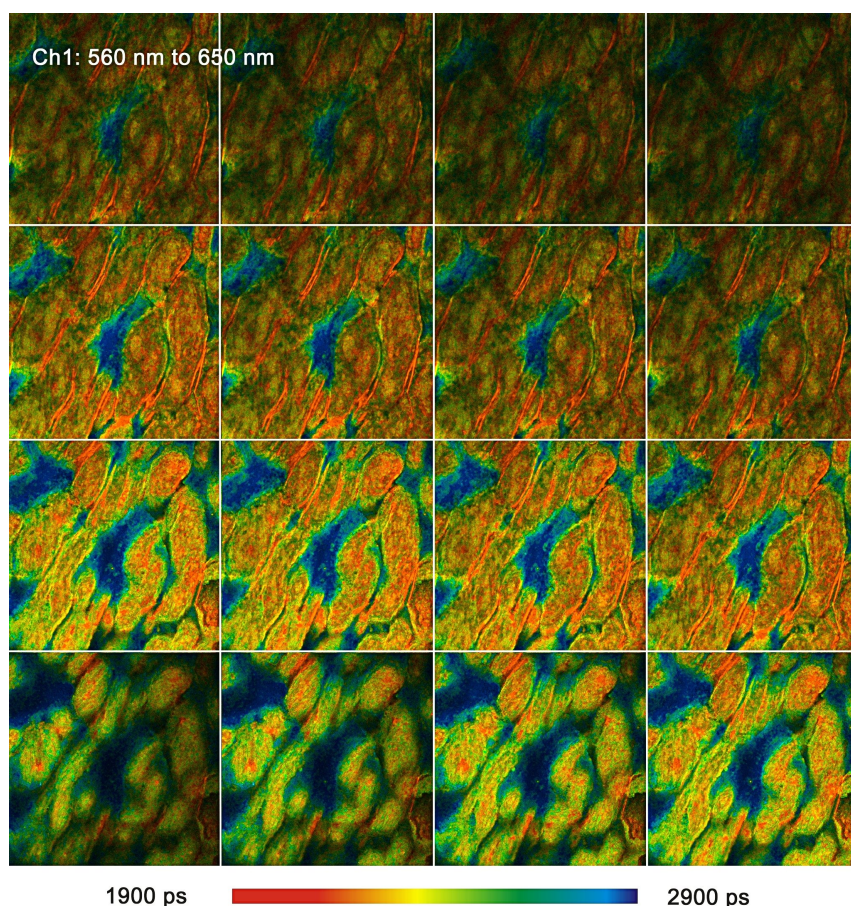


Fig. 43: FLIM Z-stack, recorded by Mosaic FLIM. Pig skin, autofluorescence. 16 planes, 0 to 30 μm from top of the tissue. Each element of the FLIM mosaic has 512x512 pixels and 256 time channels per pixel.

Z Stack Recording by Record-and-Save Procedure

Z stack recording by Mosaic FLIM is fast and efficient. However, it is restricted to about 64 Z planes. Larger stacks overwhelm even the memory capacity of 64 bit software. Z tacks larger than that can be recorded by the traditional record-and-save procedure. The sample is continuously scanned. For each plane, a FLIM image is acquired for a specified ‘collection time’. Then the data are saved in a file, the microscope is commanded to step to the next plane, and the next image is acquired. The procedure continues for a specified number of Z planes.

Fig. 44 demonstrates Z stack imaging of a fly (*Musca domestica*) with the DCS-120 DMP system. 289 Z planes of 2.5 μm Z step width, 1024 x 1024 pixels in X and Y, and 1024 channels in time were recorded. Double-exponential decay analysis was performed on these data by the batch processing function of SPCImage NG. A 3D reconstruction on the basis of the SPCImage NG results by ImageJ / FIJI is shown in Fig. 44.

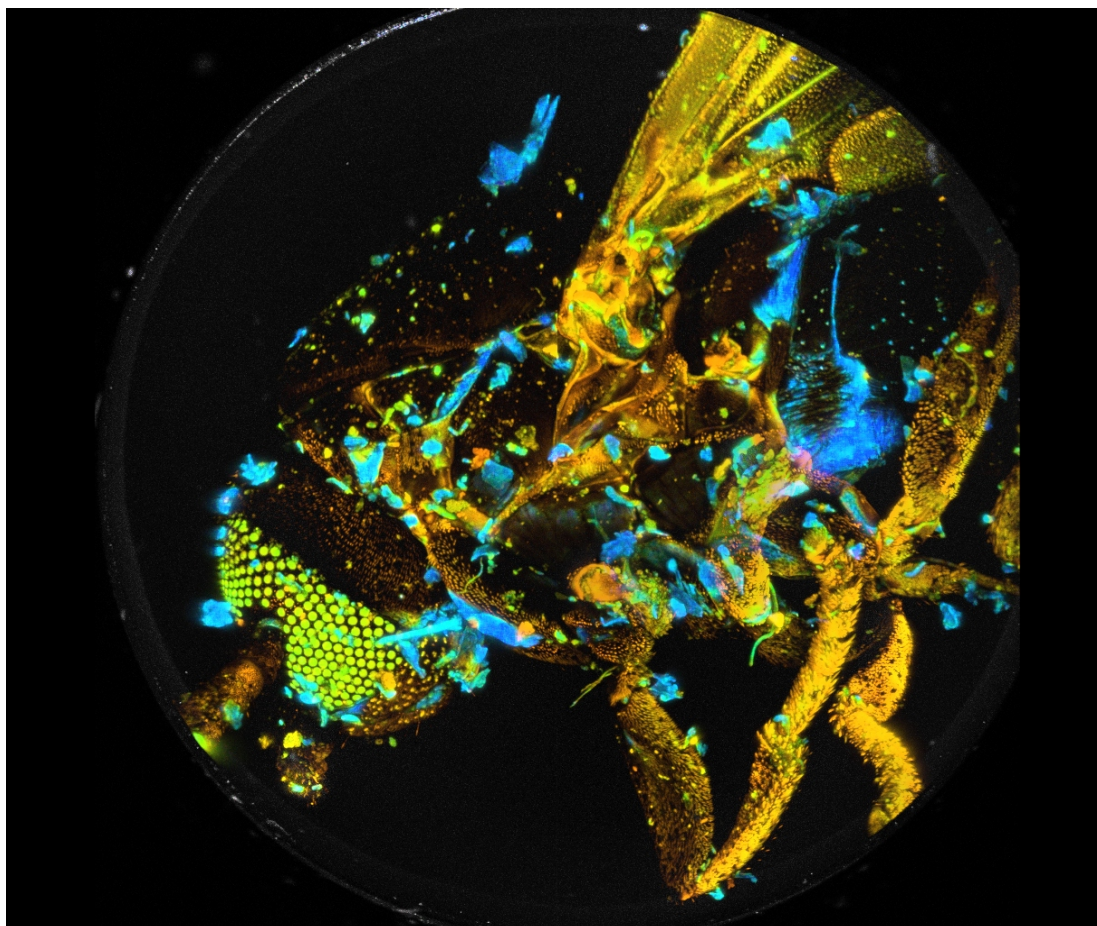


Fig. 44: Z stack recording of a fly, *Musca domestica*. 289 Z planes of 2.5 μm Z step width, 1024 x 1024 pixels per plane, 1024 time channels per pixel. Batch-processing function of SPCImage NG, 3D reconstruction by ImageJ / FIJI. Two-photon excitation, DCS-120 DMP with Toptica fibre laser.

Time-Series FLIM by Record-and-Save Procedure

Time-series FLIM by the traditional record-and-save procedure is available for all DCS-120 system versions. With the SPC-152 dual-channel systems time series as fast as 2 images per second can be obtained [310]. A time series taken at a moss leaf is shown in Fig. 45. The fluorescence lifetime of the chloroplasts changes due to the Kautski effect induced by the illumination.

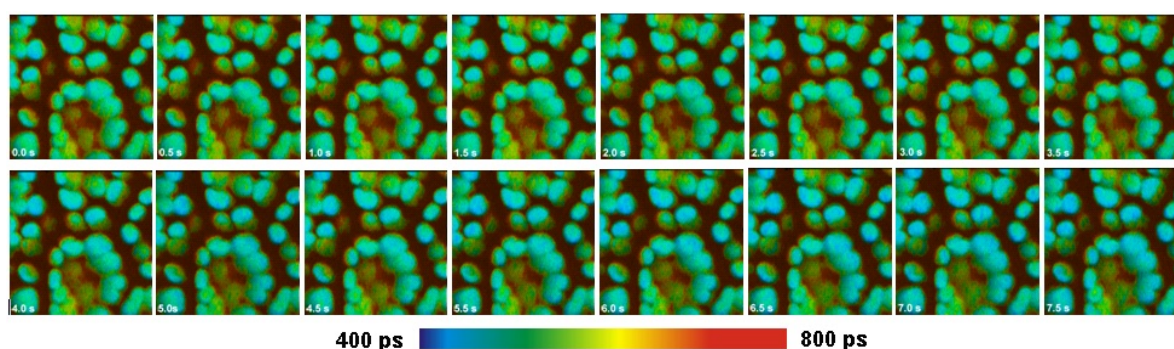


Fig. 45: Time-series FLIM, 2 images per second. Chloroplasts in a leaf, the fluorescence lifetime of the chlorophyll decreases with the time of exposure.

Time-Series FLIM by Temporal Mosaic FLIM

Temporal Mosaic FLIM uses the Mosaic Imaging function of the DCS-120 systems to record fast time series. The sample stage or the Z drive of the microscope are not moved but subsequent frames of the scan are recorded into subsequent elements of the data mosaic. The sequence can be repeated and accumulated [77, 83]. The time per mosaic element can be as short as a single frame, which can be less than 100 ms. Another advantage is that the entire array can be analysed in a single SPCImage data analysis run. Fig. 46 shows the change in the lifetime of chlorophyll in plant tissue with the time of illumination.

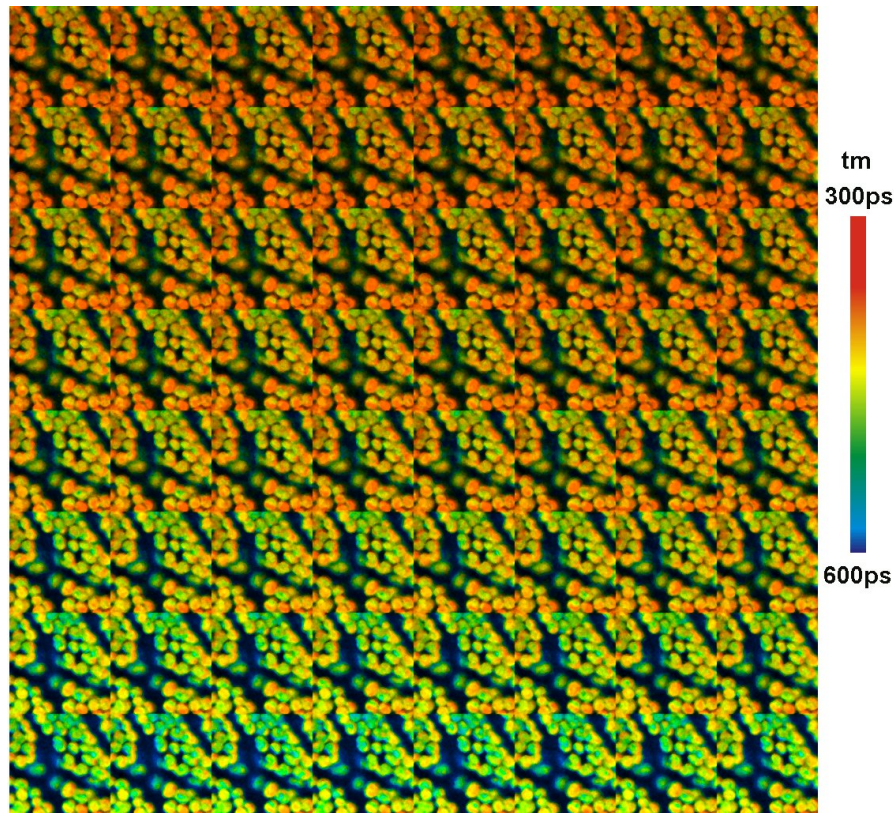


Fig. 46: Time series of chloroplasts in a leaf recorded by Mosaic Imaging. 64 mosaic elements, each 128x128 pixels, 256 time channels. Scan time per element 1s. Experiment time from lower left to upper right. Amplitude-weighted lifetime of double-exponential decay.

FLITS: Fluorescence Lifetime-Transient Scanning

FLITS records dynamic effects in the fluorescence lifetime of a sample along a one-dimensional scan. The technique is based on building up a photon distribution over the distance along the scan, the arrival times of the photons after the excitation pulses, and the experiment time after a stimulation of the sample. The maximum resolution at which lifetime changes can be recorded is given by the line scan time. With repetitive stimulation and triggered accumulation transient lifetime effects can be resolved at a resolution of about one millisecond [74, 77].

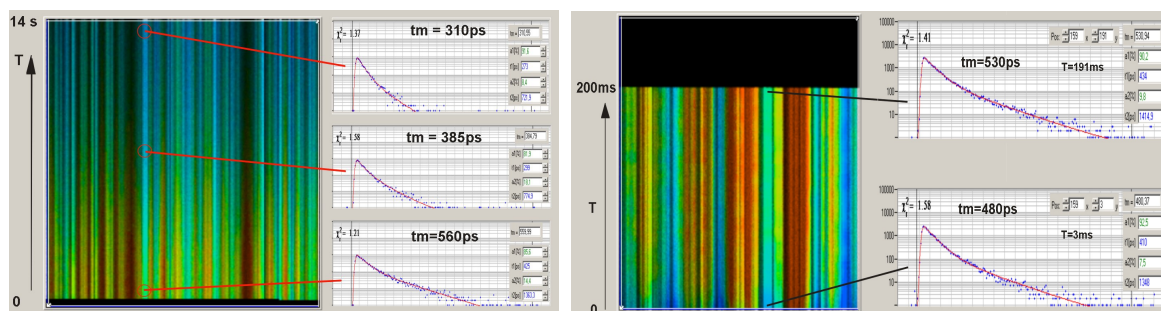


Fig. 47: FLITS of chloroplasts in a grass blade, change of fluorescence lifetime after start of illumination. Left: Non-photochemical transient, transient resolution 60 ms. Right: Photochemical transient. Triggered accumulation, transient resolution 1 ms.

The technique has been used to record Ca^{++} transients in live neurons at a resolution of 2 ms, see [76, 77, 229].

Simultaneous FLIM/PLIM

Phosphorescence and fluorescence lifetime images are recorded simultaneously by bh's proprietary FLIM/PLIM technique. The technique is based on modulating a ps diode laser synchronously with the pixel clock of the scanner [73, 77]. FLIM is recorded during the 'On' time, PLIM during the 'Off' time of the laser. The SPCM software delivers separate images for the fluorescence and the phosphorescence which are then analysed with SPCImage FLIM/PLIM analysis software. Please see page 293 for details.

Currently, there is increasing interest in PLIM for background-free recording and for oxygen sensing [19, 20, 188, 190, 294, 435, 590]. In these applications, the bh technique delivers a far better sensitivity than PLIM techniques based on single-pulse excitation. The real advantage of the FLIM/PLIM technique used in the DCS-120 is, however, that FLIM and PLIM are obtained *simultaneously*. It is thus possible to record metabolic information via FLIM of the NADH and FAD fluorescence, and simultaneously map the oxygen concentration via PLIM. An example is shown in Fig. 308.

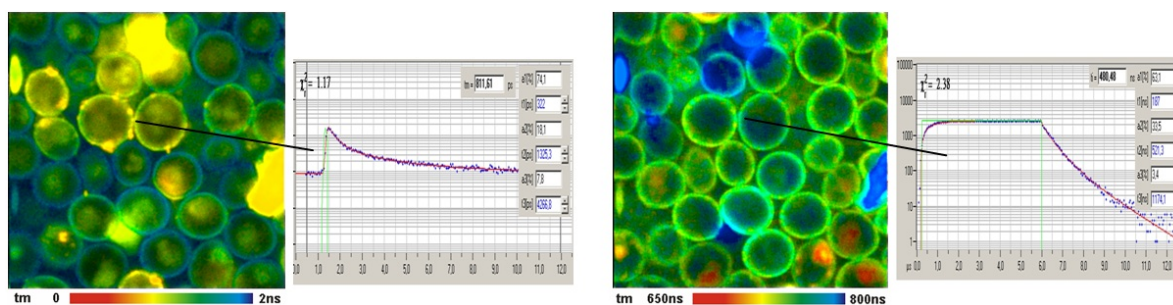


Fig. 48: Yeast cells stained with (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate. FLIM and PLIM image, decay curves in selected spots.

PLIM can also be interesting for the investigation the luminescence properties of inorganic compounds, see Fig. 49. PLIM is also used for the study of migration, possible dissolution, and chemical reaction of nanoparticles from sunscreens in skin. The results of such studies are highly important to skin cancer research.

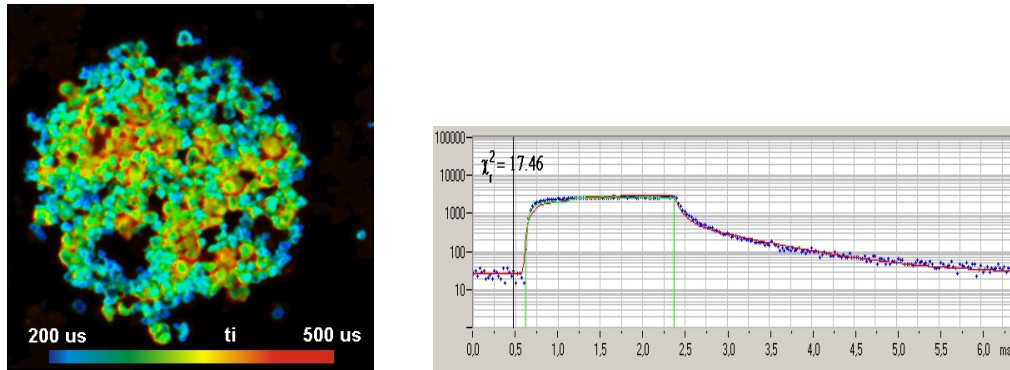


Fig. 49: Phosphorescence lifetime imaging of nanoparticles, here of the phosphor of a cathode-ray tube. Left: Lifetime image. Right: Phosphorescence decay curve at selected position within the image

DCS-120 MACRO: FLIM of cm-Size Objects

The DCS MACRO version of the DCS system scans objects directly in the focal plane of the scanner. Objects up to a size of 15 mm can be imaged at high resolution. With two lasers of 375 nm and 405 or 445 nm, the DCS-120 MACRO an excellent system for inspection of excised tumor tissue and metabolic FLIM of tumors in small animals. An example is shown in Fig. 50.

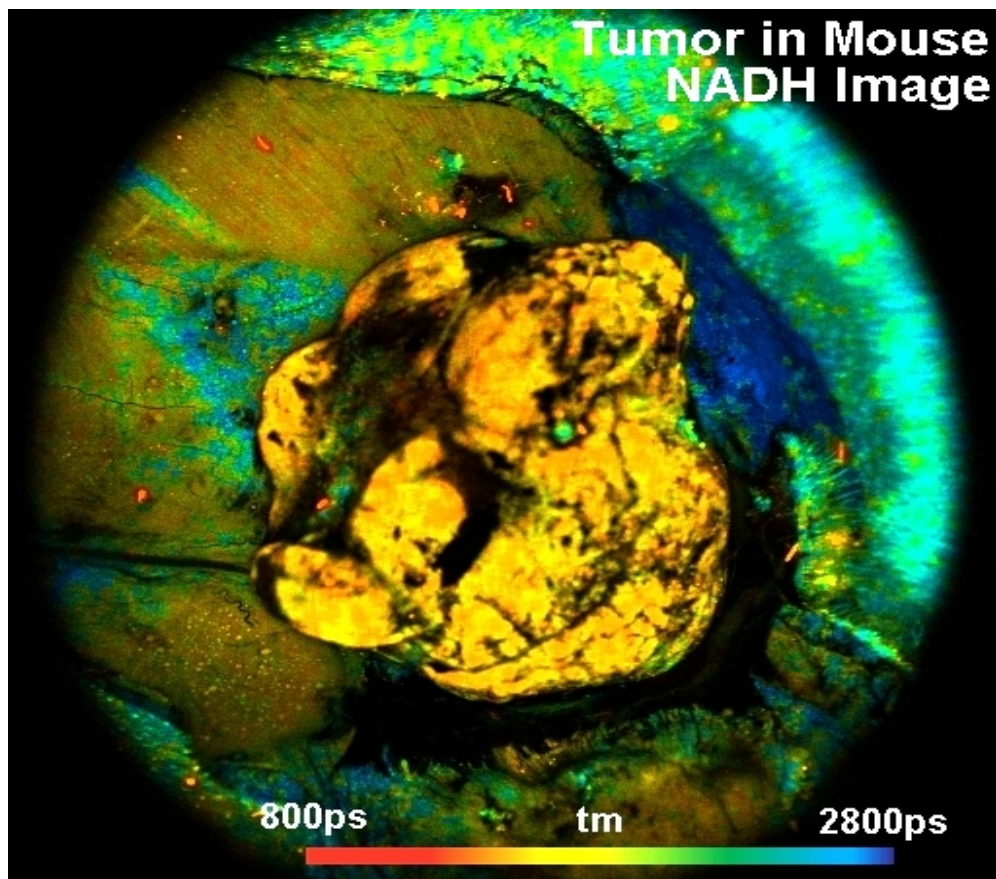


Fig. 50: Lifetime Image of a tumor in a mouse. NAD(P)H image, image size 15 x 15 mm, recorded by DCS-120 Macro system.

High-Performance FLIM Data Analysis

Data analysis is performed by bh's legendary SPCImage FLIM Data analysis. SPCImage combines Time-Domain and Phasor analysis. It uses an MLE fit procedure, resulting in superior accuracy down to low photon numbers, and unprecedented accuracy of multi-exponential decay analysis. By running the calculations on a GPU, results are obtained within seconds. This makes it possible to analyse complex data structures, such as FLIM/PLIM data, multi-wavelength data, and metabolic-FLIM data. A Global Fit procedure is included for precision analysis of triple-exponential FRET and Metabolic-FLIM data.

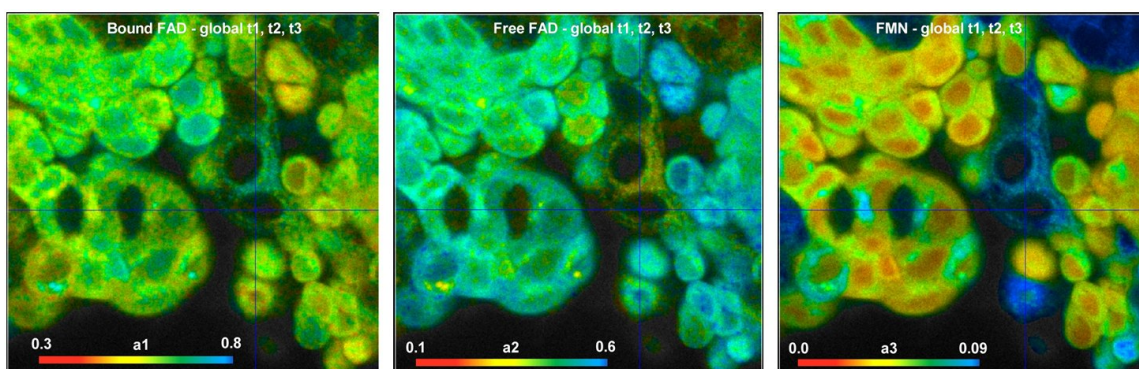


Fig. 51: Global fit, triple-exponential analysis of FAD / FMN fluorescence. Separation of bound FAD, free FAD, and FMN

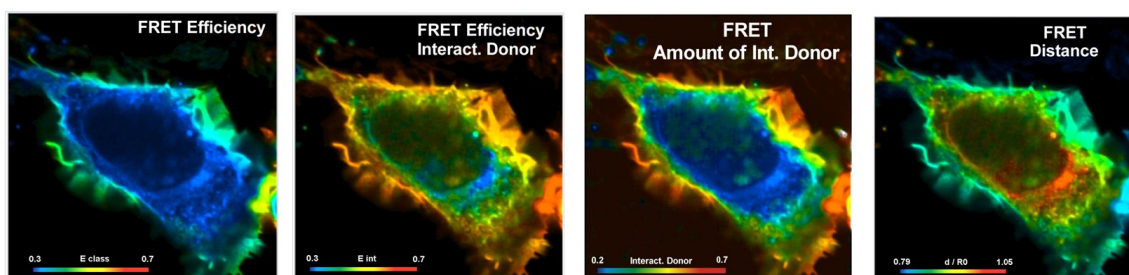


Fig. 52: Calibration-free FRET analysis by double-exponential MLE fit

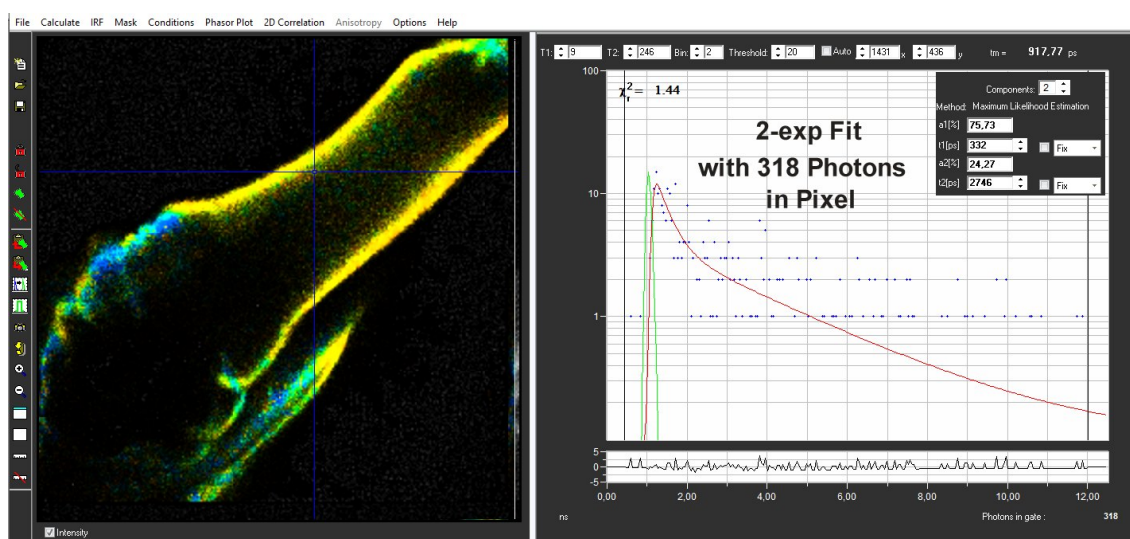


Fig. 53: Double-exponential fit on 318 photons. Decay components of FAD are correctly reproduced.

Quick Start for Beginners

General Principle of FLIM Recording

This section gives beginners a quick start to the recording of fluorescence lifetime images with the DCS-120 system. It does not require much knowledge about the underlying optical and electronical principles of the DCS-120. Just remember that the recording is based on scanning the sample with the beam of a high-frequency pulsed laser, detecting the photons of the fluorescence emitted by the sample, and building up the distribution of the photons over the pixels of the image and their times after the laser pulses. The result is an image that represents the pixels of the scan, with each pixel containing a fluorescence decay curve in the form of photon numbers in at consecutive times after the excitation pulses. The image is displayed with the fluorescence intensity as brightness, and the fluorescence lifetime of the decay curve as colour. Please see Fig. 54.

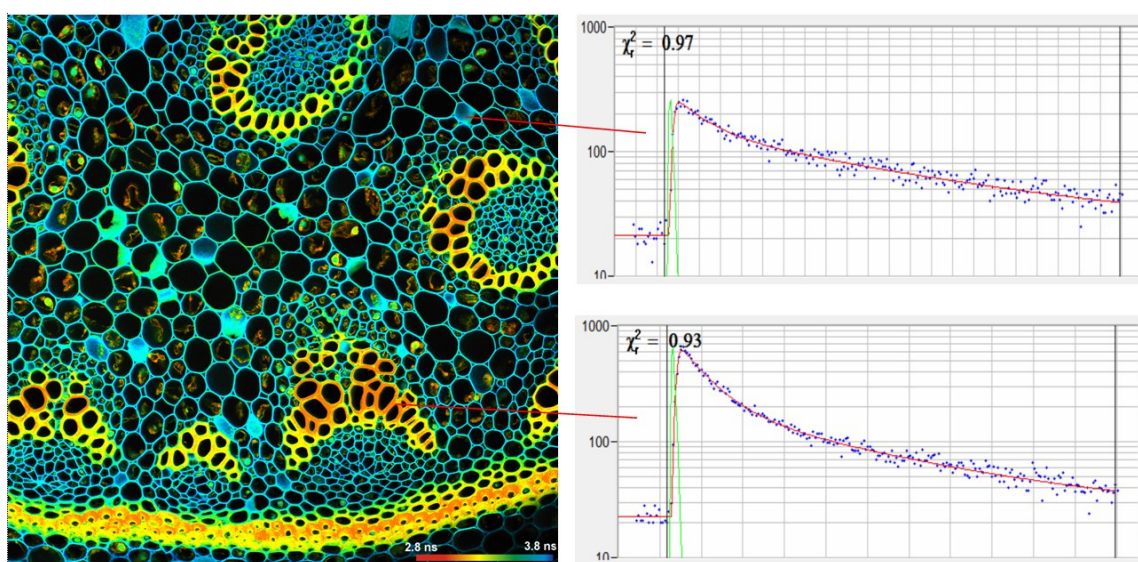


Fig. 54: A FLIM image is an array of pixels, each of which contains a fluorescence decay function in form of photon numbers in subsequent time channels after the excitation pulses. The brightness of each pixel is the fluorescence intensity, the colour the fluorescence lifetime of the decay function.

FLIM Protocol

Turn On All System Components

Turn on the System Computer, turn on the microscope, turn on the GDA-120 Galvo Driver, turn on the DCS-Switch Box, see Fig. 55. In the DCS-120 CMI (confocal molecular imaging) system, turn on the LHB-104 Laser Hub. We recommend to power all system components from a single power strip. Then you have only to turn on the power strip, and not all the individual system components.



Fig. 55: Turn on all system components. Upper left to lower right: GDA-120 Scan Amplifier, LBH-104 Laser Hub, DCS Switch Box, System Computer.

In a multiphoton system, turn on also the Ti:Sa laser or the fibre laser(s). Start the System Computer.

Start SPCM on the System Computer

Start SPCM on the system computer. SPCM will show up on the screen in a configuration like the one shown in Fig. 56. Please note that SPCM starts in a configuration and with system parameters as they were at the end of the previous session. It may therefore not come up in exactly the configuration you want.

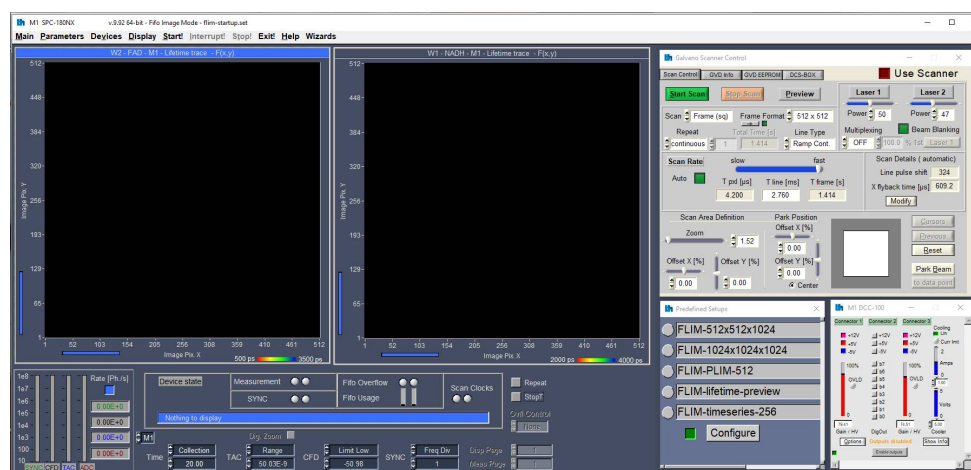


Fig. 56: SPCM Software after start of system

Load a Predefined Setup or a data file

Select a setup from the list of 'Predefined Setups', see Fig. 57, left. Use a simple setup, e.g. FLIM with 512 x 512 pixels, 1024 time channels. For your first FLIM attempts, do not use setups for complex experiments, such as Mosaic FLIM, Z-Stack FLIM or Time-Series FLIM. After loading the setup the SPCM panel will change into the configuration in the setup file, and show data which are in this file, see Fig. 57, right.

Alternatively, you can load an .sdt file of a previous measurement (see 'Saving and Loading Setup and Measurement Data', page 119). Loading this file will restore the state of the system as it was when the file was saved.

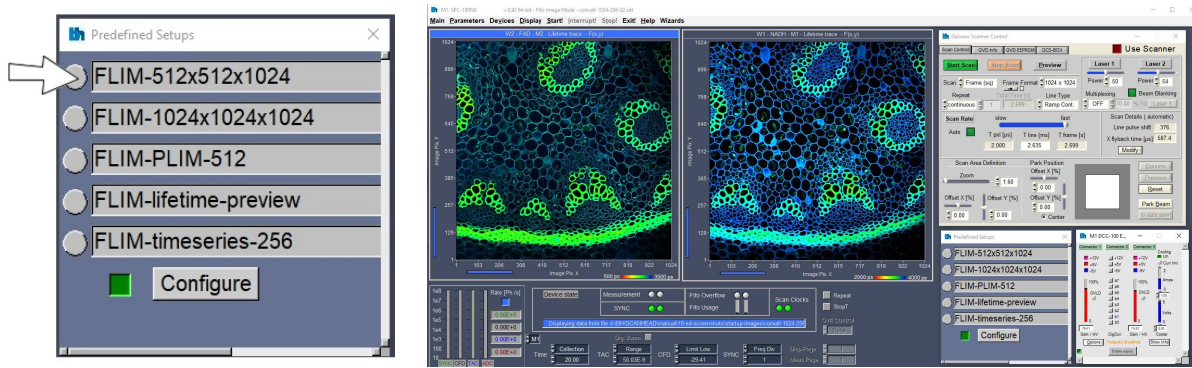


Fig. 57: Left: Predefined Setup panel. Right: SPCM software after loading as predefined setup or a data file

Ti:Sa Systems: Wait until the Laser is Running

Ti:Sa lasers need some time to warm up. In multiphoton system with a Ti:Sa laser, therefore check the Sync rate the FLIM system is receiving from the laser, see Fig. 58. Most Ti:Sa lasers have a repetition rate of 80 MHz. Therefore, the Sync rate should read 80 MHz. If it doesn't the laser is still warming up. In this case, wait until the correct Sync Rate shows up. The laser is then ready for use.

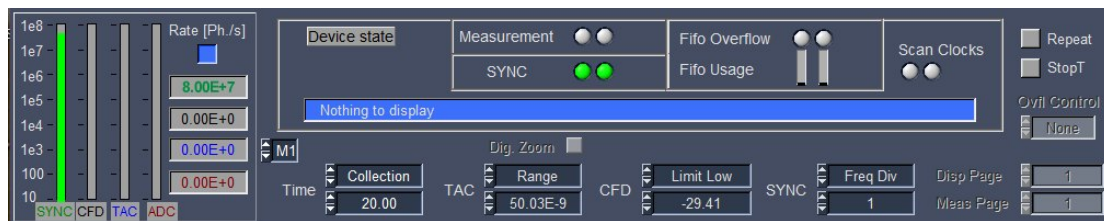


Fig. 58: Status section of SPCM main panel. Laser Sync Rate shown left.

Set up the scanner for the planned experiment

Put the secondary beamsplitter in a position which splits the fluorescence signal into the two spectral components you want, see Fig. 59. If you don't want to split the signal, use Position '100%' to upper detector. This sends the entire signal into Recording Channel 1. For initial system test, use 'Non-Polarising Beamsplitter' or 'Polarising Beamsplitter'. This sends the fluorescence into both detection channels. These actions are not needed for multiphoton microscopes. These system use a different detection beam path with its own filters and beamsplitters.



Fig. 59: Scan head with Secondary-Beamsplitter positions

Put in Correct Emission Filters.

Put correct bandpass filters in the slots of the filter sliders, see Fig. 60. The filter transmission ranges should correspond to the emission wavelength range of the sample to be imaged. If you don't know at which wavelength the sample emits use long-pass filters which block the laser wavelength but let pass longer wavelengths. Bandpass filters are marked with 'HQ', long pass filters with 'LP'. The filters are not relevant for multiphoton microscopes. These systems send the fluorescence signals through a different beam path.



Fig. 60: Filter sliders and filter slots of the scan head

Pinholes

In confocal systems, set Pinhole Size 1 in both channels. See Fig. 61. This will work for most samples. Larger pinholes yield more intensity, smaller pinholes yield higher spatial resolution. In DCS-120 MP and DCS-120 DMP multiphoton systems the pinhole size has no meaning. These systems detect through a non-descanned detection path which is independent of the confocal path. Please see 'Multiphoton FLIM Procedure' page 208.

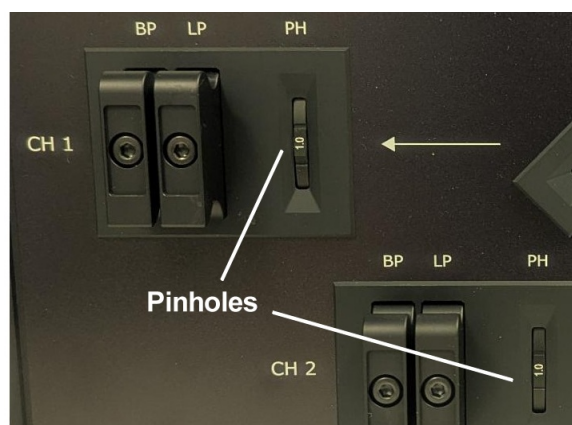


Fig. 61: Pinhole wheels

Put a Sample Under the Microscope Switch to 'Eyepiece', and focus visually

Put the sample under the microscope, see Fig. 62. Switch the microscope beam path to 'Eyepiece', see Fig. 63. Turn on the microscope lamp. By looking into the eyepieces, adjust focus and sample position. In multiphoton systems, you may also have to rotate the beamsplitter carousel of the microscope into a free position (see Fig. 63, right).

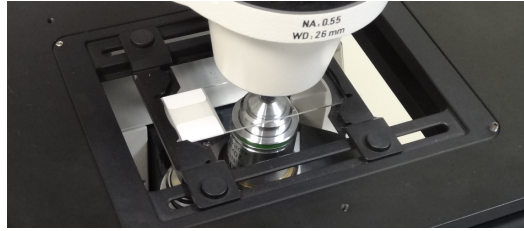


Fig. 62: Sample under the microscope. (Inverted Microscope)

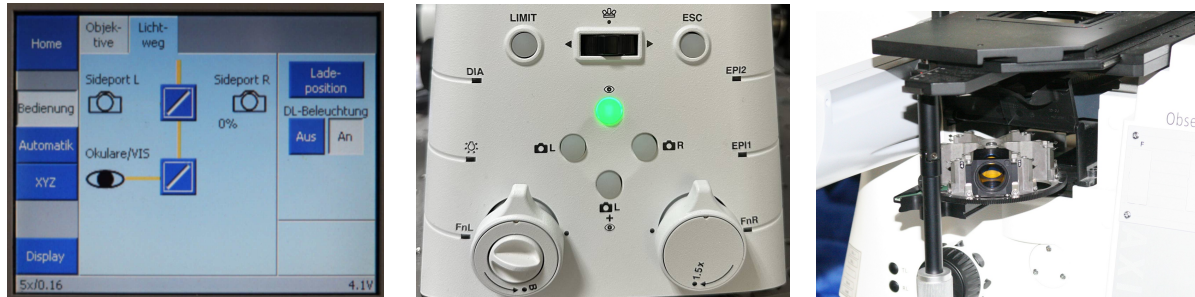


Fig. 63: Setting the beam path in the microscope to 'Eyepieces'. Left Zeiss, middle Nikon. Right: NDD beam splitter in microscope filter carousel. For visual use it has to be rotated out of the beam path. Cover removed.

Switch the microscope beam path to the scanner

Turn off the microscope lamp. Switch the microscope beam path to the port to which the scanner is attached. See Fig. 64. In a multiphoton system, also rotate the beamsplitter carousel of the microscope back into the position where the two-photon beamsplitter sits. (Please see 'Multiphoton FLIM Procedure', page 208) When the beam path is set, you are ready for imaging.

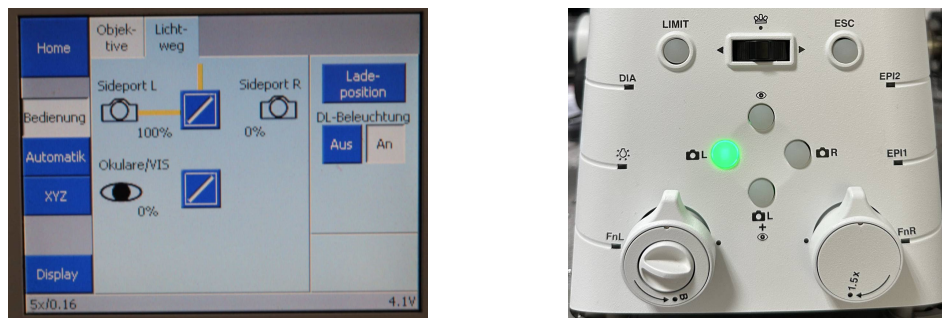


Fig. 64: Setting the beam path to 'Imaging'. Left Zeiss, right Nikon. Right: NDD beam splitter in microscope filter carousel. For confocal imaging it has to be rotated out of the beam path

Activate the Detectors, Activate the Laser

In SPCM, click into the detector-control panel, and enable the detectors. Then click into the Scanner control panel, and activate the laser you want to use. Please see Fig. 65. In a multiphoton system with non-descanned detection (NDD) you may also have to open the NDD shutter. Please see Fig. 65.

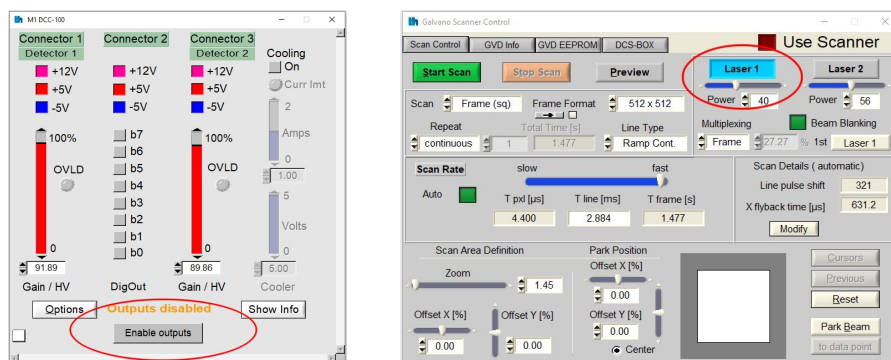


Fig. 65: Activation of Detectors and Laser

Start the Preview

In the scanner control panel, click on the 'Preview' button' Fig. 66, left. You can hear that the scanner starts working. Shortly after that, first preview images appear in the display windows of the SPCM main panel.

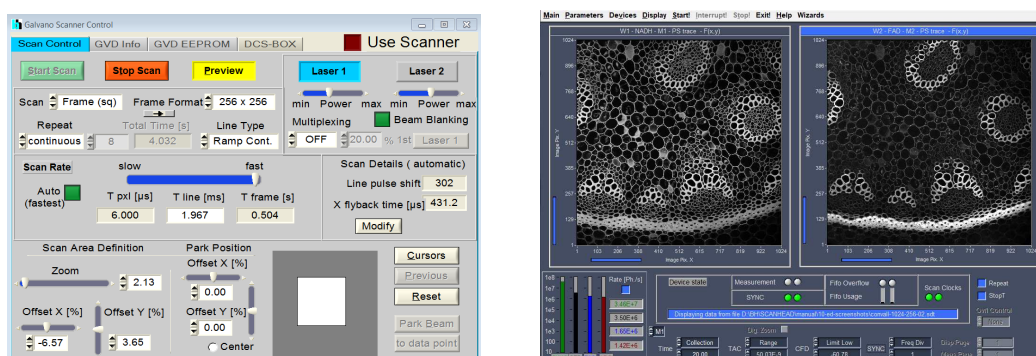


Fig. 66: Left: Start of 'Preview' in the scanner panel. Right: Preview images in the SPCM Main panel.

The Preview images are updated in intervals of the frame time of the scanner. Using the focus button of the microscope, focus into a sample plane that gives you sharp and bright images. Using the 'Zoom' slider in the scanner panel, adjust the scan field to an appropriate size. Using the controls of the sample stage, find a good lateral sample area from which looks promising to record a FLIM image from it.

When you have found the desired lateral spot in the sample, take some time to fine-focus into the absolutely best sample plane. If necessary, turn off the 'Autoscale' function in the display parameters (Fig. 67) and adjust the focus for maximum brightness. Please note that perfect focusing is a precondition for perfect FLIM data! Please see [82].

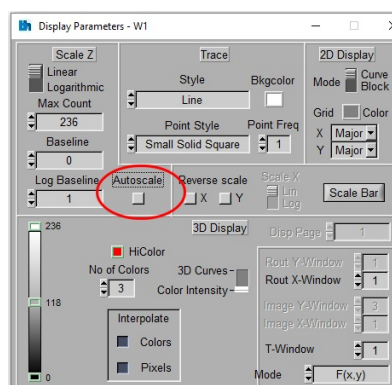


Fig. 67: Autoscale button in the Display Parameters

Laser Power

Keep an eye on the detector count rates. Using the power control wheels of the scanner (Fig. 68, left), keep the count rates in the range of a few 100,000 counts to about 2 million counts per second (Fig. 68, middle). Higher count rate can damage the sample or cause recording artefacts, lower count rate results in unnecessarily long acquisition time. Extremely high count rates may shut down one or both detectors. In that case, reduce the laser power and click the 'Reset' button in the detector control panel (Fig. 68, right).

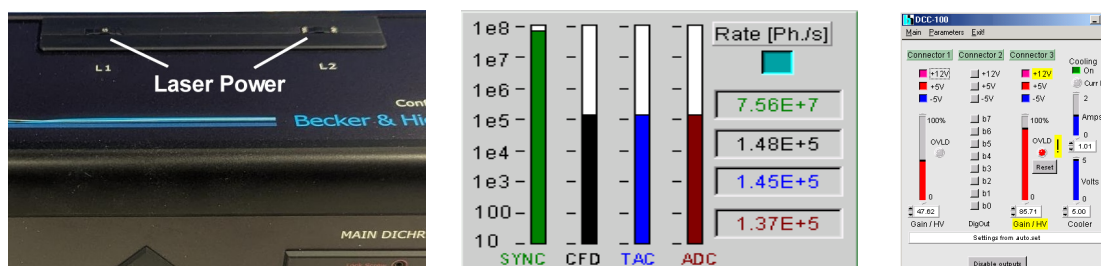


Fig. 68: Left: Wheels for controlling the laser power. Middle: Count rates. Right: Detector control panel after overload shutdown.

Stop the preview

When the correct plane is in focus, click on the Preview button again. This stops the preview. You are now ready to record the final FLIM image.

Start the FLIM Recording

Start the FLIM recording by a click on the 'Start Scan' button in the scanner control panel, see Fig. 69, left. The scanner starts working, and colour-coded lifetime images appear in the display windows of SPCM, see Fig. 69, right. With the recommended system parameter settings the system continues to accumulate photons until it is stopped by a click on the 'Stop Scan' button. The Autoscale function of the display routine will keep the image brightness on a constant level. However, as you accumulate more and more photons, the signal-to-noise ratio will become better and better. Let the process run until you are satisfied by the image quality. Then stop the recording by a click on the 'Stop Scan' button.

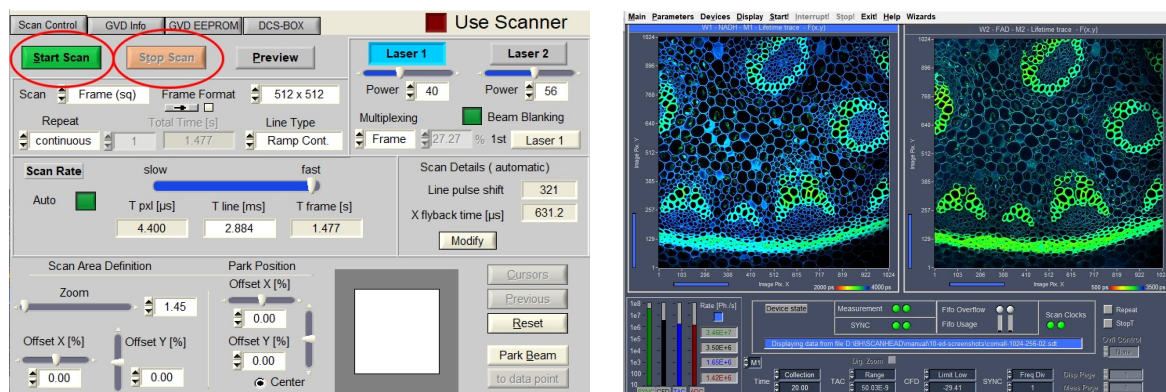


Fig. 69: Left: Display windows of SPCM during recording. Right: Stopping the recording via the 'Stop Scan' button.

Check the Decay Data

SPCM has a 'Show Decay' function to display decay data in selected spots of the images, in regions of interest, or over the entire image area. Please see Fig. 70. If you have enough screen area we recommend to keep the function active during the entire measurement and thereafter. Simple mistakes, such as shifted decay curves, wrong filters, laser leakage, or optical reflections can easily be seen in the decay data.

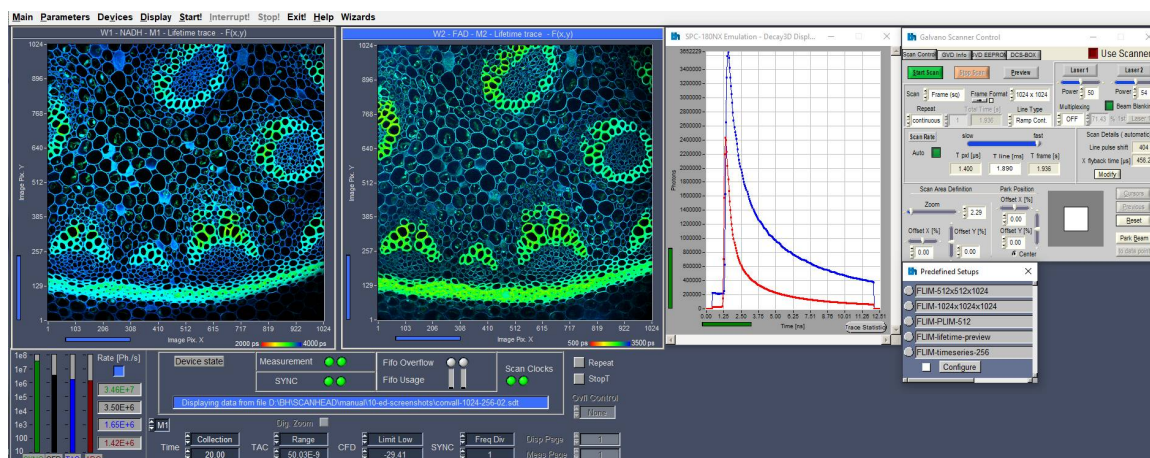


Fig. 70: Online display of decay data

Save the FLIM Data

Using the 'Save' function of SPCM, save the data into an .sdt file. Please see 'Saving and Loading Setup and Measurement Data', page 119. This step is important. It avoids that you lose your data, for example by inadvertently starting a new measurement or loading another data file. More than that: The .sdt file contains not only the FLIM data but also the system setup parameters of the measurement. By loading the .sdt file later you are able to restore all these parameters and, if necessary, repeat the measurement with exactly identical measurement parameters.

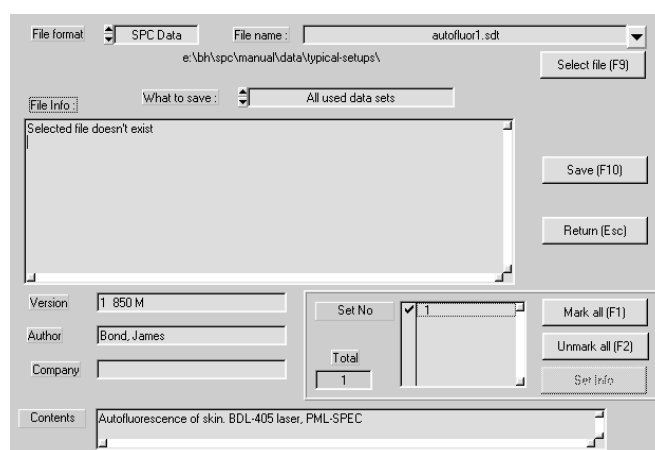


Fig. 71: Saving .sdt data

Check the Data by SPCImage NG

Right-click into the image you want to check. Select 'Send to SPCImage'. This opens SPCImage NG and sends the data to it. Initially, SPCImage shows the data as a grey-scale

image because the decay data have not been analysed yet, see Fig. 72. The lower right part of the panel shows a decay function at the position of the image cursor. Make sure that the decay function has a reasonable shape, is well centred in the display window, and has enough photons for decay analysis. If necessary, increase the spatial binning factor as indicated in Fig. 72, middle. Select the number of decay components of the fit model (Fig. 72, upper right) until you get a good fit of the data. In Fig. 72 a two-component model has been selected. When this is done, click on 'Calculate', 'Decay Matrix' to obtain a first version of the lifetime image, see Fig. 73. Please note that the image may be not final. It may still need refinements, such as adjustment of the lifetime and intensity scale. Also, you may be interested in detailed parameters of the decay function, such as component lifetimes, component amplitudes or ratios of these parameters. Please see chapter 'Analysing FLIM Data with SPCImage', page 303.

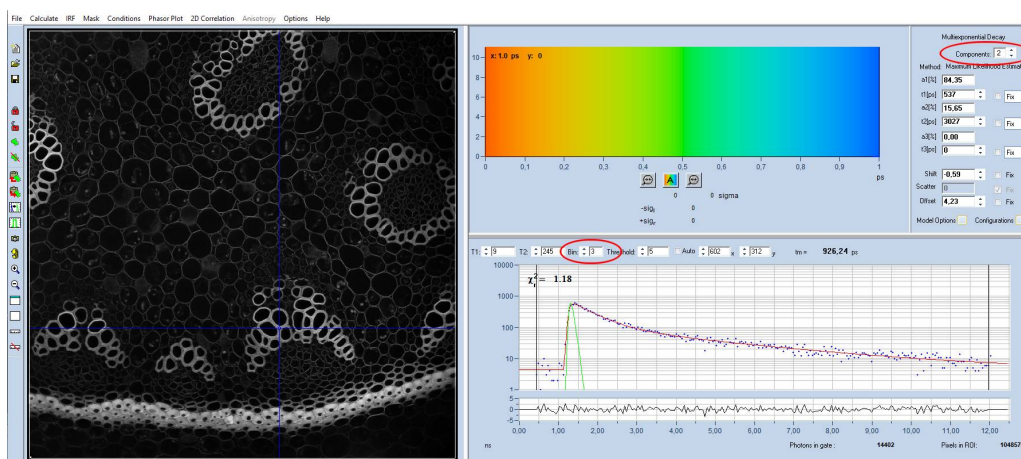


Fig. 72: SPCImage panel after receiving data from SPCM

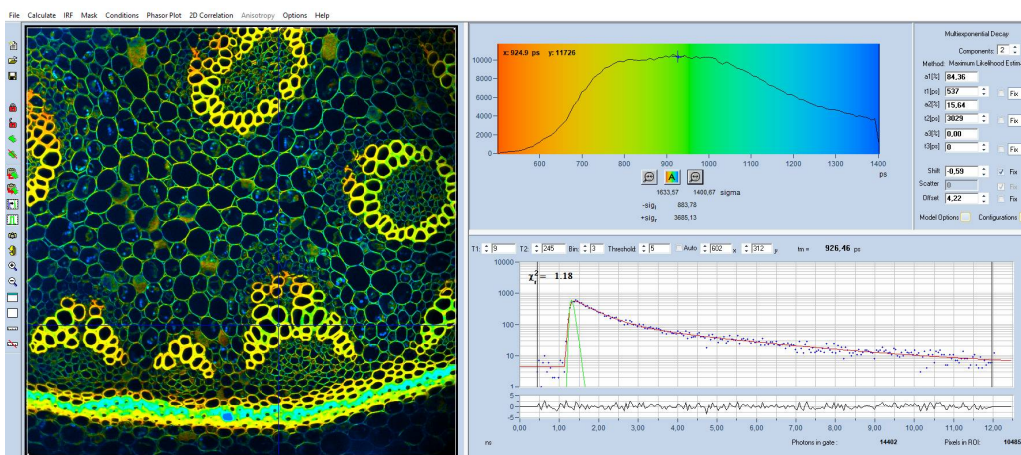


Fig. 73: SPCImage panel after data analysis

Save the SPCImage Data

Save the analysed data in SPCImage as shown in Fig. 74. The data are saved together with the fit conditions, such as model function, fit type, spatial binning factor, or IRF function as they were used in the previous analysis.

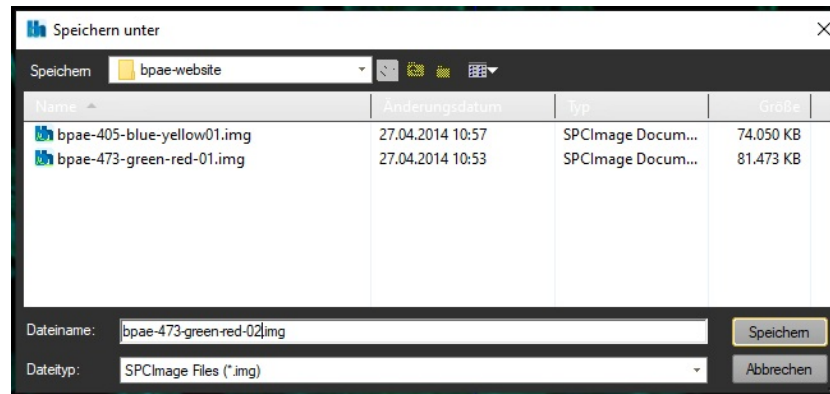


Fig. 74: Saving SPCImage data

Condensed FLIM Procedure

When you run a series of measurements of similar samples or a series of measurements in different spots of the same sample you do not have to run through all steps of the protocol described above. You also may not have to check the data of each individual measurement with SPCImage. All you have to do is this:

- Replace the sample. In a multiphoton system you may have to turn off the detectors for this step.
- Turn on the Preview Mode
- Find the correct Z position and find a good spot for the next measurement.
- Turn off the Preview.
- Start the new FLIM recording. Stop when you are happy with the images.
- Save the data

If you only want to repeat a measurement in a different spot of the same sample:

- Turn on the Preview mode.
- Find a new spot for the next measurement. Re-focus if necessary.
- Turn off the Preview
- Start a new FLIM recording. Stop when you are happy with the images.
- Save the data.

DCS-120 System Components

Scan Head

Principle

One-Photon Excitation

In a conventional fluorescence microscope fluorescence is excited by the classic one-photon process. Fluorescence is excited in a double cone throughout the full depth of the sample, see Fig. 75, left. The sharp image seen in the focal plane is therefore surrounded by out-of-focus haze from above and below this plane. A confocal microscope solves this problem by scanning the sample with a focused laser beam and detecting the signal only from a small volume element around the illuminated spot [169, 181, 407, 442]. The principle is called 'Confocal Detection', see Fig. 75, right.

However, scanning does more than that - it also avoids that light scattered in the sample causes crosstalk between the pixels. Surprisingly, this is rarely mentioned in the microscopy literature. Consider an imaging system that illuminates the entire sample at once and detects an image from the focal plane by a camera. In all pixels of this image the camera will detect scattered light not only from this pixel but from all other pixels in the illuminated area. A scanning system - scanning the sample by a focused laser beam and detecting light only from the excited spot - records only light from the current pixel. In other words, already scanning alone - without the help of confocal detection - improves the image quality dramatically [77].

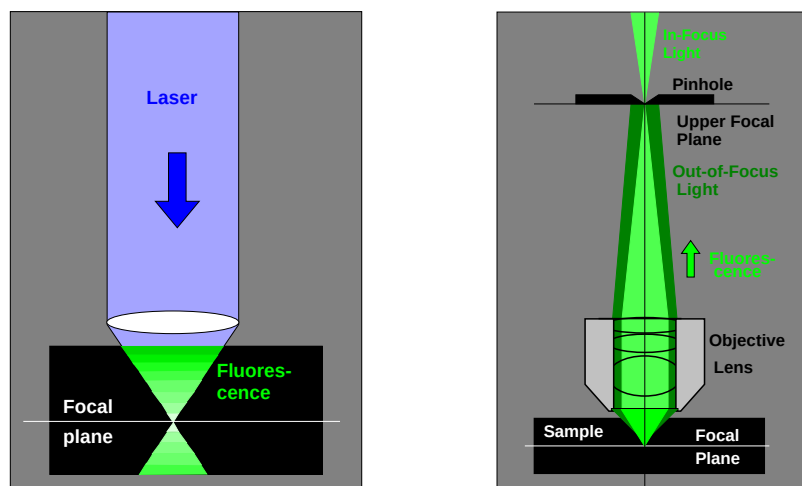


Fig. 75: Left: The laser excites fluorescence in a double-cone throughout the depth of the sample. Right: Confocal detection. A pinhole in the upper focal plane of the microscope lens suppresses light which does not come from the focal plane.

Confocal Detection

The principle of confocal detection is shown in Fig. 75, right. The basis is a pinhole in the upper focal plane of the microscope, Fig. 75, left. Consider a point source located in the focal plane in the sample and on the optical axis. The light from this point (shown bright green) will be focused into a diffraction-limited spot in the upper focal plane of the microscope. A pinhole placed in this plane and on the optical axis will transmit the light from this particular point of the sample [169, 181, 407, 442, 625]. Light coming from points above or below the focal plane (shown dark green) is focused into a plane below or above the pinhole. As a re-

sult, only a small fraction of it can pass. The pinhole thus suppresses light from sample planes that are not exactly in the focus of the microscope lens.

A system as the one shown in Fig. 75, right, does, of course, not deliver an image of the sample. To obtain an image the point in which the light is excited and detected must be scanned over the sample. Scanning can be achieved by moving the sample or by moving the light beam. Moving the sample has the merit that the point from which the system detects is always on the optical axis. The drawback is, however, that sample scanning is slow, and exerts forces to the sample. Sample scanning is therefore not used in laser scanning microscopy for biological objects. Instead, the microscopes use optical beam scanning by galvanometer-driven mirrors (often called ‘galvos’). The principle is shown in Fig. 76, left. The collimated laser beam is deflected by the galvanometer mirrors. It then passes the ‘scan lens’. This lens is the equivalent of the eyepiece of a conventional microscope. In a scanning system, it focuses the laser into the upper focal plane, which is conjugate with the focal plane of the microscope objective lens in the sample. Thus, the angular motion of the galvos scans a focused spot of laser light over the focal plane in the sample.

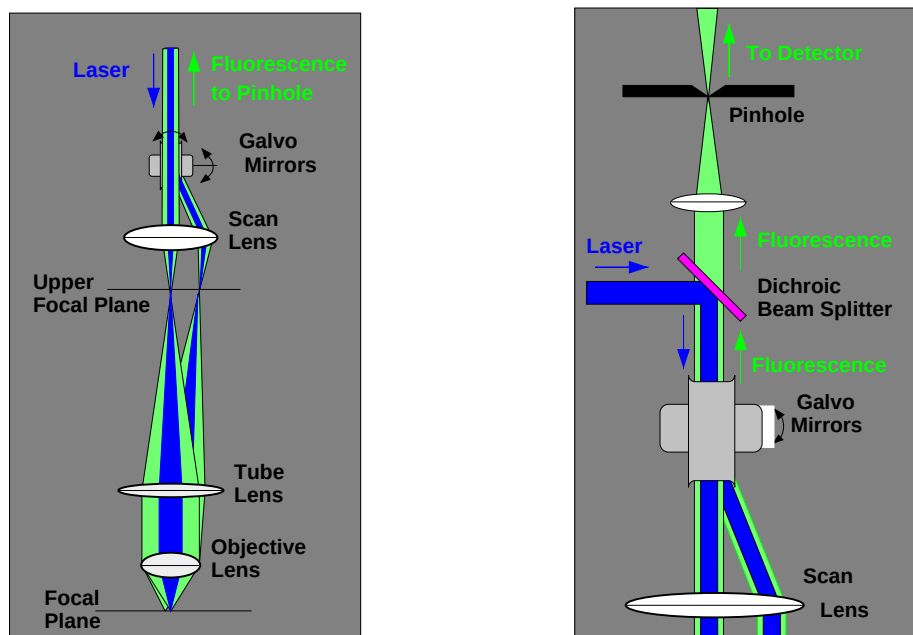


Fig. 76: Left: Scanning system. Right: Separation of excitation and fluorescence light and projection into pinhole.

Fluorescence light emitted in the focal plane is returned via the same beam path. After being reflected at the galvanometer mirrors the fluorescence signal from the focal plane forms a stationary, collimated beam of light, see Fig. 76, right. It is separated from the laser beam by a dichroic mirror, and focused into a stationary pinhole. Only light that passes the pinhole is transferred to the detector.

Multiphoton Excitation

Multiphoton excitation uses a near-infrared femtosecond laser to excite the sample by simultaneous absorption of two or more photons. Simultaneous absorption of several photons has been theoretically predicted by Maria Göppert-Mayer [255]. It has been suggested for laser scanning microscopy by Wilson and Sheppard in 1984 [629] and practically introduced by W. Denk and J.H. Strickler [179] in 1990. In most cases excitation by just two photons is used, but excitation by three photons can be used in special cases, e.g. for excitation of fluorophores with absorption wavelengths deep in the ultraviolet region.

The efficiency of two-photon excitation increases with the square of the excitation power density. It becomes especially efficient when a femtosecond pulsed laser beam is focused by a high-NA microscope lens. Due to its nonlinearity multiphoton excitation occurs only in the focus of the microscope lens, see Fig. 77, left. There is no substantial out-of-focus excitation, and, consequently, no need to suppress out-of-focus light by a pinhole. Therefore, the fluorescence light is not fed back through the beam path of the scanner. Instead, it is diverted from the excitation beam behind the microscope lens, and directly fed to the detector, see Fig. 77, right. The principle is called 'Non-Descanned Detection', or 'NDD'. Non-descanned detection does not only avoid losses on the way through the scanner beam path, it also detects photons which are scattered on the way out of the sample. Despite the photons are scattered the TCSPC process assigns these photons to the correct x-y position in the FLIM data, and a sharp image is obtained.

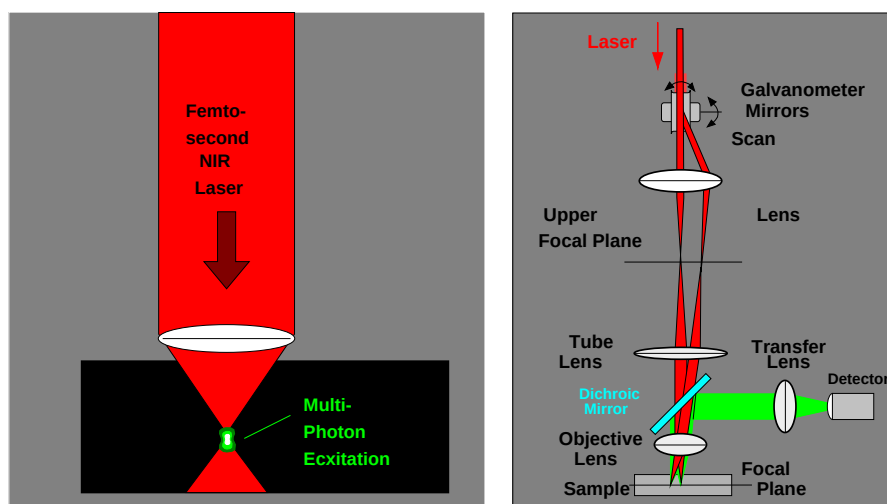


Fig. 77: Multiphoton Excitation. Left: Excitation occurs only in the focus of the laser. Right: The fluorescence light is diverted from the excitation beam path immediately behind the microscope lens and fed to a detector.

Opto-Mechanical Architecture of the Scan Head

The internal design of the DCS-120 scan head is shown in Fig. 78, a schematic drawing in Fig. 79.

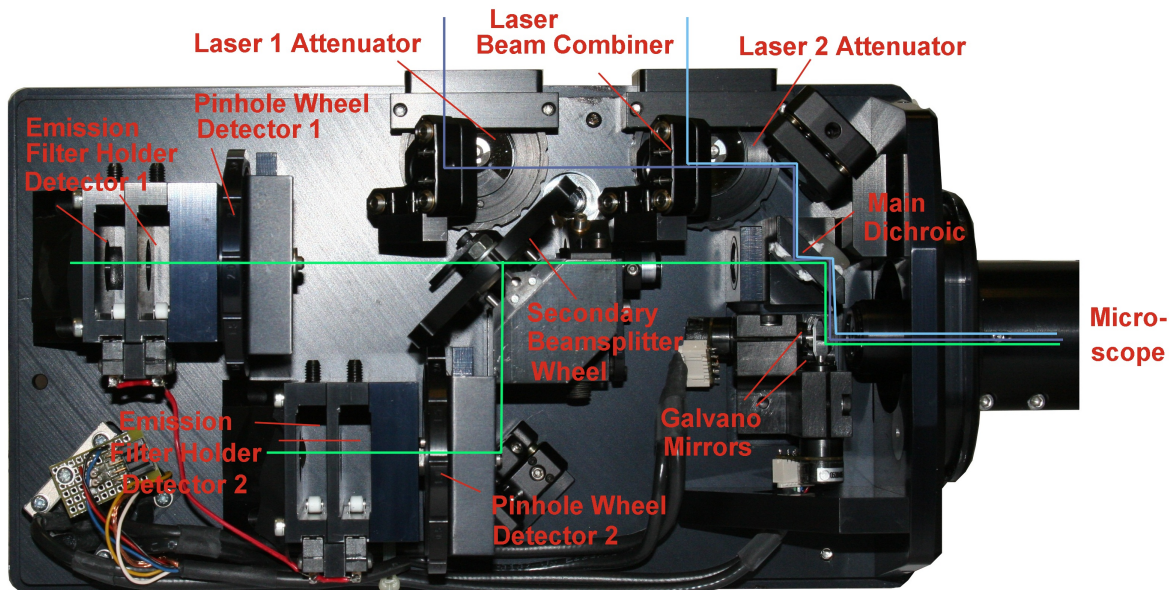


Fig. 78: Internal optics of the DCS-120 scan head

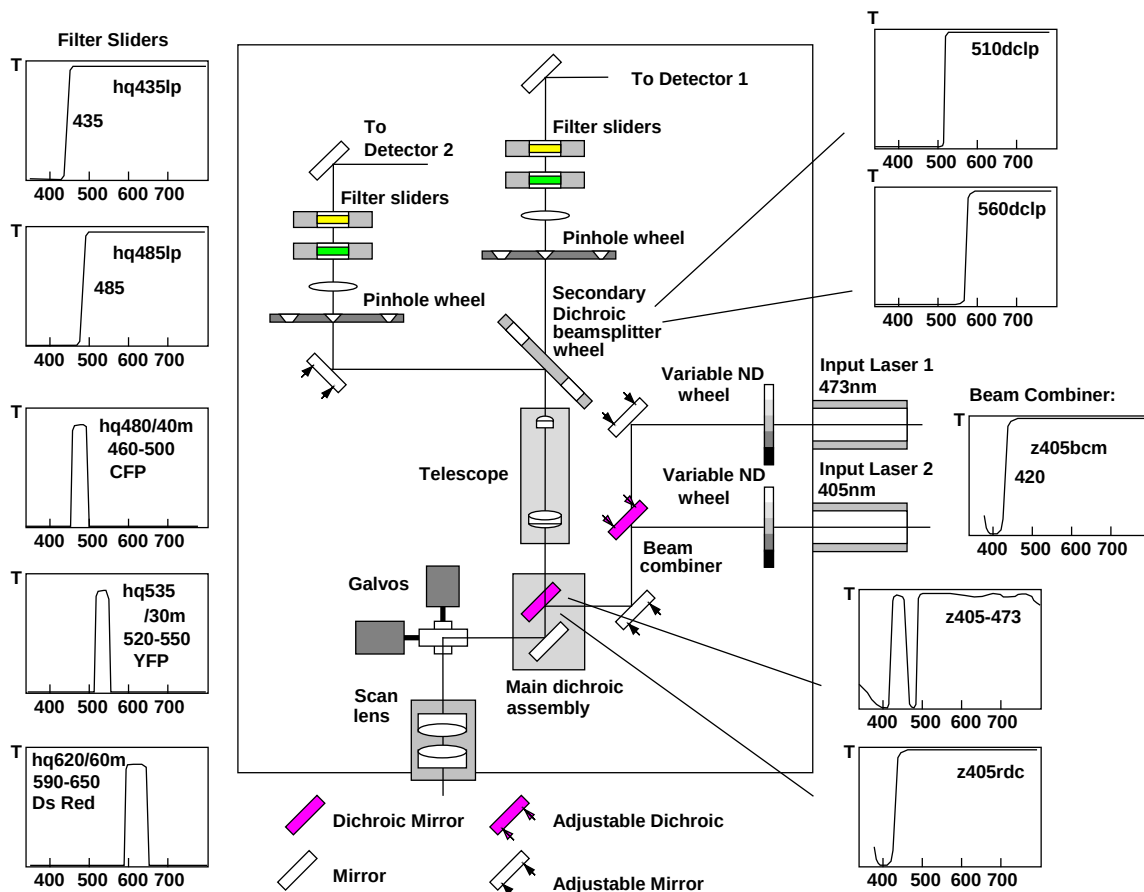


Fig. 79: Schematics of the DCS-120 scan head with filter characteristics of standard filters and dichroics

Laser Inputs

Diode lasers and other lasers for one-photon excitation are connected to the scan head via single-mode optical fibres. The DCS-120 uses special fibres that have collimators at their outputs, see Fig. 80, left. The collimators are inserted into fibre manipulators which allow the lateral position and the angular alignment to be changed in the sub- μm range see Fig. 80, middle. The adjustments are used to centre the laser beam on the back aperture of the microscope lens and to align the image of the excited spot of the sample with the pinholes. The benefit of this design is that slight realignments are possible without opening the scan head. For multiphoton excitation, the beam of a titanium-sapphire laser is coupled into the scanner by sending it through the centre of the fibre manipulator, see Fig. 80, right.

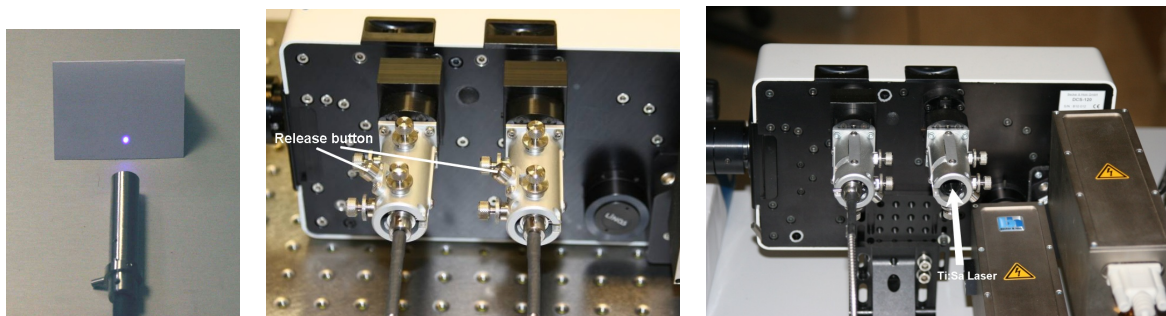


Fig. 80: Coupling of the lasers into the DCS-120 scan head. Lasers for one-photon excitation are coupled via single-mode fibres that deliver a collimated beam (left). The fibres are attached to the scanner via fibre manipulators (middle). For multiphoton excitation the beam of the titanium-sapphire laser is sent into the scanner directly (right)

Internally, both laser-input beams are combined via a dichroic mirror. A conventional mirror sends the combined laser beams down to the main dichroic beamsplitter assembly.

Laser Attenuators

Both laser input channels have neutral-density filter wheels, see Fig. 81. By these wheels the laser intensity can be varied within a range of more than 1:100.



Fig. 81: Optical power control wheels at the DCS-120 scan head

Main Dichroic Beamsplitter Assembly

The main dichroic beamsplitter separates the beam of fluorescence coming back from the sample from the excitation beam going to the sample. The main beamsplitter not only has to be perfect in terms of optical quality but also needs extraordinary mechanical stability: The fluorescence beam is projected into a small pinhole of diffraction-limited size. The slightest deviation in the angle of the beamsplitter moves the beam away from the centre of the pinhole and thus causes substantial loss in sensitivity and spatial resolution.

In DCS systems made until 2025 the main dichroic assembly consists of a solid block that holds a conventional mirror and the dichroic. The assembly is solidly fixed to the scanner base plate. The main dichroic assembly is shown in Fig. 82. A number of different main dichroics are available for the DCS-120. Customer-specific versions can be made on request. However, there is a problem: The beamsplitter shown in Fig. 82 cannot be replaced without opening and re-aligning the scanner.

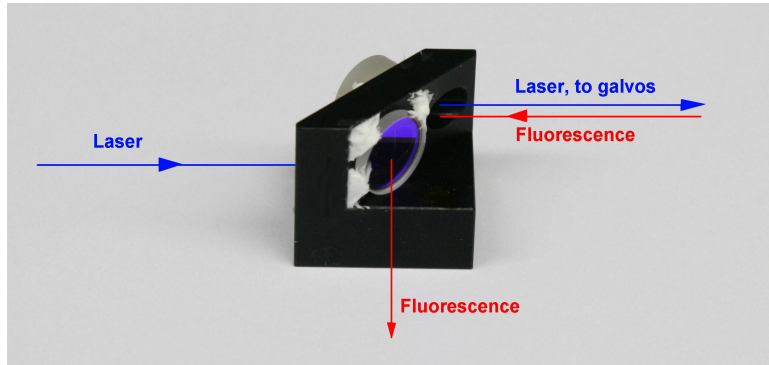


Fig. 82: Main dichroic beamsplitter assembly before 2026

To address this problem, from 2026 on a new dichroic assembly is used. It contains an additional mirror that compensates any angular movement of the fluorescence beam when the angle of the entire dichroic assembly changes. As a result, the dichroic assembly can be replaced by the user. Several beamsplitter assemblies for different wavelength and the place where the assembly is inserted in the scan head are shown in Fig. 83.



Fig. 83: Left: Main dichroic beamsplitter assemblies for different wavelength. Right: The beamsplitter assembly is located behind a flap in the cover of the scan head.

Galvanometer Mirrors

After being reflected at the main dichroic the laser beam is deflected by the galvanometer mirrors. The galvanometer mirrors are shown in Fig. 84. The mirrors have small size and, consequently, small moments of inertia. Moreover, optimised electronic control and optimised flyback trajectories minimise mechanical resonance. This way, the scanner achieves line times down to 0.6 ms and pixel times down to 0.6 μ s, see page 94.

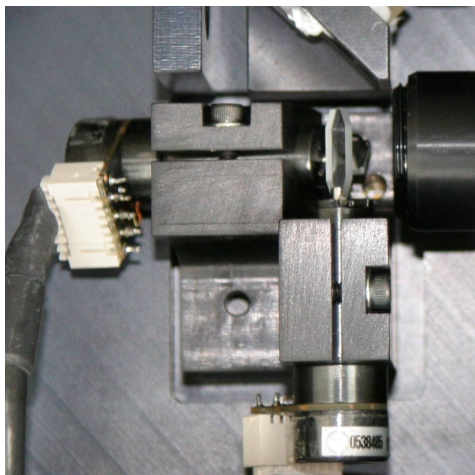


Fig. 84: Galvanometer mirrors of the DCS-120 scan head

Scan Lens

The excitation light leaves the scanner through the scan lens. The lens focuses the laser into the upper focal plane of the microscope and simultaneously projects the galvo rotation axis on the back aperture of the microscope lens (see Fig. 76, right). To bring the image plane of the scan lens into coincidence with the upper focal plane of the microscope the complete scanner can be moved in and out with respect to the microscope. The scan lens is shown in Fig. 85. It is important that the front side of the lens is kept free of fingerprints or dust. Such contaminations can cause shadowing or fluorescence artefacts in the images.



Fig. 85: Scan lens

Fluorescence Light Path

The fluorescence light returned from the sample enters the scanner back through the scan lens. The scan lens collimates the light into a narrow beam. The motion of the beam is ‘descanned’ by reflection at the galvanometer mirrors. The descanned beam passes the main dichroic. The result is a stationary beam of fluorescence light that is largely free of the laser light. The beam of fluorescence light passes a telescope that adds additional magnification to the optical path from the sample to the pinholes, see Fig. 86. The advantage of this additional magnification is that the physical size of the pinholes becomes much larger than it would be in the upper image plane of the microscope. The alignment of the optical elements behind the telescope is therefore less critical.

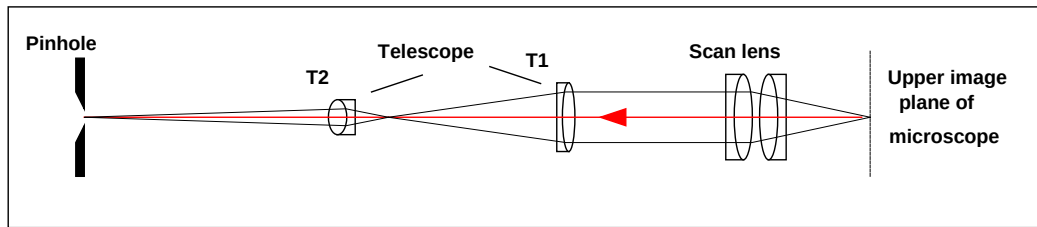


Fig. 86: Function of the telescope in the fluorescence light path. Together with the scan lens the telescope adds additional magnification to the system so that the physical pinhole sizes for diffraction-limited operation are in range from 0.25 to a few mm. Not to scale, aperture of light bundles exaggerated. Main dichroic beamsplitter not shown.

Secondary Beamsplitter

Before the beam enters the pinholes it is split into two components by the secondary beamsplitter. This is a wheel containing two dichroics, a 50:50 beamsplitter, a polarising beamsplitter, a mirror, and a glass plate. Thus, the light can be split into two different wave-length components, components of 0° and 90° polarisation, or the full signal can be sent into either detection channel.



Fig. 87: Scan head with Secondary-Beamsplitter positions

Emission Filters

Each detection channel has two positions to insert emission filter. The filters are contained in sliders which fit into corresponding slots of the scan head, see Fig. 88. Both bandpass and long-pass filters are delivered with the DCS-120 scanner. For general characteristics of the standard filters please see Fig. 79. Different filters and empty filter sliders are available on request.



Fig. 88: Emission filters. Left: Filter sliders. Right: Filter positions in DCS-120 scan head

Pinholes

The pinhole wheels contain 11 pinholes from 0.25 to 5 mm in diameter, and a ‘closed’ position. For the commonly used objective lenses these diameters correspond to approximately 0.5 to 10 Airy disc diameters in the focal plane. The effect of the pinhole size on the recorded image is shown in Fig. 89 and Fig. 90. In a thick sample (Fig. 89), the image intensity increases dramatically with the pinhole size. Simultaneously, the image definition decreases. The reason is that a large pinhole transmits photons from a larger depth interval.

In a thin sample (Fig. 90) the effect is less pronounced. Once the depth interval exceeds the thickness of the sample there is almost no further increase in image intensity. For normal FLIM experiments we recommend to start with a pinhole size of 1. If there is enough image intensity and you want to squeeze out the last bit of resolution, decrease the pinhole to 0.5. If the intensity is low and cannot be increased by more laser power, increase the pinhole size.

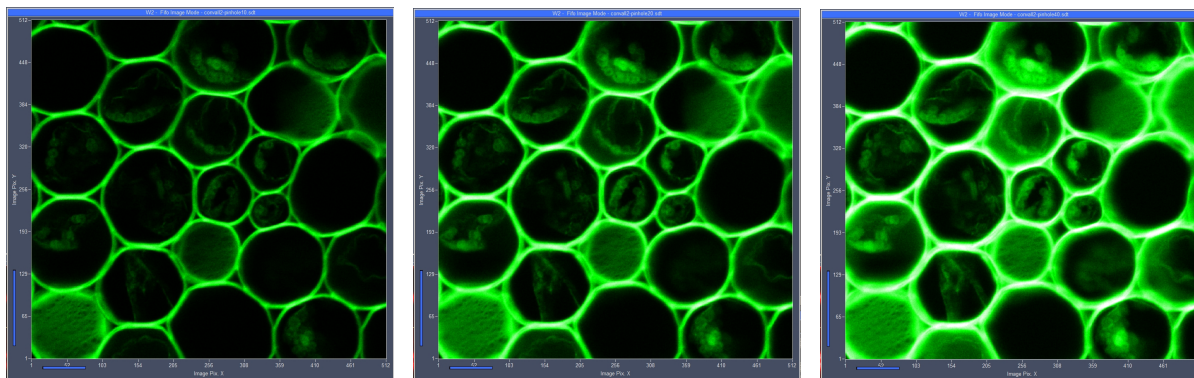


Fig. 89: Effect of the pinhole size, sample thickness about 30 μm . Left to right: Pinhole size = 1, 2, 4.

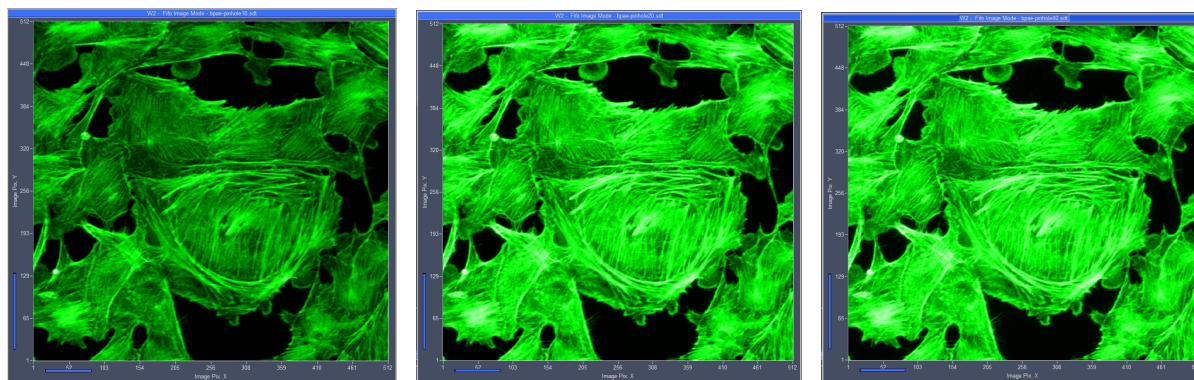


Fig. 90: Effect of the pinhole size, sample thickness about 5 μm . Left to right: Pinhole = 1, 2, 4.

Electronic Pinhole Alignment

From 2012 on the DCS-120 system has a piezo-driven pinhole alignment. The alignment is performed manually via the hand controller shown in Fig. 91.



Fig. 91: Hand controller for pinhole alignment

The two outer knobs aligns the beam in the pinhole in X and Y, the centre knob alignes it in Z. Alignment is done in the preview mode. Start with a pinhole size of 1 in both channels. Put a sample in, start the Preview mode, and focus correctly by the microscope focus drive (this is important!). Open the display parameters, and turn off 'Autoscale'. Then maximise the intensity by turning the X and Y controls. If the display gets saturated click 'Autoscale' on and off, if the count rate exceeds 10^6 reduce the laser power. When you have obtained maximum intensity optimise the Z axis (the focus into the pinhole). For best results, repeat the procedure with a pinhole size of 0.5 or 0.25, and with a smaller piezo step width, see below. When the system is aligned it stays in the selected position, even if the hand controller or the entire DCS-120 system are switched off.

The piezo hand controller has variable step width. You can change it by pushing the control knobs. We recommend to start with a step width of 10 in X and Y, and 40 in Z, see Fig. 92, left. In the final alignment steps the piezo step width can be reduced to 2 in X and Y, and 10 in Z. The hand controller has also options to change the voltage at the actuators, and the speed of the individual steps. You get into these modes by pushing a button and rotating it. We recommend not to change these settings. Please use the parameters shown in Fig. 92, middle and right. The current mode of the controller is indicated by small triangles on the left of the settings that are going to be changed. To get back to the pinhole adjust mode push the buttons until the triangles have disappeared.



Fig. 92: Piezo hand controller in step width selection mode (left) and voltage selection mode (middle), and speed selection mode (right)

Even minor misalignment has a noticeable effect on the sensitivity and the image quality. Fig. 93 shows three examples.

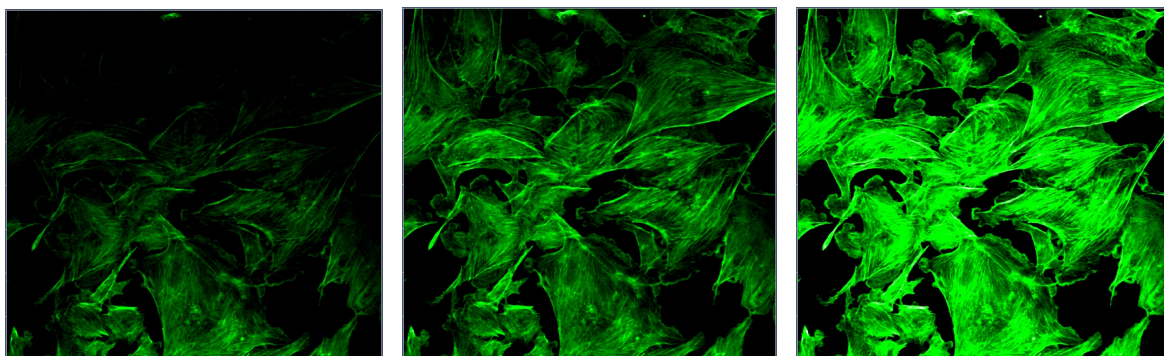


Fig. 93: Effect of pinhole alignment on the image. Left: 24 steps (12 clicks with step width 2) off in Y. Middle: 240 steps (24 clicks with step width 10) off in Z. Right: Perfectly aligned in all three axis. Pinhole size 0.5.

Scanner Control

The galvanometer mirrors of the DCS-120 scan head are controlled via a bh GVD-120 or GVD-140 scan controller card. In addition to the scanner the GVD-120 also controls two lasers, the GVD-140 four lasers. Both modules are compatible, therefore the GVD-140 can also be used in systems with two lasers.

The GVD generates the ramp signals for the x and y deflection. The signals are generated by the hardware of the GVD module, the software is only used to load different waveforms or scan parameters in the scan controller. The waveforms are therefore independent of the computer speed and of software reaction times. The DCS-120 uses a cycloid waveform for the flyback portion of the signals (see Fig. 94, right). This minimises mechanical resonances and thus allows the DCS-120 to be operated at extremely high scan rates (see Table 1 and Table 2, page 95).

The GVD module also generates the scan synchronisation pulses for bh TCSPC modules, and the beam blanking, multiplexing, and intensity control signals for the excitation lasers. The scanner-control is fully integrated in the in the DCS-120 data acquisition software. For detailed description please see 'Scanner and Laser Control', page 93. A GVD-120 card is shown in Fig. 94, left, the software control panel in Fig. 94, right. The GVD-140 card and its control panel are shown in Fig. 95.

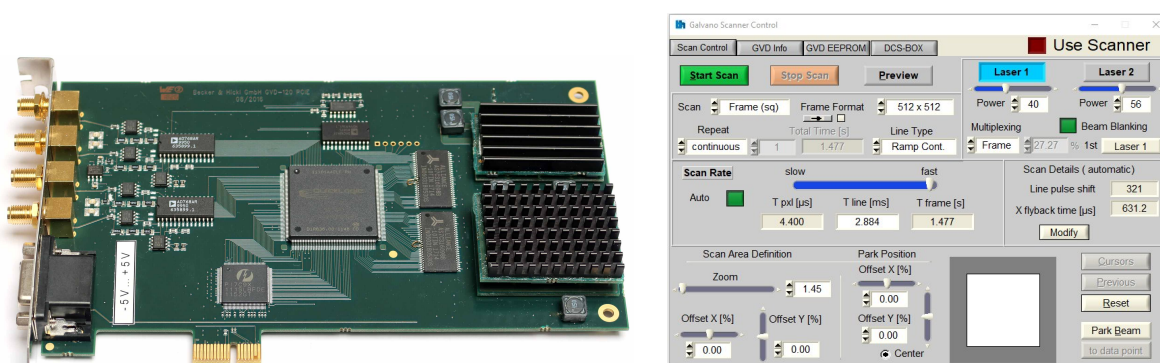


Fig. 94: GVD-120 scan controller card, PCI express version. The card controls the scanner and two lasers. Software panel shown on the right.



Fig. 95: GVD-140 Scan controller card. The card controls the scanner and four lasers. Software panel shown on the right.

GDA-120 Galvo Driver Amplifier

Driving the galvo mirrors at high scan rates requires more electrical power than a PC card can deliver. The scan signals from the GVD-120 or GVD-140 are therefore amplified by a GDA-120 dual channel galvo amplifier. The amplifier box is shown in Fig. 96.



Fig. 96: GDA-120 dual-channel driver amplifier for the galvanometer scanner

Excitation Sources

Picosecond Diode Lasers

Picosecond diode lasers provide excitation pulses in the ultraviolet and visible spectral range. They are available with wavelengths of 375 nm, 405 nm, 445 nm, 473 nm, 488 nm, 515 nm, and 640 nm [31, 32]. Lasers of longer wavelengths are available for imaging with special NIR fluorophores. Pulse repetition rates are 80 MHz, 50 MHz, and 20 MHz. The average power at 80 MHz is on the order of a few mW. This is more than enough for FLIM of biological objects. The pulse width is on the order of 50 to 80 ps FWHM, or 20 to 40 ps RMS. All diode lasers of the DCS-120 system feature excellent timing stability and power stability. Moreover, the lasers have fast on/off switching capability for phosphorescence lifetime imaging (PLIM) and for imaging of laser-induced dynamic lifetime changes.

Until 2017 DCS-120 systems used BDL-SMC and BDL-SMN series lasers [31]. In 2018 the BDL lasers were replaced with BDS-SM family lasers [32]. The electrical and optical parameters of the BDL and BDS lasers are mostly similar. However, the BDS lasers have smaller size, which makes them suitable for direct attachment to the DCS-120 scan head, see DCS-MACRO system, and as parts of the BH Laser Hub, see section below. The lasers are shown in Fig. 97. Typical pulse shapes are shown in Fig. 98.

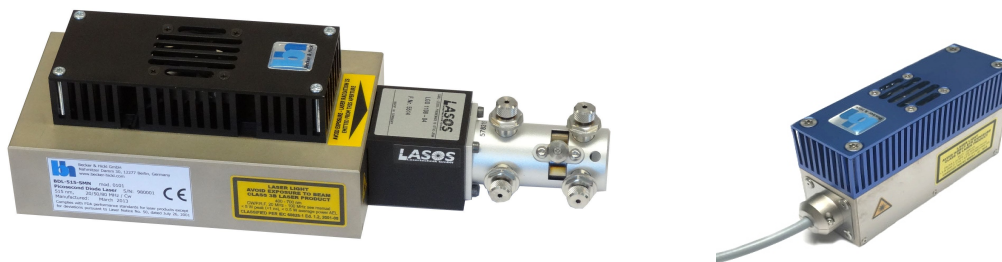


Fig. 97: BDL-SMN (left) and BDS-SM (right) picosecond diode lasers

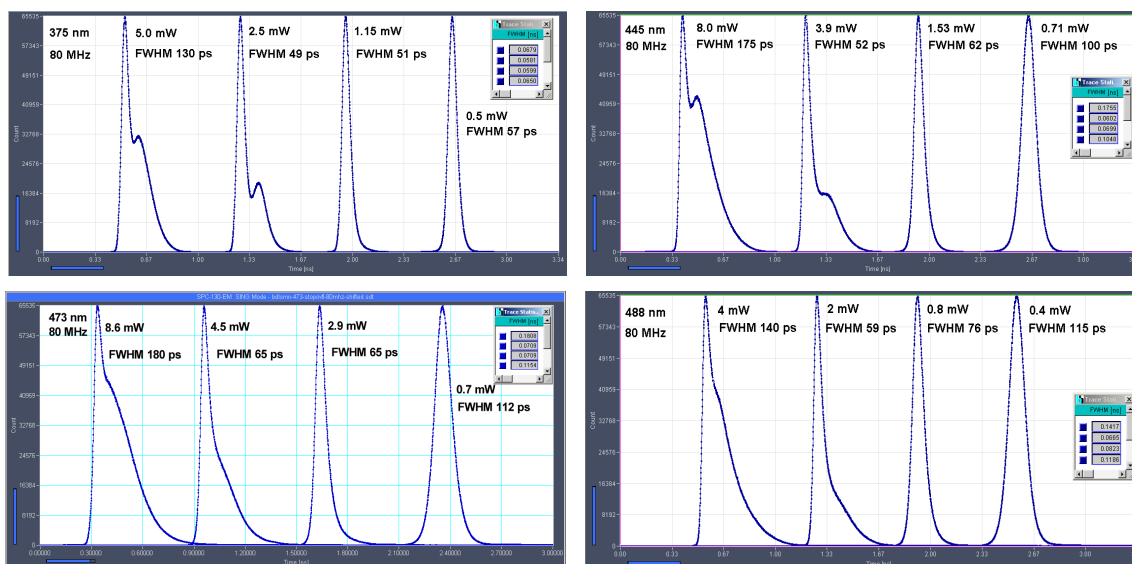


Fig. 98: BDS-SM lasers, pulse shapes for four typical laser wavelengths. 80 MHz, power measured in free beam.

LHB-104 Laser Hub

The LHB-104 'Laser Hub' [33] is used in the DCS-120 CMI molecular imaging system. It contains four bh BDS-SM picosecond-diode lasers [32]. Any combination out of 375 nm, 405 nm, 445 nm, 473 nm, 488 nm, 515 nm, and 640 nm can be used. The optical outputs of the individual lasers are combined into one single-mode output fibre. The LHB-104 Laser Hub is shown in Fig. 99.



Fig. 99: LHB-104 Laser Hub, with four lasers of 405 nm, 488 nm, 515 nm, and 640 nm. The photo shows 4-wavelength output from a single fibre. For demonstration, the beams were separated by an optical grating.

Femtosecond Fibre Lasers

The DCS-120 MP and DCS-120 DMP multiphoton systems use femtosecond fibre lasers as excitation sources. Fibre lasers are virtually maintenance-free, compact in size, and do not need warm-up time. The lasers deliver pulses of 100 to 150 fs pulse width at a wavelength of 780 nm. Wavelengths of 880 nm and 920 nm are available as well. Depending on the laser version, the repetition rate is in the range of 40 to 80 MHz.

A frequent concern is that fibre lasers are not tuneable. However, the lack of tuneability is less a problem than commonly believed. A wavelength of 780 nm not only excites NADH efficiently but also a large number of other fluorophores. Two-photon absorption spectra are broad, and excitation is obtained even if the laser wavelength does not match the absorption maximum exactly. Especially, on the short-wavelength side there is usually enough two-photon absorption to obtain reasonable excitation efficiency.

The performance of the DCS-120 MP system with a 780-nm Toptica Femto Fibre Pro laser has been demonstrated successfully, please see [87, 93, 94, 94, 96, 97] and section 'Ultra-Fast FLIM of Biological Material', page 218 of this handbook. The papers not only demonstrated the feasibility of two-photon excitation with fibre-lasers but also the fact that FLIM with fs lasers delivers extremely high time resolution. FLIM systems with fs fibre lasers and HPM-100-06 and -07 detectors deliver instrument responses of less than 20 ps FWHM, or less than 10 ps RMS [52].

Recent DCS-120 MP systems use Toptica FemtoFibre Smart lasers, preferentially with 780 nm. DCS-120 DMP dual-excitation systems use two synchronised Toptica FemtoFibre Ultra lasers of 788 nm and 920 nm. The lasers have internal AOMs for intensity control and multiplexing. Multiplexing is the basis for metabolic imaging. By multiplexing 780 nm and 920 nm, the signals of NADH and FAD can be cleanly separated, providing the basis for imaging the metabolic state of live cells, organoids, and tissues.

Titanium-Sapphire Lasers

The Titanium-Sapphire laser is the traditional excitation source for multiphoton excitation in laser scanning microscopes. The pulse width is on the order of 80 to 120 fs, the power is on the order of 2 Watts. The repetition rate is fixed to typically 80 MHz. Due to the short pulse width and high peak power high efficiency of multiphoton excitation is obtained. In contrast to fs fibre lasers, Ti:Sa lasers are tuneable over a wide range. With the ultra-fast HPM-100-06 and -07 detectors a TCSPC instrument-response function of less than 20 ps FWHM is obtained. A disadvantage is that Ti:Sa lasers cannot be modulated or power-controlled electronically. DCS-120 systems with Ti:Sa lasers therefore use an AOM (acousto-optical modulator) to control the intensity and to modulate the laser for simultaneous FLIM/PLIM. The control of the laser and the AOM is fully integrated in the SPCM data acquisition software. Please see 'Ti:Sa Laser and AOM Control', page 113.

Due to their tuneability, Ti:Sa lasers are sometimes used in the DCS-120 MP system instead of the standard fs fibre lasers. However, the main use is in the DCS-120 Super MPC combined Multiphoton / Confocal system. This system simultaneously provides fs one-photon excitation and fs multiphoton excitation via the bh SHG / AOM unit. Please see below.

SHG / AOM Unit

The SHG / AOM unit combines an AOM (Acousto-Optical Modulator) and an SHG (Second-Harmonic Generator) device in a single case. A photo is shown in Fig. 100. From a single

Ti:Sa laser beam, the SHG / AOM unit delivers a fundamental-wavelength NIR beam and a frequency doubled VIS beam. The visible-range beam is injected into an optical fibre (coupler shown left in Fig. 100), the NIR beam is delivered in a free beam (output shown right in Fig. 100).



Fig. 100: SHG / AOM Unit.

In the DCS-120 MPC system, the SHG / AOM unit provides both visible-range radiation for one-photon excitation and NIR radiation for multiphoton excitation. With these capabilities, the DCS-120 MPC system is the only system that combines tuneable multiphoton excitation and tuneable single-photon excitation in one and the same system. It is also the only system that offers sub-ns visible range excitation, and, consequently, sub-20 ps FWHM system IRF width for confocal FLIM.

Data Acquisition System

TCSPC-FLIM Modules

The DCS-120 systems came to the market in 2007. Since then, they have been constantly updated with new excitation sources, optical systems, detectors, software, and TCSPC modules. Over the time the DCS systems were around a number of different TCSPC modules have been used. Early systems used two SPC-150 modules. In 2013 the SPC-150 modules were replaced with SPC-150N modules. The SPC-150N has ultra-fast discriminators, reaching an IRF width of less than 20 ps for multiphoton systems with HPM-100-06 hybrid detectors.

Current DCS-120 systems use either two ultra-fast SPC-180 NX modules or one triple-channel SPC-QC 104 module. The SPC-180 NX and the SPC-QC 104 are shown in Fig. 101.

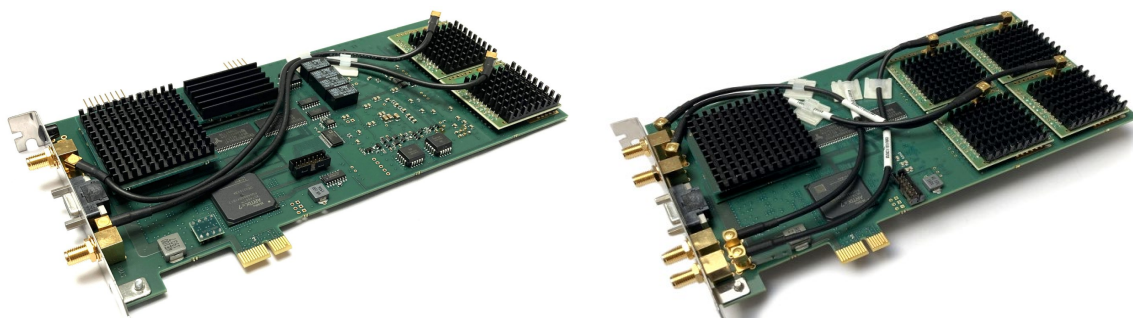


Fig. 101: SPC-180NX (left) and SPC-QC-104 (right) TCSPC/FLIM modules

A comparison of the dual-SPC-180NX system versus the SPC-QC-104 system is given in the table below.

	SPC-180NX	SPC-QC-104
IRF width, electrical, FWHM	3.5 ps	30 ps
IRF width, electrical, RMS	1.8 ps	14ps
Min. Time-Channel Width	400 fs	4 ps
Saturated Count Rate	10 MHz per module	120 MHz per channel
Discriminator Bandwidth	5 GHz	5 GHz
IRF width with fs laser and HPM-100-40	90 ps	110 ps
IRF width with fs laser and HPM-100-06	20 ps	70 ps
IRF width with ps laser and HPM-100-40	120 ps	130 ps
IRF width with ps laser and HPM-100-06	80 ps	90 ps
Preferred use for	multiphoton systems	one-photon systems

Both modules types work well both in one-photon and in multiphoton systems. The differences are mainly in the IRF width and in the saturated count rate. In terms of time resolution, the SPC-180NX has a clear advantage in multiphoton systems, where the resolution is not limited by the laser pulse width. With HPM-100-06 detectors the SPC-180 NX reaches an IRF width of 20 ps. A high-resolution multiphoton FLIM result with the SPC-180NX and the HPM-100-06 detector is shown in Fig. 24, page 14.

In one-photon systems the time resolution is limited by the laser pulse width. The IRF is therefore almost the same for the two module types. An exception is the DCS-120 MPC system. Here, the excitation light for one-photon operation is derived from a Ti:Sa laser beam by SHG. As are result, the pulses for visible-range excitation have sub-ps width.

In terms of saturated count rate the SPC-QC-104 has an advantage over the SPC-180NX. However, the importance of high saturated count rate should not be over-estimated. Count rates in excess of 10 MHz are extremely rare in molecular imaging applications. If such count

rates occur at all, than mostly in one-photon (confocal) systems. Therefore, the dual-SPC-180NX systems are used preferentially in multiphoton-excitation systems, SPC-QC-104 systems in one-photon excitation (confocal) systems.

Detectors

HPM-100 Hybrid Detectors

All DCS-120 systems use hybrid detectors. A hybrid detector consists of a photocathode, an electron acceleration system, and an avalanche diode into which the accelerated electrons are injected. The total gain is on the order of 10^6 . That means a single detected photon yields an output pulse which, after passing a fast amplifier, easily triggers the input discriminator of a TCSPC module. Hybrid detectors feature high sensitivity, high time resolution, a clean IRF shape free of tails or bumps, and low dark count rate. The most significant advantage of the Hybrid detector is the absence of afterpulsing. Afterpulsing is the major source of background in FLIM experiments. In comparison to conventional PMTs a HPM therefore delivers a substantially better dynamic range of the decay curves. Fluorescence decay parameters can be derived from a given number of photons at a higher accuracy [40, 72, 77, 320].

As usual, there is no detector that perfectly matches the requirements of *all possible applications*. Consequently, there are different versions of hybrid detectors, which differ in spectral range, IRF width, and sensitivity. An overview on the HPM-100 versions used in the DCS system is given in the table below.

	HPM-100-40	HPM-100-06	HPM-100-50
Cathode type	GaAsP	Bialkali	GaAs
Spectral range	300 to 700 nm	300 to 650 nm	400 to 900 nm
Intrinsic IRF width	90 ps	<20 ps	180 ps
Detection efficiency	50%	20%	20%

The standard detector of the DCS-120 systems is the HPM-100-40. It covers the spectral range the endogenous and exogenous fluorophores typically used in molecular-imaging applications, it is fast enough to resolve the decay profiles of these fluorophores at high accuracy, and it has an extraordinarily high detection efficiency.

The HPM-100-06 is the detector for ultra-fast decay applications. The trade-off for the fast IRF is a slightly lower detection efficiency.

The HPM-100-50 is used for FLIM with NIR fluorophores. Its GaAs cathode is sensitive up to about 900 nm but has lower sensitivity than the GaAsP cathode of the HPM-100-40. As for all NIR detectors, its dark count rate is higher than for visible-range detectors. Typical dark count rates at room temperature are about 2000 counts per second, i.e. 5 to 10 times more than for the -40 and -06 versions. However, this should not be over-estimated. Compared to conventional PMTs, SPADs, or SIPMs the effect on the signal background is negligible because HPMs have no afterpulsing background. A HPM-100-40 detector and the IRF is shown in Fig. 102.

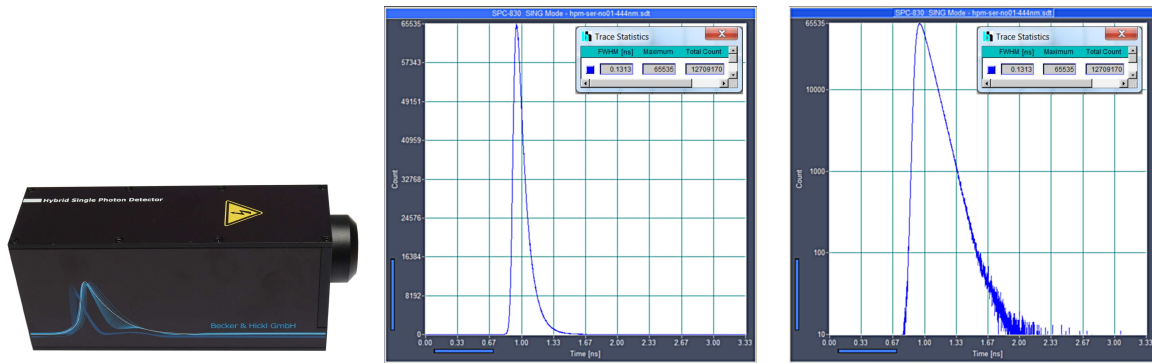


Fig. 102: HPM-100 hybrid PMT module. The housing contains the tube, the high-voltage power supplies, and a low-noise preamplifier. Middle and right: IRF of the HPM-100-40, linear and logarithmic scale.

MW-FLIM GaAsP Multi-Wavelength FLIM Detector

The MW FLIM detection system [27] detects the fluorescence simultaneously in 16 wavelength channels [69]. The optical principle is shown in Fig. 103, left. The fluorescence light is focused into the slit plane of a polychromator. The polychromator projects a spectrum of the fluorescence light on a 16-channel PMT tube inside a bh PML-16 multichannel detector [27]. Until 2013, multi-wavelength systems used the PML-16C, based on a 16-channel PMT with a conventional multi-alkali cathode. Since 2014, the multi-wavelength detector is available with a GaAsP-cathode PMT, providing about 5 times the sensitivity of the PML-16C.

Multi-wavelength FLIM uses the multi-dimensional recording process described in section 'Multi-Wavelength FLIM', page 143. For every photon, the routing electronics of the PML-16 delivers a timing pulse and a 'channel' data word. These signals are, together with the scan clocks, used by the TCSPC module to build up a multi-dimensional photon distribution over the image coordinates, the time in the fluorescence decay, and the wavelength [67, 77]. In other words, the TCSPC module 'routes' photons of different wavelength into separate life-time images. Apart from unavoidable loss in the polychromator, the recording process itself works without loss of photons. For electronic principles please see [27] or [77]. A photo of the MW FLIM assembly is shown in Fig. 103, right.

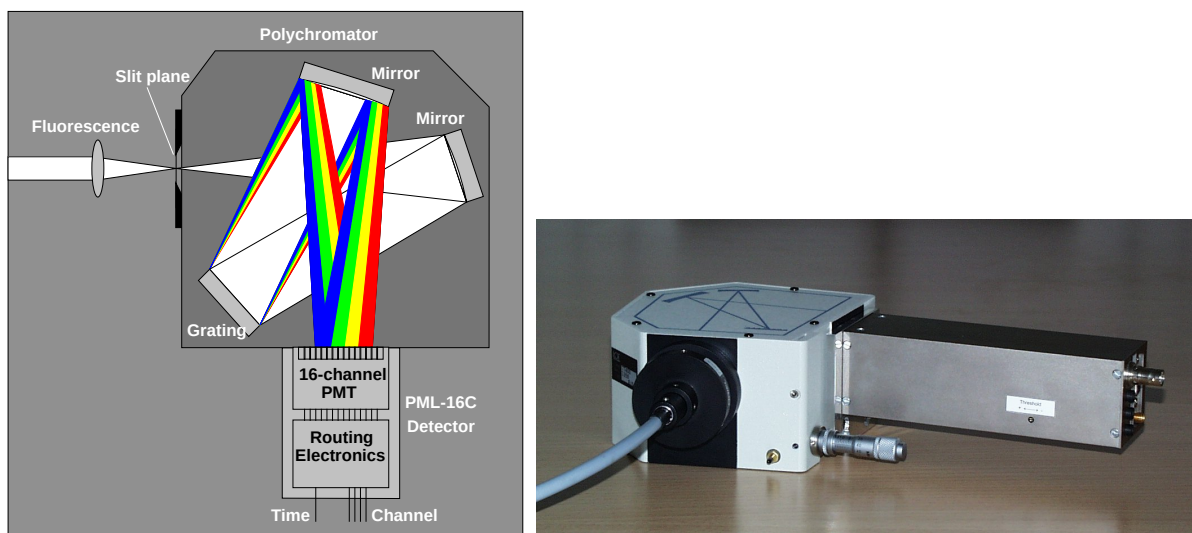


Fig. 103: Multi-wavelength FLIM detector assembly. Left: Optical principle. Right: Photo

As the other FLIM detectors, the MW FLIM detector has its own internal high-voltage generator. No external high-voltage power supply unit is required. The detector is controlled via a DCC-100 or DCU-400 detector controller module which provides for power supply, gain control, and overload shutdown.

Detector Controller

The detectors of the of standard DCS-120 systems are controlled via a bh DCC-100 detector controller card [28, 77]. The card supplies the power to the detectors, drives the coolers of actively cooled detector modules, provides software control of the detector gain and shuts down the detectors in the event of overload. A DCC-100 detector controller card and the software control panel are shown in Fig. 104.

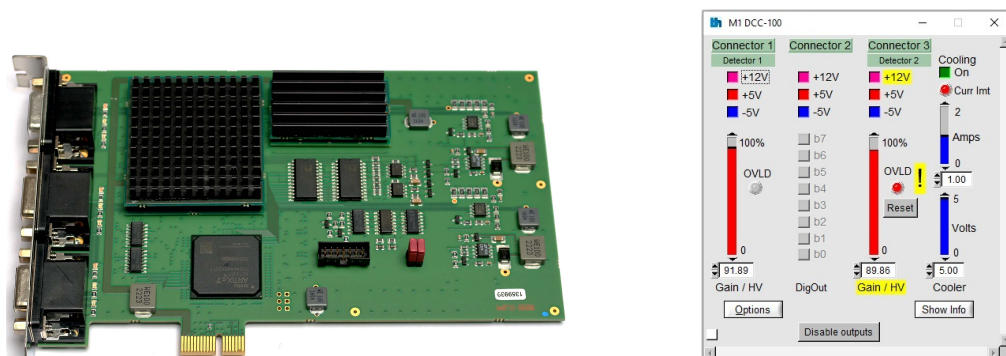


Fig. 104: DCC-100 detector controller card, PCI-Express version. Control panel shown on the right. The detector on the right is in overload shutdown.

Detectors of DCS-120 systems with more than two detectors are controlled by an external DCU-400 or DCU-800 controller module. Different than the DCC-100, the DCU modules are connected to the system computer via a USB interface. The DCU-400 controls four detectors, the DCU-800 up to eight. The detector control is integrated in bh's SPCM software. Please see 'Detector Control', page 116.



Fig. 105: DCU-400 Detector Controller with USB interface. Software panel shown right.

Important: Do not use the detector gain to control the image intensity. The detector gain is set up for efficient detection of the single-photon pulses from the detector and for best time resolution. Reducing it, e.g. to record at high signal intensity, can degrade the time resolution, the linearity, and the signal-to noise ratio. If the detected signals are too strong, reduce the laser power.

Interface Components

DCS Switch box

The DCS switch box distributes scan clock signals, routing signals, and detector and laser control signals throughout the system, and switches between different sources and destinations of these signals. It also provides trigger and marker inputs to the TCSPC modules for special FLIM modes and FLIM procedures. Moreover, it provides the power supply to the ps diode lasers, and allows the user to switch between different laser pulse repetition rates and CW operation. A photo of the DCS switch box is shown in Fig. 106.



Fig. 106: DCS switch box

GVD-BOB-02 Box

In multiphoton systems switches for the laser repetition rate and power supply to diode lasers are not needed. These systems therefore use a simplified signal distribution box. The GVD-BOB-02 box is shown in Fig. 107. The GVD-BOB-02 box does not provide the combination of routing signals of a multi-wavelength detector into the scan-signal cables to SPC Modules. This is no problem for SPC-180 NX modules because these have separate input connectors for scan pulses and routing signals. For SPC-QC modules please use the 'DCS-Switch Box'.



Fig. 107: GVD-BOB-02 for scan-signal distribution in multiphoton systems

SYNC Delay Box

we provide a USB-controlled delay switch box for the Sync signal from the lasers to the SPC modules. The box allows the user to electronically adjust the temporal relationship of the detector signals and the laser reference pulses [77]. The delay box is also able to switch between two sources of the Sync signal. Such changes may be required if the FLIM system is used with different lasers, with different detectors, or in different optical configurations. Setups for the different configurations can then be defined, and be stored in the 'Predefines Setup' panel of the SPCM software. The correct Sync delay is set when the setups are loaded. The delay box is shown in Fig. 108. It is accessed via the 'Sync' settings in the main panel, or via the Sync section of the system parameter panel.



Fig. 108: Delay switch box. It is used to set the correct temporal relationship between the detector signals and the synchronisation signals from the lasers. The correct Sync delay for different system configurations is set by loading setup data via the SPCM software.

SPCM Data Acquisition and Control Software

The SPCM data acquisition and control software is an integral part of the DS-120 system. It controls the scanner via the GVD-120 or GVD-140 card, it controls the detectors and the lasers, it configures the TCSPC module(s) for the desired type of measurement, starts and stops the measurement, and reads the single-photon data from the TCSPC modules. It builds up images or other results from the photon data stream and displays the data as intensity images, colour-coded lifetime images, decay curves, or correlation curves. It records spatial mosaics of FLIM data, Z-stacks, time series, and multi-wavelength data from the MW FLIM detector [77]. This section gives an overview on the SPCM software functions related to the DCS-120 system. A typical configuration of the SPCM main panel is shown in Fig. 109.

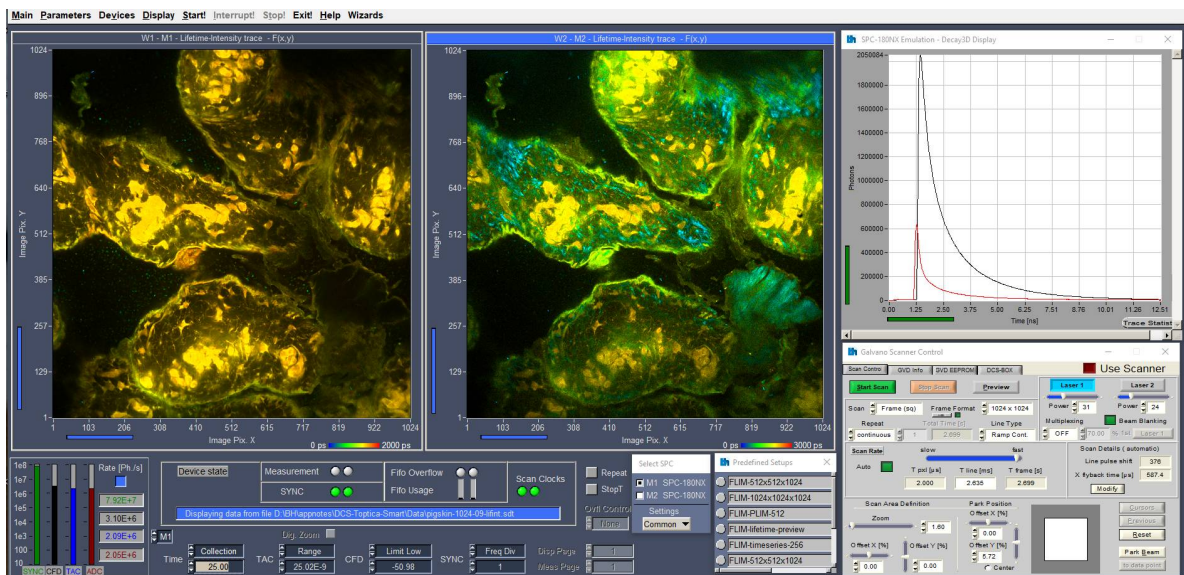


Fig. 109: Main panel of SPCM Data acquisition and control software. DCS-120, FLIM images from two parallel channels, decay curves in ROIs, scanner control panel

User Interface

The DCS-120 confocal and multiphoton FLIM systems are complex multi-modal systems which acquire data in a complex multi-parameter space. The data produced by the systems can be fluorescence lifetime (FLIM) Images, single fluorescence decay curves, multiple decay curves for different wavelength or different times after the start of an experiment, FCS curves, fluorescence decay data along a line in the sample, fluorescence and phosphorescence intensity images, multi-wavelength lifetime images, or even arrays of lifetime images for different sample position in x, y or z, or different experiment times after the start of an experiment or times after a periodic stimulation of the sample. To run all these different experiments and to display the results in a user-compatible way the bh SPCM software has a user interface which is configured according to the particular operation mode of the TCSPC system and the requirements of the experiment the user is performing. There are up to eight display windows for TCSPC/FLIM data. The data in the windows may be fluorescence decay curves, time-series of decay curves, decay and FCS curves, images from different TCSPC channels or within different time gates of the same TCSPC channel, images within different wavelength intervals of a multi-wavelength detector, or arrays of images from different spatial locations or different times within an experiment or two-dimensional projections of such data. Examples are shown in Fig. 110.

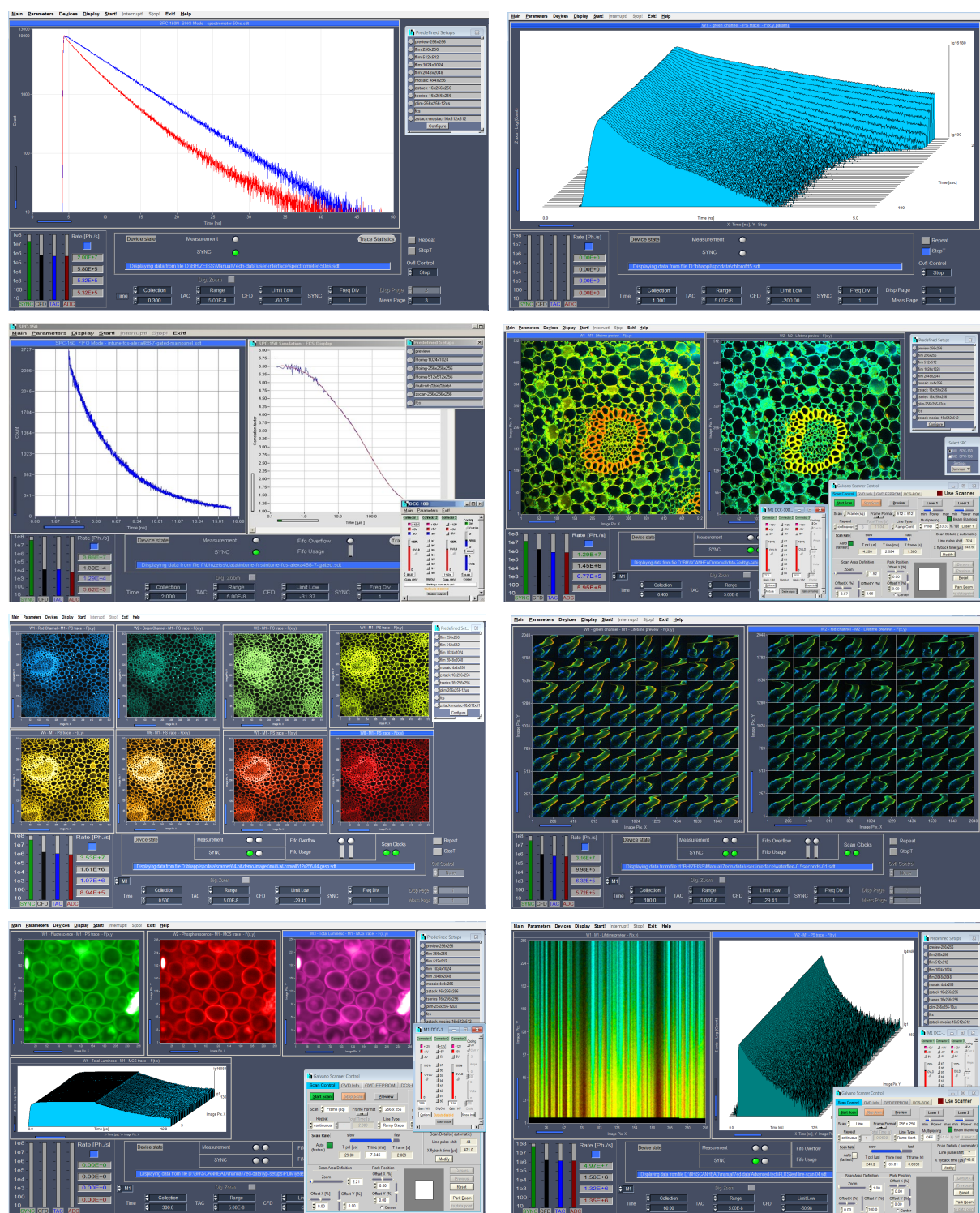


Fig. 110: Examples of user interface configurations of the bh SPCM software

Basic Measurement Control

Predefined Setups

Setup parameters for different FLIM formats, different measurement procedures or personal user-interface configurations can be loaded from the 'Predefined Setups' panel. Please see 'Switching Between Different Instrument Configurations', page 108, and 'Creating Predefined Setups', page 121.

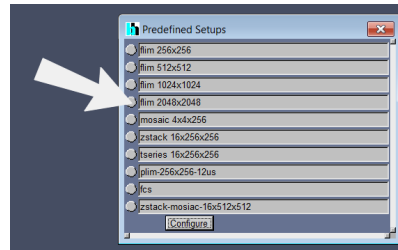


Fig. 111: Loading a setup from the 'Predefined Setup' panel

Access to Frequently Used Measurement Parameters

Frequently used measurement parameters are accessible via the status information field at the bottom of the main panel. The parameters are slightly different for the SPC-180N and the SPC-QC-104. The parameters field for a dual-SPC-180N system is shown in Fig. 112, the parameter field for the SPC-QC-104 in Fig. 113.

Timing Control Parameters are accessible via the 'Time' field. Collection Time is the acquisition time for the measurement, Display Time is the period at which intermediate results are displayed, and 'Repetition Time' is the time after which a measurement is repeated, e.g. for recording a time series.

The timing parameters work in conjunction with the Repeat and Stop T options in the upper right. When Stop T is set (upper right) the measurement stops after a defined Collection Time (lower left). A measurement can be repeated (upper right) after a defined Repetition Time (lower left). During FLIM acquisition, intermediate results are displayed in intervals of Repetition Time.

The 'TAC' or 'TDC' parameters determine the time scale of the measurement and the location of the observation-time interval within the laser pulse period. The parameters are different for the SPC-180N and the SPC-QC-104. For the SPC-180N the parameters include a 'TAC Range', 'TAC Gain', a 'TAC Offset', and two 'Limit' parameters. For the SPC-QC-104, there are a 'Range' parameter, an 'Offset' parameter and a 'Limit Low' parameter. The timing parameters are set during the installation of the system. Unless you have to change the time scale of the FLIM recording (see Ultra-Fast FLIM of Biological Material, page 218) they need not normally be changed.

Under 'CFD' and 'Sync' the discriminator thresholds and zero-cross levels for the detector and synchronisation inputs are accessible. Additionally, the 'Sync' parameters contain a frequency divider setting (used for recording data over several laser pulse periods) and the Delay of the Sync pulses in an external delay switch box (used for adjusting the proper sequence of detector and synchronisation pulses).

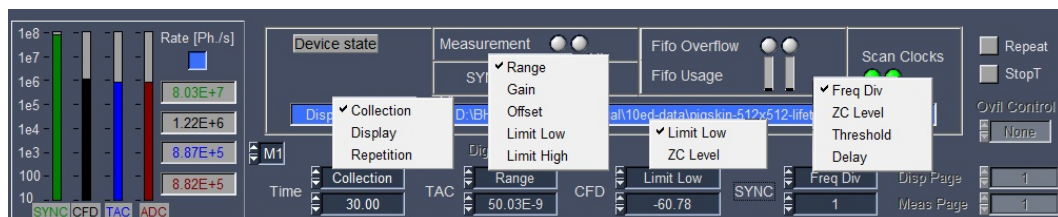
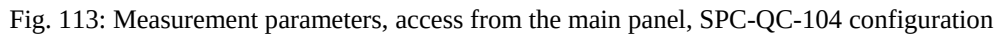


Fig. 112: Measurement parameters, access from the main panel, SPC-180N configuration.



Count rates are important indicators of the efficiency of a FLIM experiment, including the sample itself, the excitation and imaging conditions, laser power and laser wavelength, filter wavelengths, pinhole sizes, and the TCSPC process running in the TCSPC / FLIM system. The count rates are displayed in the status window of main panel of the SPCM data acquisition software, see Fig. 114 and Fig. 115.

The CFD, TAC and ADC rates indicate the rate of the detected, converted, and stored photons, respectively [77]. Extremely low detected count rates indicate that either the sample does not behave as expected, or something is wrong with the imaging conditions. Large differences between the CFD, TAC and ADC rates indicate that not all of the detected photons contribute to the buildup of the lifetime image. That means something in the TCSPC parameter setup is not optimal. The count rates are displayed for one module only. To switch from one module to the other use the Module Select panel, see Fig. 196, page 112.

The equivalent count-rate panel for the SPC-QC-104 is shown in Fig. 115. The Sync rate is shown on the right, the detection rates on the left. The standard SPC-QC-104 has three input channels, two of which are used for the detection channels of the DCS-system. The third may, however, be used in customer-specific systems with an additional spectral channel or a special SHG channel.



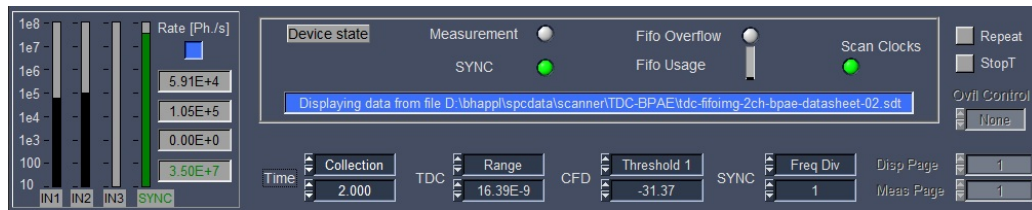


Fig. 115: Synchronisation and photon count rates in an SPC-QC-104 system

Status Information

Device State

The 'Device state' field of the SPCM main panel (see above, Fig. 114 and Fig. 115) displays information about the status of the SPC module. It shows the presence of the SYNC signal from the laser and the scan clocks, and shows whether or not a measurement is running. The information displayed includes progress of the measurement, the cycle number in a time-series, or the number of photons recorded in the FIFO or FIFO Imaging mode.

Measurement Status

The 'Measurement' indicator(s) turn on when a measurement is running. They are yellow when the system is armed for a measurement but a condition for the start of the measurement is not fulfilled yet. It may be that the system is waiting for an experiment trigger, for a frame, line or pixel clock, or for the end of the 'Repeat Time' in a series of measurements.

Status of the Scan Clock Signals

The 'Scan Clocks' lamps turn on when all scan control pulses are present. To display the state of the individual clocks, move the mouse cursor on the 'Scan Clocks' lamp. This opens a window with indicators for the individual clocks.

The software checks the Scan Clock status every one second. For frame rates slower than one frame per second it may therefore happen that the frame clock status changes between red and green. This does not indicate a malfunction, it just means that the software does not see a frame clock every one second.

FIFO Overflow

FIFO overflow occurs when the computer is not able to read the photon data from the internal first-in-first-out (FIFO) buffer at a speed higher than the rate at which the photons are detected. The FIFO then runs full, photons and scan clocks are lost, and the software is no longer able to build up correct lifetime images. The current state is displayed by the 'FIFO usage' bars. FIFO overflow is rarely a problem with recent software versions and state-of-the-art computers. Should FIFO overflow occur, the reason is usually excessively high count rate by high laser power or incomplete suppression of the laser wavelength by wrong or missing emission filters. Another reason may be that too many online-display functions are active. In that case, turn off computation-intensive online functions, such as online-lifetime display or decay-curve display from extremely large images.

Starting and Stopping a Measurement

A FLIM recording is started and stopped via the 'Start Scan' button in the Scan control panel. Please see Fig. 116, left. In addition, a measurement can be started or stopped by a click on the 'Start' or 'Stop' button in the SPCM main panel, see Fig. 116, right.

Stopping a measurement

The photon rates in FLIM measurements are vastly different, and so are the acquisition times required to reach the desired lifetime accuracy. It is therefore recommended to watch the buildup of the FLIM data by the display function of SPCM and stop the measurement when a sufficiently high signal-to noise ratio has been reached. To stop a measurement manually, click on the 'Stop Scan' button in the scan control panel (Fig. 116, left) or on the 'Stop' button in the top bar of the SPCM main panel.

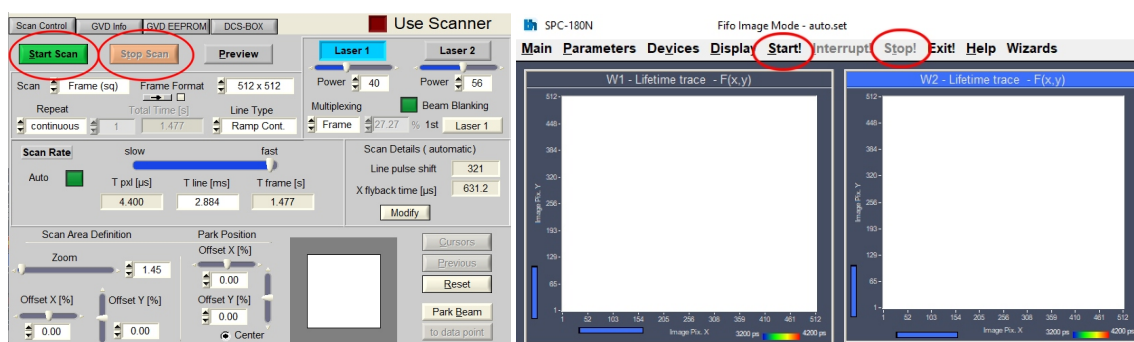


Fig. 116: Starting and stopping a measurement. Left: Via the Start and Stop buttons of the SPCM main panel. Right: Via the Start Scan and Stop Scan buttons of the scan control panel.

'Stop on T' Option

Stopping a measurement manually is not an option when you are running a series of measurements the intensities of which are to be compared. It also does not work for a time-series of measurements. To run a measurement with a defined acquisition time, activate the 'Stop T' button and define a 'Collection Time' in the measurement-control part of SPCM main window (see Fig. 117).

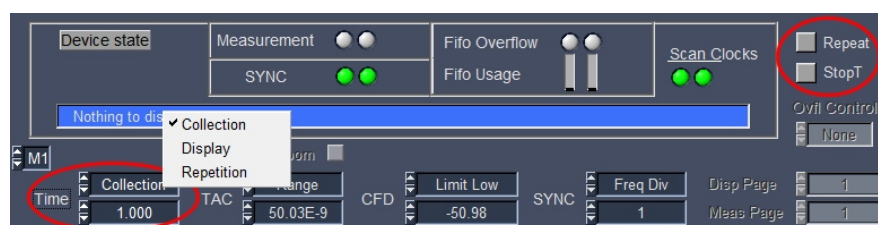


Fig. 117: Measurement Control Parameters at the bottom of the main panel of SPCM

Start-Stop Synchronisation with the Scanning

The start and the stop of a FLIM acquisition is automatically synchronised with the scanning. No matter whether you start the FLIM system first or the scanning: The measurement will not start until the TCSPC system receives the first frame clock pulse from the scanner. When you stop the measurement the FLIM acquisition continues until the scanner has finished the current frame. Consequently, the FLIM data always contain a number of complete frames. Horizontal steps in brightness caused by recording incomplete frames result are thus avoided.

It can happen that the scan stops within the acquisition time of the FLIM system. The reason can be that the scanning has been stopped without stopping the measurement, or a limited number of frames have been defined in the 'Acquisition' window of the ZEN software. In that case, the FLIM system will wait for the current frame to be completed. This does not happen,

however, and so the measurement does not complete. A corresponding message will be displayed in the status window. A second click on the 'Stop' button resolves the conflict by forcing an unconditional stop of the measurement. The FLIM data obtained in this situation are correct. However, if the scan stopped within a frame the image may contain a horizontal step in brightness. Please note that this has only aesthetic consequences: The decay data in the image are correct.

Preview Function

The DCS-120 systems have a 'Preview' function for sample positioning, focusing, finding the optimal excitation power, pinhole sizes, and other experimental parameters. The 'Preview' is started by a click on the 'Preview' button of the Scanner control panel, see Fig. 118, upper right. It is stopped by clicking the Preview button a second time.

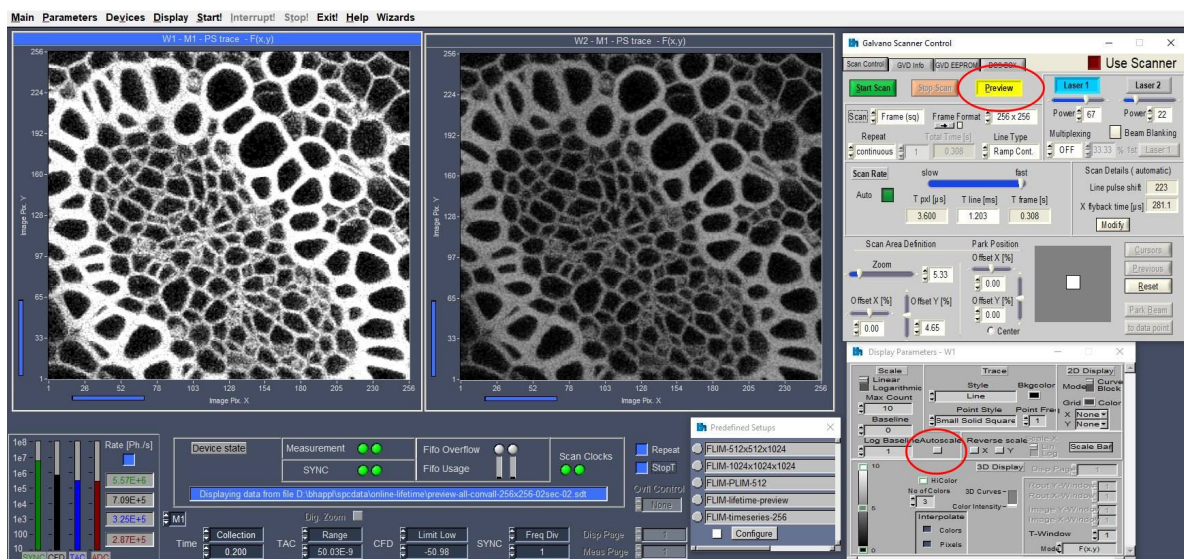


Fig. 118: SPCM software in the Preview mode.

The preview mode repetitively displays images at the maximum speed the scanner can run. The images are intensity images with a format of 265 x 256 pixels. The data are used for image display only, they do not contain decay data, they are not stored, and they cannot be saved as normal FLIM data.

When the preview starts the display parameters are in the 'Autoscale' state. This guarantees that an image is displayed (even if it contains only noise) under all conceivable count-rate conditions. Once you have found a promising area in the sample and a reasonable focal plane we recommend to turn off 'Autoscale', as shown in Fig. 118, lower right. Then fine-adjust the focus to obtain maximum intensity from the image details you are interested in. When this is done, stop the preview and start the final FLIM acquisition.

Display of FLIM Data

FLIM data are multi-dimensional photon distributions. In the basic FLIM applications the data are arrays of pixels, with each pixels containing photon numbers for consecutive times after the excitation pulses in consecutive time channels. In SPCM such data can be displayed in various ways. The usual way to display the data is as intensity images or as colour-coded lifetime images.

Intensity images represent the total number of photons in the individual pixels of the image. They are generated by summing up the photon numbers in consecutive time channels of each pixel and displaying this number as the brightness of the corresponding image pixel. An intensity image can be assigned a colour. The colour is usually selected according to the spectral range of the detector channel in which the photons were recorded. An example is shown in Fig. 119.

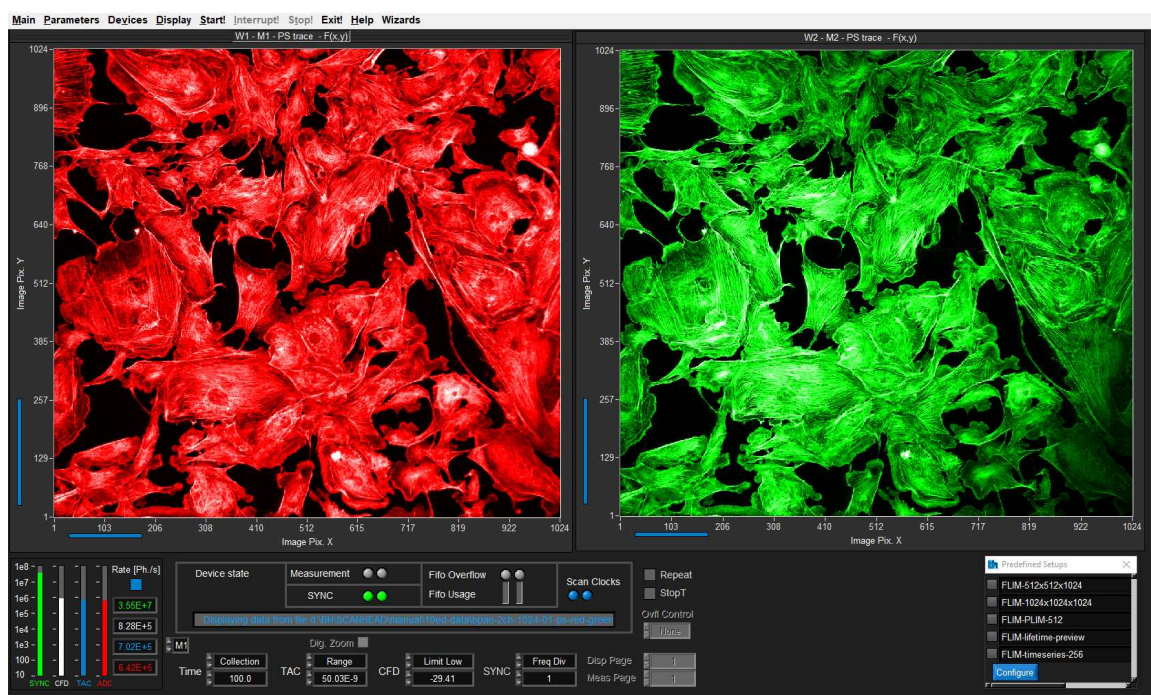


Fig. 119: Intensity image generated from TCSPC-FLIM data. Brightness represents intensity / photon counts in the pixels. Colour is assigned to the images by the user. Here, the long-wavelength channel is displayed red, the short-wavelength channel is displayed green.

Colour-coded lifetime images represent the fluorescence lifetime in the individual pixels of the image. Lifetime images are generated by calculating the first moment of the photons over the time channels of the pixels, subtracting the first moment of the IRF, and displaying the result as colour. The brightness comes from the total number of photons in this pixel. An example is shown in Fig. 120.

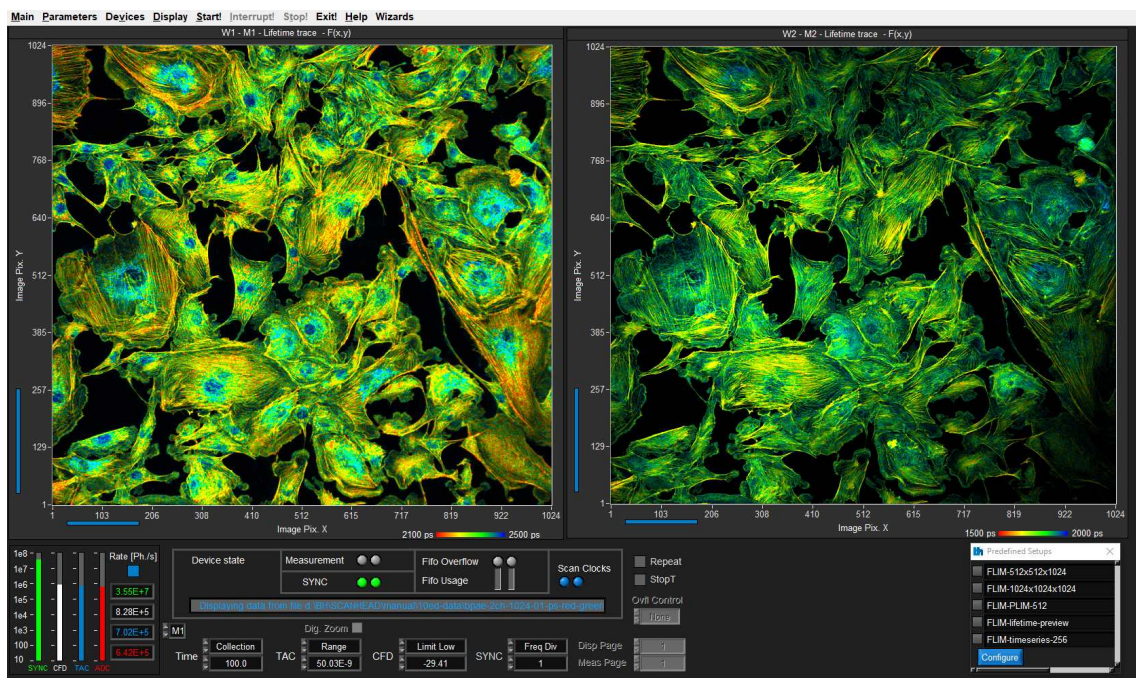


Fig. 120: Lifetime image generated from TCSPC FLIM data. Colour represents the fluorescence lifetime, brightness represents intensity / photon counts.

In early times of TCSPC FLIM intensity images were the only way to display FLIM data online. The computation power available at this time was simply insufficient for online-display of colour-coded lifetime images, especially when these contained large numbers of pixels and time channels. Online-lifetime display became available in 2015, when 64-bit Windows and multi-core processing provided the necessary computation power [47]. Since then, online-lifetime display has become the standard of FLIM display. Whether you use intensity or lifetime display is merely a matter of personal preference.

Configuring the FLIM Data Display

3D Trace, Window and Display Parameters

TCSPC-FLIM data are multi-dimensional photon distributions. In the simplest case, a FLIM image is a three-dimensional data array. The data represent an array of pixels, each of which contains fluorescence decay data in form of photon numbers for consecutive time channels in the laser pulse period. Please see Fig. 1 and Fig. 2, pages 1 and 2 of this handbook.

However, FLIM data can contain additional dimensions. These may be the wavelength of the photons, the time within the period of a stimulation of the sample, or the Z coordinate of a FLIM Z stack. The display of such data is controlled by the '3D Trace Parameters', the 'Window Parameters' and the 'Display Parameters'. The function of these parameters is described in the sections below.

3D Trace Parameters

The 3D trace parameters define how many display windows are opened, which kind of data are displayed in the individual windows, from which TCSPC module the data are taken, and in which way multidimensional data are projected into the individual display windows. Examples are shown in Fig. 121 and Fig. 123.

Fig. 121 shows a 3D trace parameter setup for displaying images of different data type. The first two windows, W1 and W2, display intensity data of ps FLIM data from two TCSPC

modules, M1 and M2. The next two windows, W3 and W4, display the same data as lifetime images. W5 and W6 display MCS mode (PLIM) images from M1 and M2. Finally, W7 and W8 display intensity images from the direct counter channels of two SPC-180N modules.

Window	Name	Active	Type	Module	Bank	Disp Page	Disp Mode	Route	X-Wind	Y-Wind	T-Wind	Image	X-Wind	Y-Wind
W1	green channel	☒	PS	M1	0	1	F(x,y)	2	1	1	1	1	1	1
W2	red channel	☒	PS	M2	0	1	F(x,y)	1	1	1	1	1	1	1
W3	green channel	☒	LIFET	M1	0	1	F(x,y)	1	1	1	1	1	1	1
W4	red channel	☒	LIFET	M2	0	1	F(x,y)	1	1	1	1	1	1	1
W5	green channel	☒	MCS	M1	0	1	F(x,y)	1	1	1	1	1	1	1
W6	red channel	☒	MCS	M2	0	1	F(x,y)	1	1	1	1	1	1	1
W7	green channel	☒	INT	M1	0	1	F(x,y)	3	1	1	1	1	1	1
W8	red channel	☒	INT	M2	0	1	F(x,y)	2	1	1	1	1	1	1

Fig. 121: 3D Trace parameters for the display of FLIM intensity images, FLIM lifetime images, MCS (PLIM) images, and intensity images from the fast counter channels of two SPC-180 modules

For all definitions shown in Fig. 121 the ‘Display mode’ is F(x,y). That means, the data shown in the individual display windows are intensity distributions over the two spatial coordinates, x and y.

However, this is not the only way the data can be displayed. FLIM data are three-dimensional x-y-t arrays. That means, when the FLIM results are displayed different projections of the data can be used. The way the data are projected is selected by ‘Display Mode’. The principle is shown in Fig. 122.

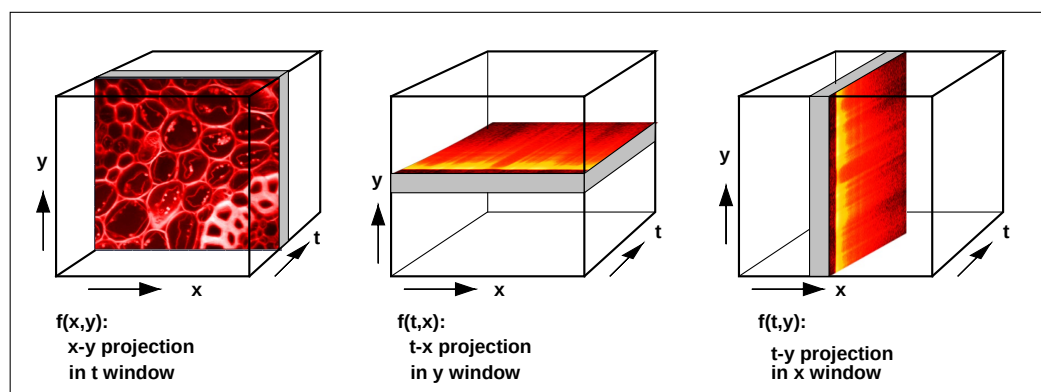


Fig. 122: Different projections of FLIM data

$F(x,y)$ projects the intensities (photon numbers) of a selected time window in the x-y plane. The result is a time-gated image, or, if the time window covers the entire t axis, an intensity image of the sample.

$F(t,x)$ projects the photon numbers of a selected y window into a t-x plane. The result displays the intensity decay along the t axis versus the location in x for a selected window in y. $F(t,y)$ projects the photon numbers of a selected x window into the t-y plane. The result displays the intensity along the t axis versus the location in x for a selected window in y. In both cases, the spatial window can also cover the entire x or y coordinate. Examples of trace parameters with different projection are shown in Fig. 123.

A special case is the direct display of the fluorescence lifetime. In this case, the display routine calculates the first moment of the decay data along the t axis of the individual pixels and converts it into the fluorescence lifetime. When ‘Lifetime Display’ is selected (W3 and W4 in Fig. 121) the colour of the image represents the lifetime, the brightness the total or gated intensity.

So far, things are still relatively straightforward. However, TCSPC data can easily become four-dimensional. This happens especially when the routing function is used. Data in the indi-

vidual routing channels then represent different detection wavelengths, different excitation wavelengths, or different states of an external signal applied to the sample. The ‘Routing Window’ defines a range of routing channels the data of which are projected into individual x-y, t-x, or t-y planes and displayed as described above. Trace-parameter examples for these situations are shown in Fig. 123. Please note that time windows cannot be defined for intensity image from the SPC-180 counters and for lifetime display. The counter images do not contain temporal data, and lifetime images are not $f(t,x)$ or $f(t,y)$ functions.

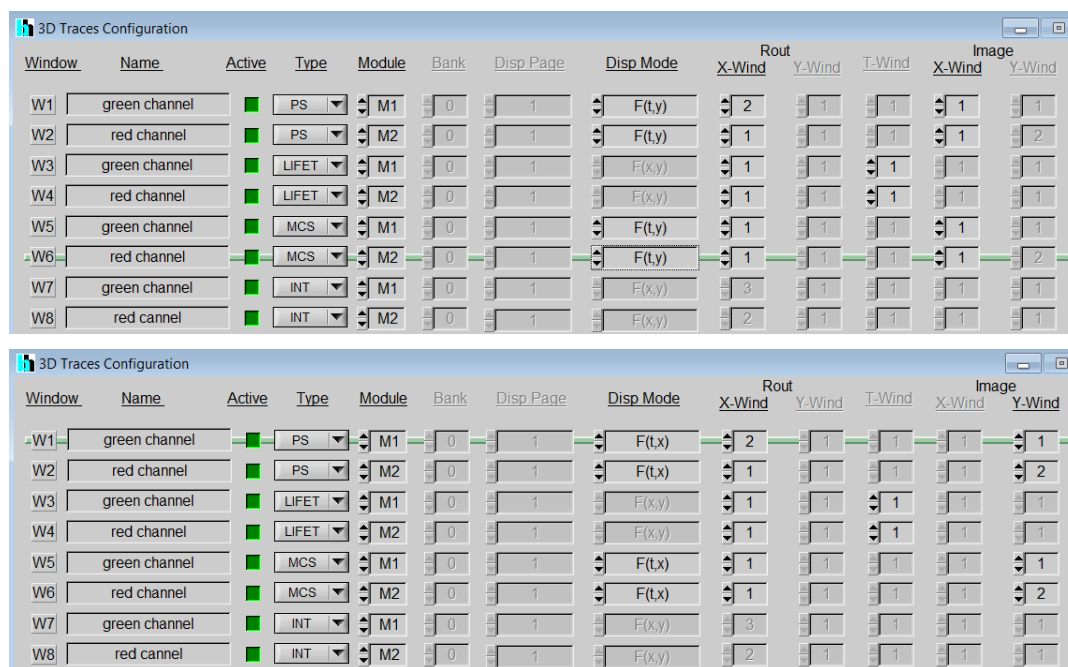


Fig. 123: 3D Trace parameters for the display of decay functions over one spatial and one temporal coordinate of spatially two-dimensional data

Window Parameters

The window parameters define time windows for FLIM, routing windows, time windows for PLIM (MCS windows) and spatial windows in X and Y for displaying multi-dimensional data. Fig. 124 shows definitions for 8 windows along each coordinate.

The figure shows the 'Window Intervals' window for SPC-160. It contains five panels: Time Windows, Rout X Windows, MCS Time Windows, Image X Windows, and Image Y Windows. Each panel has a 'Time Windows' section with a 'No of Windows' set to 8 and a table of window intervals. The 'Time Windows' section is set to 'Equidistant'.

Wind	From	To
1	1	32
2	33	64
3	65	96
4	97	128
5	129	160
6	161	192
7	193	224
8	225	256

Wind	From	To
1	1	2
2	3	4
3	5	6
4	7	8
5	9	10
6	11	12
7	13	14
8	15	16

Wind	From	To
1	1	32
2	33	64
3	65	96
4	97	128
5	129	160
6	161	192
7	193	224
8	225	256

Wind	From	To
1	1	32
2	33	64
3	65	96
4	97	128
5	129	160
6	161	192
7	193	224
8	225	256

Wind	From	To
1	1	32
2	33	64
3	65	96
4	97	128
5	129	160
6	161	192
7	193	224
8	225	256

Fig. 124: Window Parameters. Definitions shown for 8 FLIM time windows, 8 routing windows, 8 PLIM time windows, and 8 windows in x and y.

Basic FLIM and PLIM images are often displayed without the gating functions of the SPCM display routines. In that case, it is enough to define just one windows along each coordinate, covering the entire time axis, all routing channels, and the entire image in x and y. Definitions for data of 256 FLIM time channels, 256 PLIM time channels, and 256 x 256 pixels without routing are shown in Fig. 125.

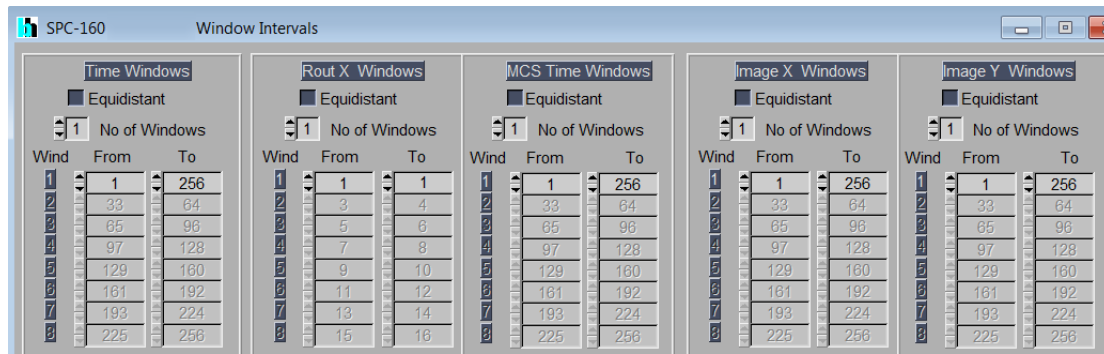


Fig. 125: Window parameter definitions for 256 FLIM and PLIM time channels 256 x 256 pixels for FLIM and PLIM display without gating functions.

Display Parameters

The display parameters define how the data are displayed in the individual display windows of the SPCM software. Every display window has its own set of display parameters. The parameters define the display range, the colours, the display style (curves or colour-intensity plot), the display mode ($f(x,y)$, $f(t,x)$ or $f(t,y)$), and the t , x and y windows in which the data are displayed. Examples are shown in Fig. 126.

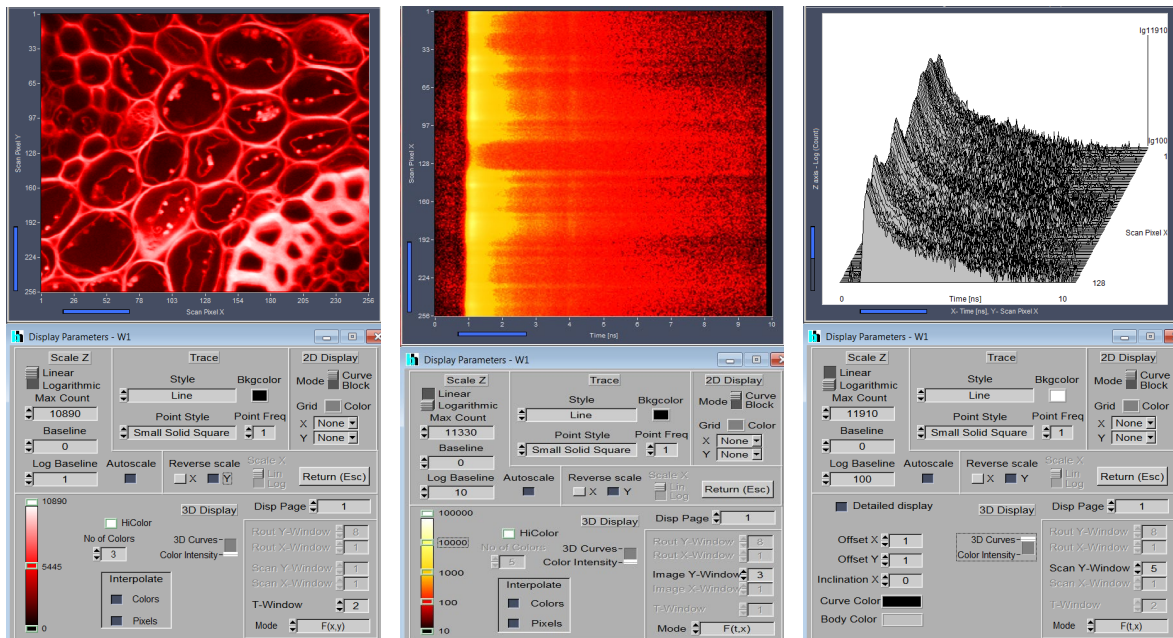


Fig. 126: Influence of display parameters on the data display. Left: Image displayed in a selectable time window. Middle: Decay functions over x coordinate in a selectable y window displayed in colour-intensity mode, Right: Decay functions over x coordinate in a selectable y window displayed in curve mode

For lifetime display ('LIFET' selected in the 3D trace parameters) the display parameter panel is expanded by the definitions for the lifetime display. These include the lifetime range, a reference moment of the IRF, and contrast and brightness of the image displayed. An example is shown in Fig. 127.

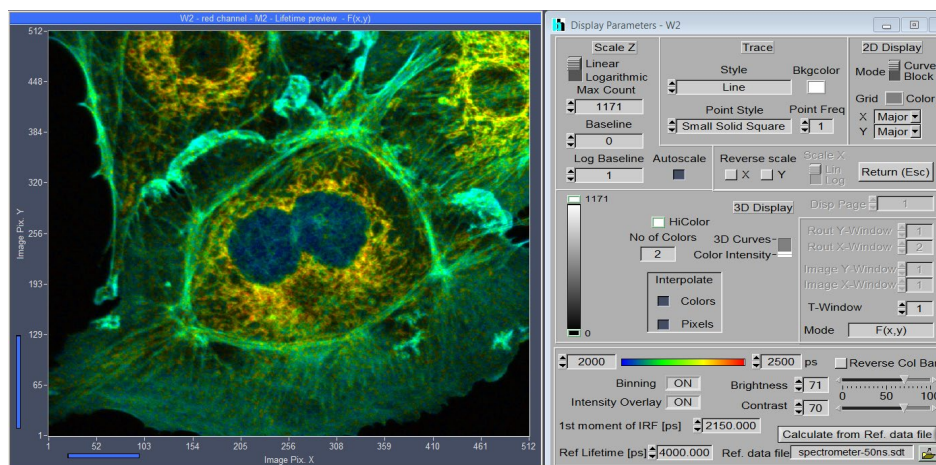


Fig. 127: Display parameters for lifetime image display

Autoscale Function

An autoscale function is available for all display modes of the SPCM software. We recommend to keep 'autoscale' active during the measurement and during possible data operations, see Fig. 126 and Fig. 127. Only for images containing extremely bright spots (e.g. from contaminations) it should be switched off and the display range defined manually.

Examples for Display of FLIM Data

2D trace parameters, window parameters, and display parameters for frequently used imaging modes are shown in the sections below. For description of the parameters of the SPCM display functions please see section above, or refer to the 'Software' section of the bh TCSPC Handbook [77].

Intensity Images

Configuration parameters for the display of intensity images from the two SPC modules of a DCS-120 system are shown in Fig. 128 through Fig. 130. Similar parameters are used for systems with SPC-QC modules.

The Trace Parameters (Fig. 128) define two display windows. The data type to be displayed is 'ps FLIM', the data in W1 and W2 come from TCSPC module M1 and M2, respectively. The display mode is F(x,y).

3D Traces Configuration											
Window	Name	Active	Type	Module	Bank	Disp. Page	Disp. Mode	Route	X-Wind	Y-Wind	Image
W1	Red Channel	☒	PS	M1	0	1	F(x,y)	1	1	1	3
W2	Green Channel	☒	PS	M2	0	1	F(x,y)	1	1	1	3
W3		☐	PS	M1	0	1	F(Lx)	2	1	1	1

Fig. 128: 3D Trace Parameters for the display of the images from two SPC modules, M1 and M2

The Window Parameters (Fig. 129) define windows through which the data are seen. For intensity images recorded in the basic FLIM modes these parameters can be used to display time-gated images, see Fig. 135 and Fig. 136. If the gating function is not used, there is only one time window, see Fig. 129, containing the photons of all TCSPC time channels from 1 to 1024. Routing Windows are not available because no routing was used when the data were recorded. MCX time windows are irrelevant because the data were not recorded in the MCS

mode. Image X and Image Y windows are irrelevant as well, because data are not displayed in the $f(t,x)$ or $f(t,y)$ display mode.

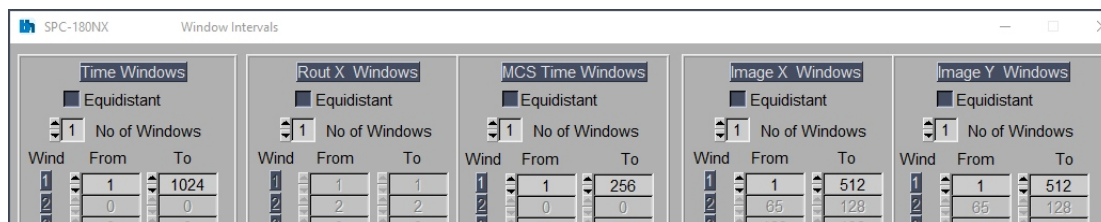


Fig. 129: Window parameters for the display of the images from two SPC modules

The Display Parameters, Fig. 130, define two intensity images for the two SPC modules. There are separate sets of display parameters for the two display windows, W1 and W2, see Fig. 130.

The intensity is coded by colour. For W1 the colour scale goes from black over red to white, for W2 from black over green to white. Both display windows contain the photons of Time Window 1, i.e. photons of all time channels (from 1 to 256, see Fig. 129). The SPCM main panel with the two images is shown in Fig. 131.

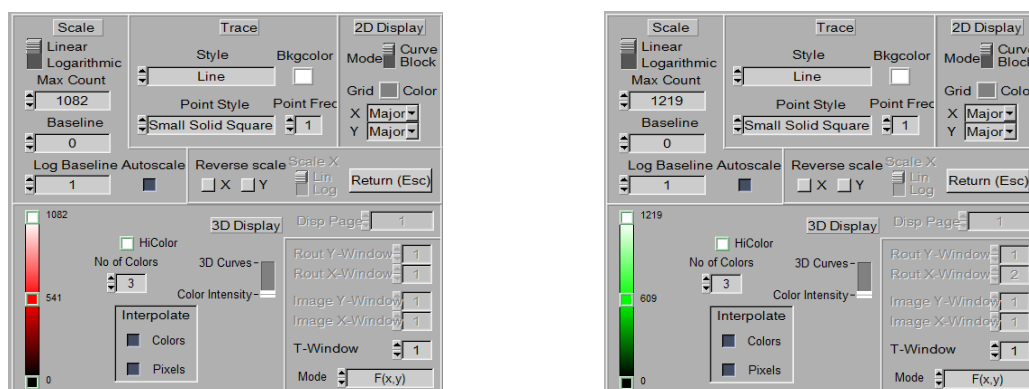


Fig. 130: Display parameters for the two images defined by the parameters in Fig. 129

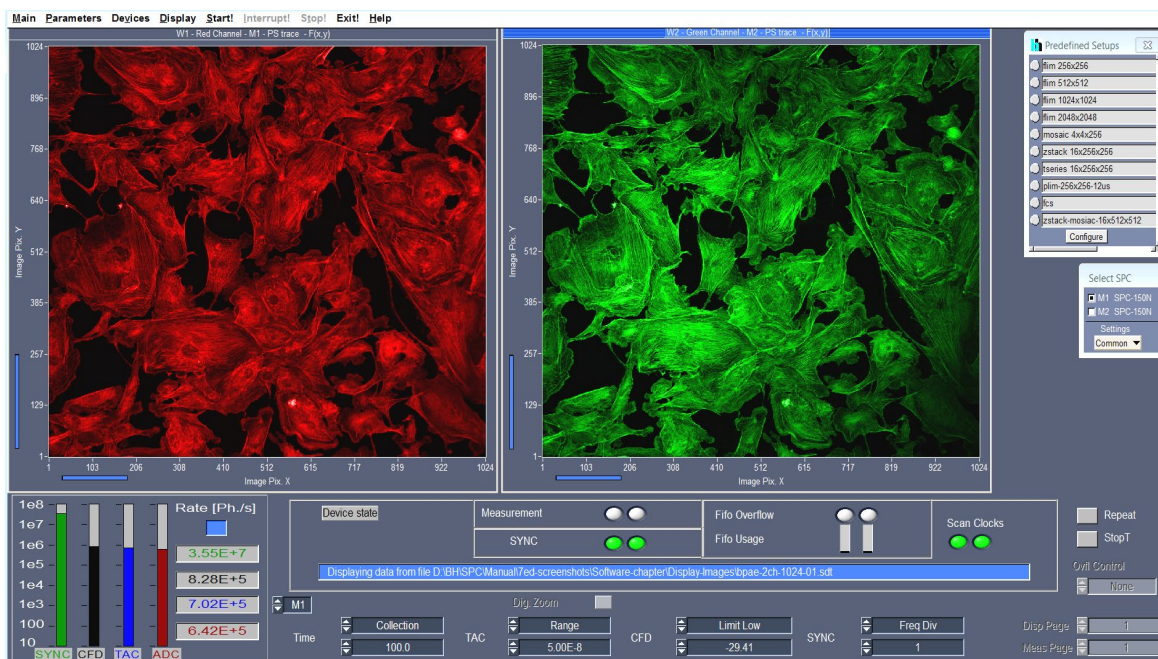


Fig. 131 SPCM Main panel. Two intensity images for two separate SPC modules.

Time-Gated Intensity Images

Time-Gated intensity images can be displayed by defining time windows in the Window Parameters. Fig. 133 shows the parameters for the display of four time-gated images from two TCSPC modules. The 3D trace parameters (Fig. 132) define eight display windows for the data of module M1 and module M2 in four T windows, 1 to 4.

Window	Name	Active	Type	Module	Bank	Disp Page	Disp Mode	Route	X-Wind	Y-Wind	T-Wind	Image	X-Wind	Y-Wind
W1	Red Gate 1	☒	PS	M1	0	1	F(x,y)	1	1	1	1	1	1	1
W2	Red Gate 2	☒	PS	M1	0	1	F(x,y)	2	1	1	2	1	1	1
W3	Red Gate 3	☒	PS	M1	0	1	F(x,y)	1	1	1	3	1	1	1
W4	Red Gate 4	☒	PS	M1	0	1	F(x,y)	1	1	1	4	1	1	1
W5	Green Gate 1	☒	PS	M2	0	1	F(x,y)	1	1	1	1	1	1	1
W6	Green Gate 2	☒	PS	M2	0	1	F(x,y)	1	1	1	2	1	1	1
W7	Green Gate 3	☒	PS	M2	0	1	F(x,y)	1	1	1	3	1	1	1
W8	Green Gate 4	☒	PS	M2	0	1	F(x,y)	1	1	1	4	1	1	1

Fig. 132: 3D Trace parameters for display of time-gated images

The Time Windows are defined in the Window Parameter panel, see Fig. 133. The numbers under 'Time Windows' are the start and the end points of the individual time windows.

Time Windows				Route X Windows				MCS Time Windows				Image X Windows				Image Y Windows			
Equidistant				Equidistant				Equidistant				Equidistant				Equidistant			
No of Windows				No of Windows				No of Windows				No of Windows				No of Windows			
Wind	From	To		Wind	From	To		Wind	From	To		Wind	From	To		Wind	From	To	
1	50	100		1	1	1		1	1	256		1	1	1024		1	1	1024	
2	100	150		2	1	1		2	0	0		2	0	0		2	0	0	
3	150	200		3	1	1		3	0	0		3	0	0		3	0	0	
4	200	250		4	1	1		4	0	0		4	0	0		4	0	0	
5	0	0		5	1	1		5	0	0		5	0	0		5	0	0	

Fig. 133: Window parameters (bottom) for the display of four time-gated images of SPC module M1.

The images displayed in W1 through W8 have separate display parameters. Display parameters for the images from Module M1 are shown in Fig. 134. The SPCM main panel with the eight gated images is shown in Fig. 135.

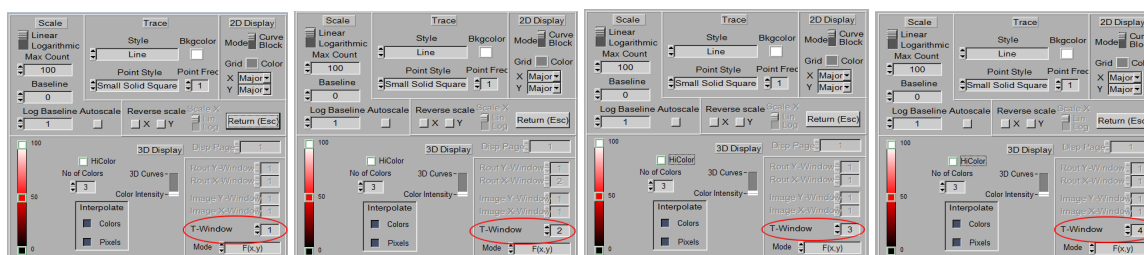


Fig. 134: Display parameters for the four gated images

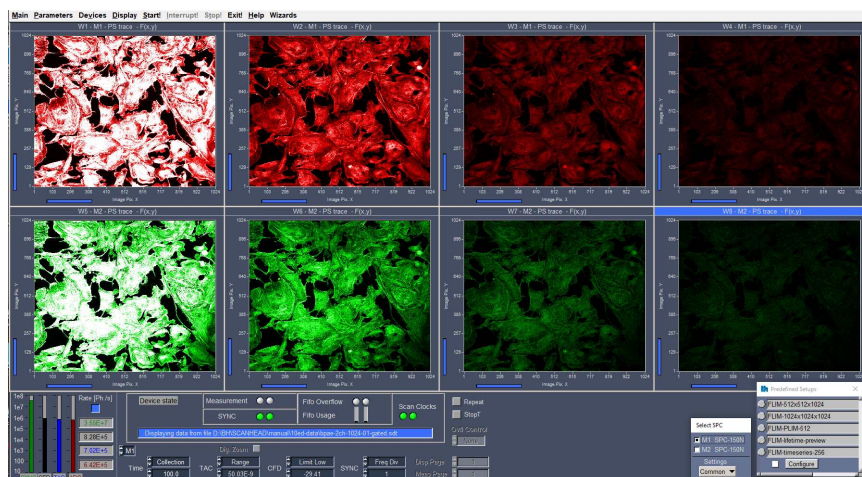


Fig. 135: Eight time-gated images in the main panel of SPCM

Time-gated images were commonly used in early times of TCSPC FLIM. With the introduction of colour-coded lifetime images time-gating has come largely out of use. However, time-gates images are still useful to display SHG images or images of extremely fast decay effects on a slow-decay background. An example is shown in Fig. 136.

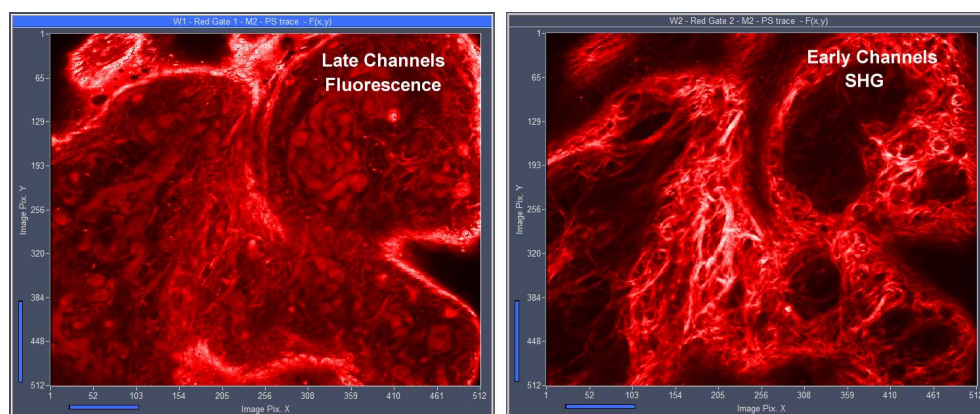


Fig. 136: Pig skin, 2-photon excitation by femtosecond laser, gated images. Left: Late time channels, showing fluorescence of NADH. Right: Early time channels, showing SHG of collagen.

Display of Lifetime Images

Fig. 137 and Fig. 138 show the definition of the parameters for online lifetime display for a dual-channel SPC system. In the 2D trace parameters, two display windows are defined, one for each module. Data Type is 'Lifetime'.

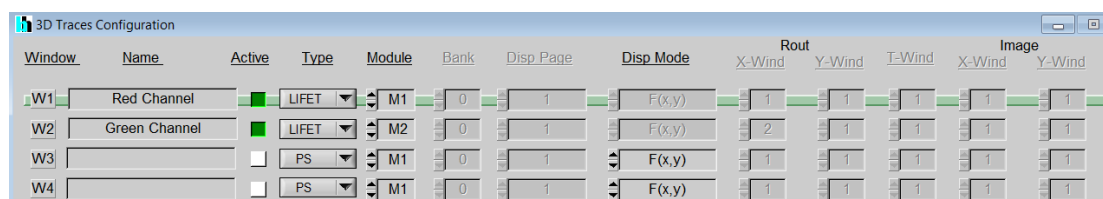


Fig. 137: 3D trace parameters for run-time display of lifetime images

The display parameters are shown in Fig. 138. As usual, there is a separate set of display parameters for each image. In the lower part of the panels, the lifetime range, the direction of the colour bar, the brightness and the contrast, and a reference moment for the IRF is defined. The reference moment can be determined by SPCImage (see SPCImage NG Data Analysis Software, page 293) or calculated from a reference FLIM file of a sample with known fluorescence lifetime. For principle of run-time lifetime calculation please see bh TCSPC Handbook [77]. The main panel of the SPCM software with online lifetime display is shown in Fig. 139.

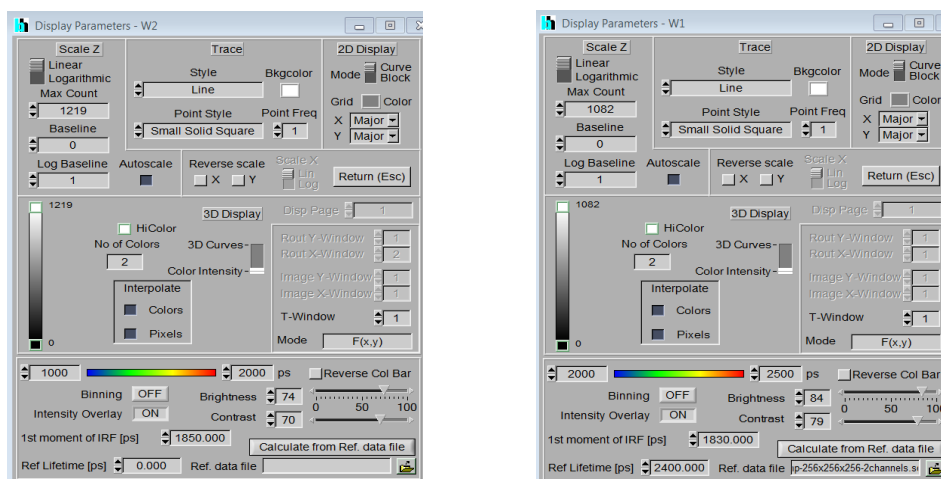


Fig. 138: Display parameters for online lifetime display

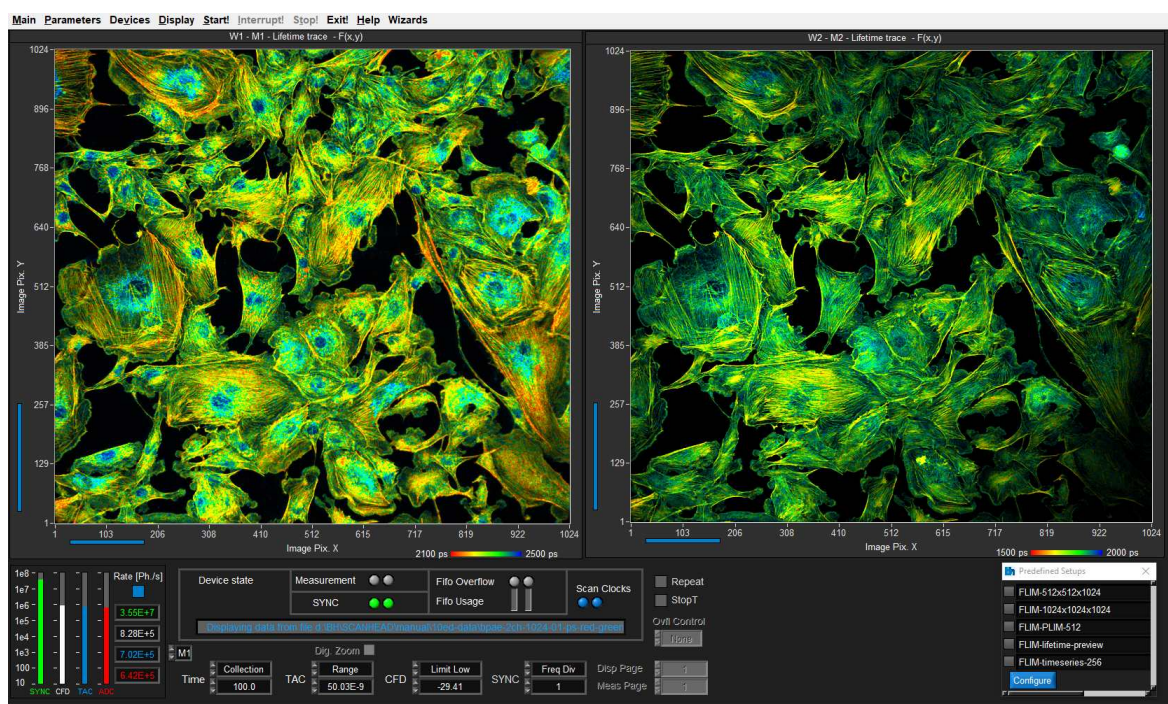


Fig. 139: SPCM Main panel with online lifetime Display. Two SPC channels.

Lifetime - Intensity Images

SPC-180 series TCSPC / FLIM modules have a fast counter implemented which counts the detected photons in parallel with the TAC/ADC timing electronics. The dead time of the counter channel is much lower than that of the timing electronics. 'Lifetime-Intensity' images are built up from the counter data and the timing data. Photon numbers determined by the counter are virtually free of counting loss. Lifetime-Intensity images therefore provide better contrast at high count rates. The 3D Trace parameters for Lifetime-Intensity display are shown in Fig. 140. 'Type' for Lifetime-Intensity display is LIFET+I. With the settings shown a lifetime-intensity image and a normal lifetime from the same recording channel are displayed. The corresponding images are shown in Fig. 141.

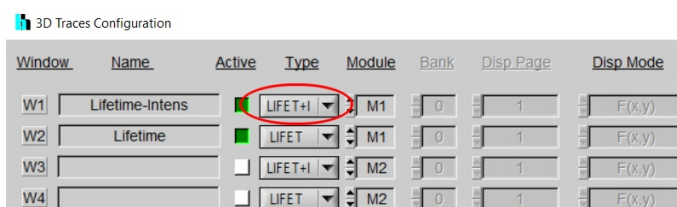


Fig. 140: 3D trace parameters for Lifetime-Intensity display.

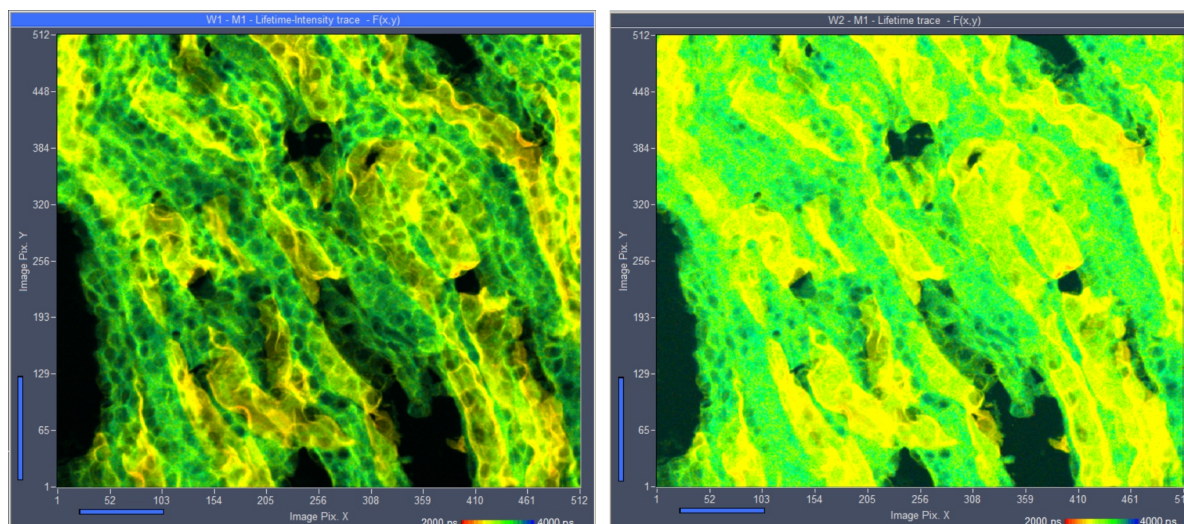


Fig. 141: Lifetime-Intensity image (left) in comparison to normal lifetime image (right). SPC-180NX, average count rate 5.5 MHz.

Excitation-Wavelength Multiplexed FLIM

Excitation-wavelength multiplexing is used when the sample contains two fluorophores which cannot be excited at the same excitation wavelength or the fluorescence of which cannot be separated by filtering on the detection side alone. In these cases two lasers of different excitation wavelength are multiplexed in time, and the photons excited by each laser are routed in separate memory blocks, see Fig. 239, page 138.

With two TCSPC modules detecting in different wavelength intervals the recording process delivers four combinations of excitation and detection wavelengths. The corresponding images are selected by the 3D Trace Parameters (Fig. 142) in combination with the routing information from the Window Parameters (Fig. 143). The 3D Trace Parameters define four display windows for different combinations of excitation wavelength (Routing X Window) and detection channel (M1 or M2). Display type is 'Lifetime'.

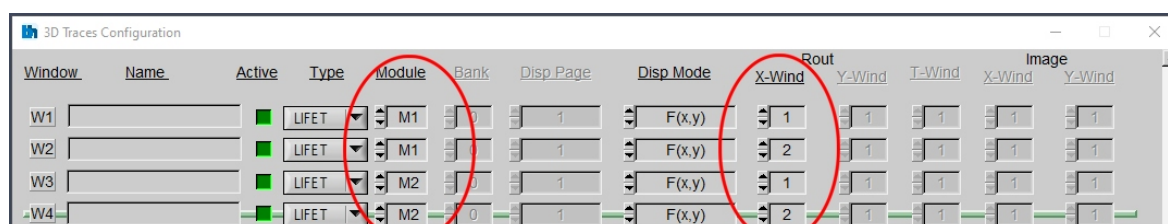


Fig. 142: 3D Trace Parameters for Laser Wavelength multiplexing

The Window Parameters are shown in Fig. 143. There are two 'Routing X Windows'. Each one contains only one routing channel. Window 1 is laser 1, Window 2 is laser 2.

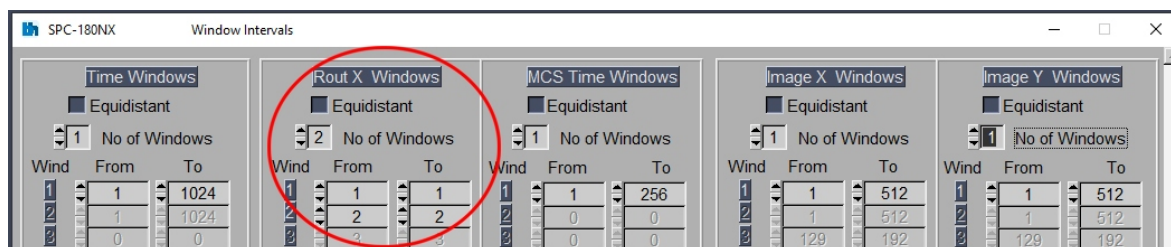


Fig. 143: Window Parameters for wavelength multiplexing

The display parameters are the same as for online-display of lifetime images in two detection channels. Except for different lifetime ranges the display parameters are the same as in Fig. 138.

The SPCM Main Panel with these parameters is shown in Fig. 144. The NADH FLIM image is shown on the left, the FAD FLIM image on the right.

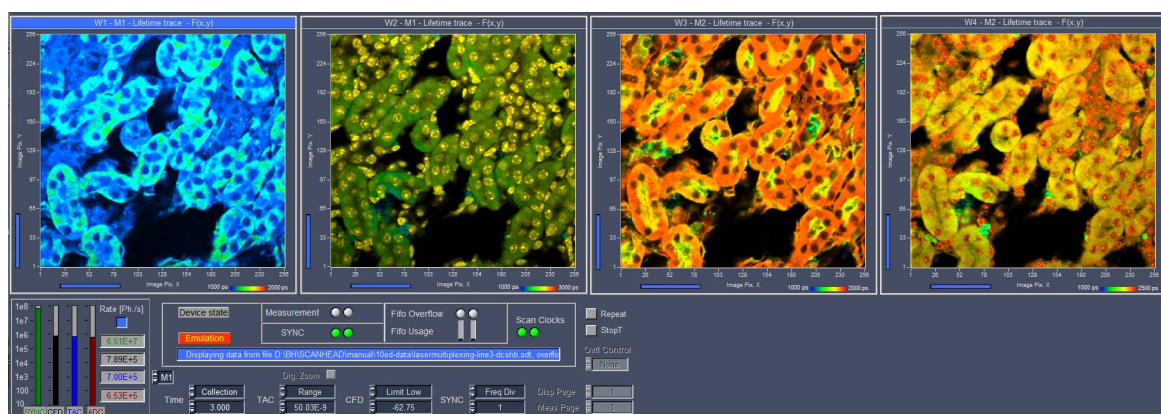


Fig. 144: Main Panel for laser-multiplexed FLIM. Two lasers and two TCSPC modules.

Multi-Wavelength FLIM Data

The classic way of displaying data from a 16-channel multi-wavelength FLIM detector is shown in Fig. 145 through Fig. 148. Each wavelength channel has 512 x 512 pixels and 256 time channels. The trace parameters define eight display windows. Data type is 'ps FLIM', i.e. the images shown in the windows are intensity images. The individual images in the display windows are derived from subsequent 'Routing Windows'. Each routing window contains the data of two subsequent routing channels, see Fig. 146. This way, data of every two subsequent wavelength channels are combined in one image.

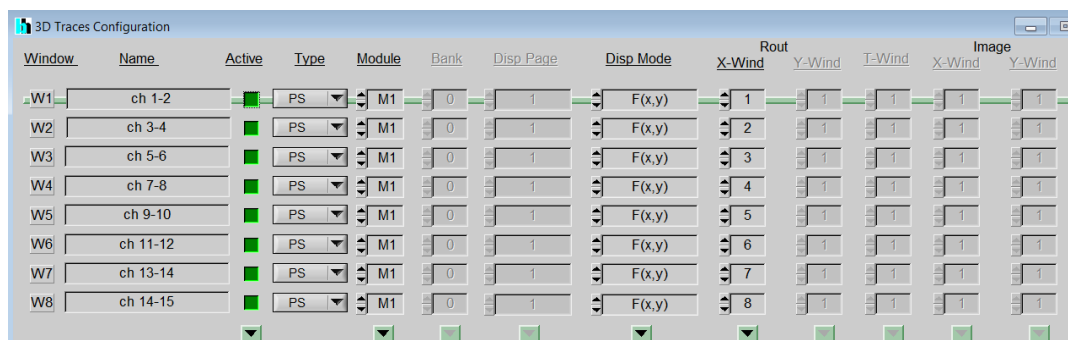


Fig. 145: Trace parameters for multi-wavelength FLIM with the PML-16C or PML-16 GaAsP detector

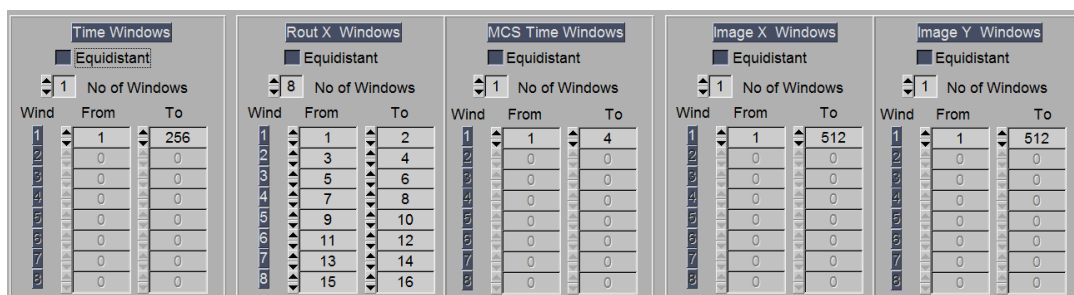


Fig. 146: Window parameters for multi-wavelength FLIM

As usual, the individual images have separate display parameters. The display parameters for the first three images are shown in Fig. 147. They contain the colour definition for the individual images, and the Routing Window. Since the intensities in different wavelength channels can vary over a wide range Autoscale is turned on for all images. The main panel of SPCM with the eight intensity images is shown in Fig. 148.

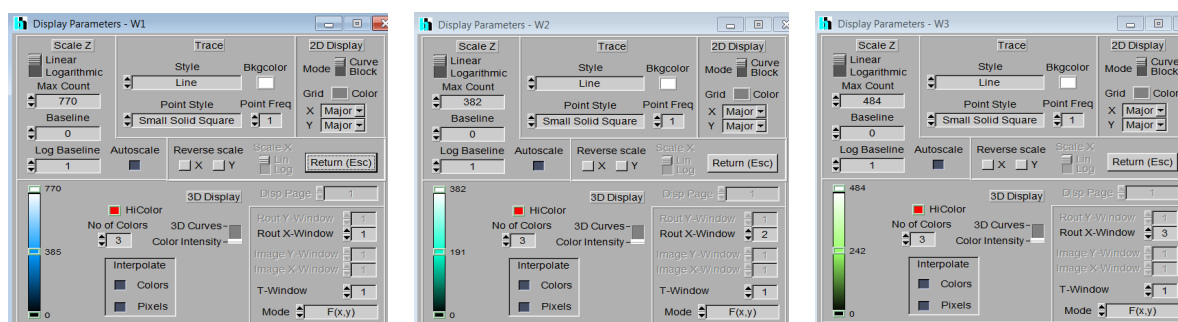


Fig. 147: Display parameters in the first three display channels of a multi-wavelength measurement

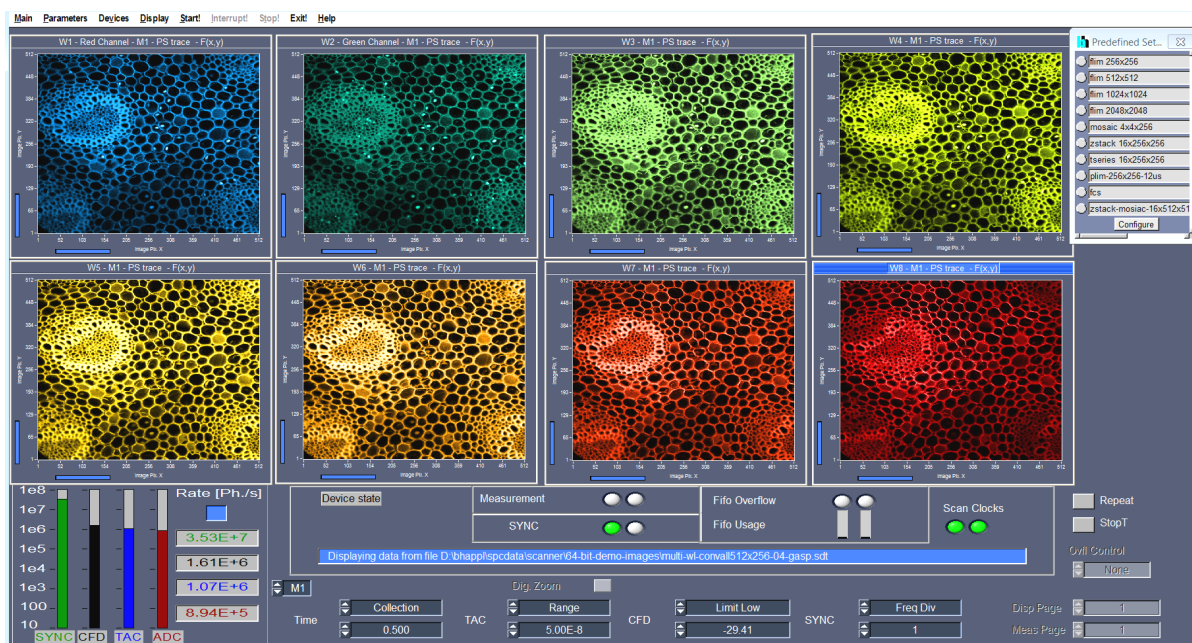


Fig. 148: SPCM Main panel for multi-wavelength FLIM, intensity display

Multi-wavelength FLIM data can also be displayed as lifetime images. In that case, the data type in the 3D Trace Parameters is 'Lifet'. The 3D trace parameters for multi-wavelength lifetime display are shown in Fig. 149, the corresponding Main Panel in Fig. 150.

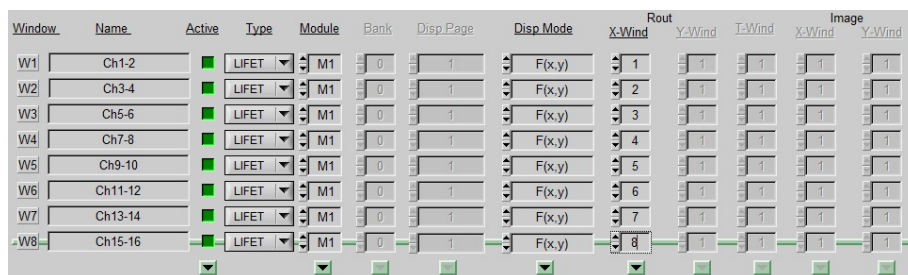


Fig. 149: 3D Trace parameters for multi-wavelength lifetime display

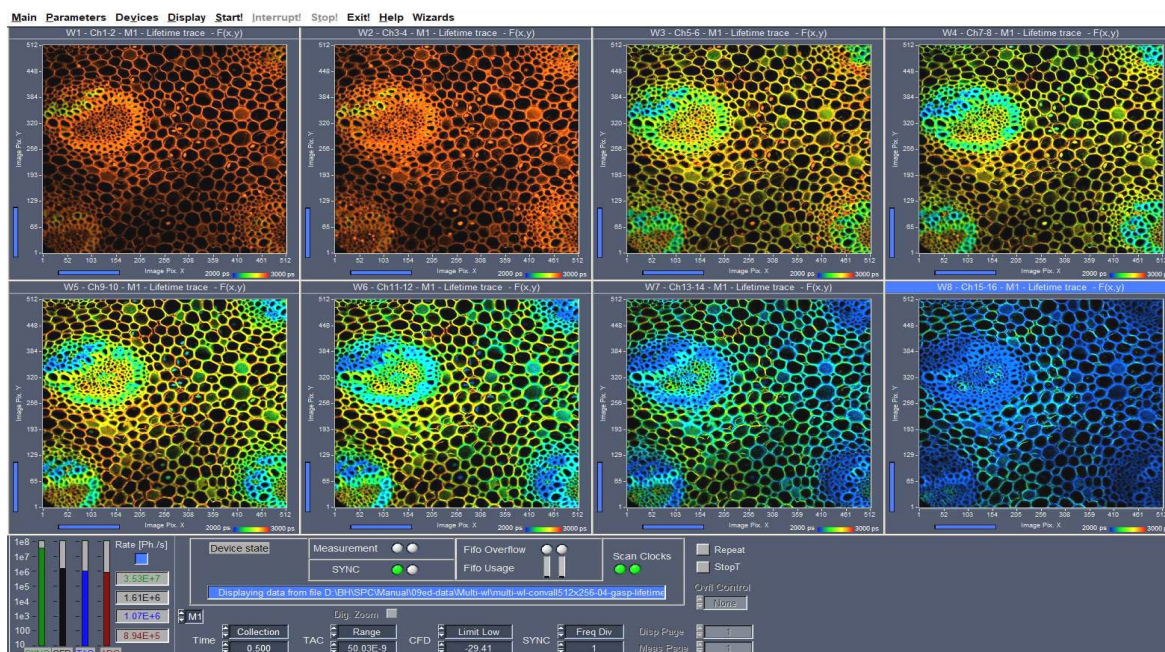


Fig. 150: SPCM Main panel for multi-wavelength FLIM, lifetime display

Multi-Wavelength display by the display functions shown above is limited to a maximum of eight display windows. Data from detectors with more than 8 wavelength channels have to be binned down to 8 channels as shown in Fig. 145, or only a selection of channels is displayed at a time. For displaying all 16 channels of a multi-wavelength detector simultaneously use the 'Mosaic FLIM' function, see section below.

Mosaic FLIM Data

Mosaic FLIM was originally introduced to record FLIM images with a lateral size larger than the field of view of a microscope lens. However, this is not all. Mosaic FLIM can be considered a general way to record FLIM data with more than three dimensions. Additional dimensions can be spectral (the wavelength channels of a multi-wavelength recording), spatial (the dimensions of an x-y mosaic or the z axis of a z scan), temporal (the time within a time series of recordings) or electrical (an electrical or a magnetic field applied to the sample). Details and system-parameter setup are described in section 'Mosaic Imaging Function', page 107. In terms of image display a FLIM mosaic is nothing than a single, large FLIM image. Consequently, a FLIM mosaic can be displayed by the functions described under 'Configuring the FLIM Data Display', page 71.

Multi-Wavelength Mosaic

Fig. 151 shows the parameter setup for a multi-wavelength FLIM mosaic. In the System parameters, 'Mosaic' must be enabled (Fig. 151, left). Mosaic Type is 'Routing Channels'. Please see also 'System Parameters', 'Mosaic Imaging Function' page 107.

In the 3D Trace parameters, 'Display Mode' is 'Mosaic' (Fig. 151, right). Only display window 1 is used, the other windows are disabled.

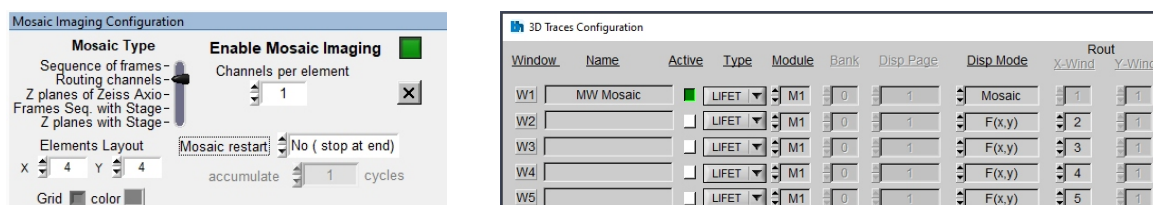


Fig. 151: Activation of Mosaic Imaging in the System parameters (left) and in the 3D Trace Parameters (right).

An example of a multi-wavelength mosaic is shown in Fig. 152. From upper left to lower right, it shows the images from the 16 wavelength channels of a MW-FLIM detector. The upper left image is empty because the wavelength is blocked by the laser-blocking filter.

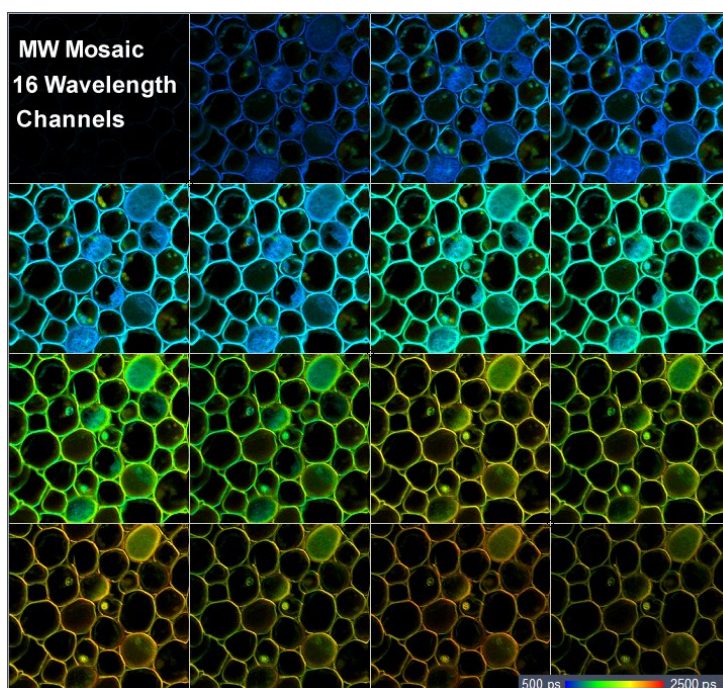


Fig. 152: Examples of Mosaic Imaging. Left: Multi-wavelength mosaic of a convallaria sample. Right: x-y mosaic of a BPAE sample.

X-Y Mosaic with Sample Stage

An X-Y mosaic recorded with an X-Y sample stage is shown in Fig. 153. It is a mosaic of $4 \times 4 = 16$ individual images recorded one after another by shifting the sample by the stage. The total size of the mosaic is 2048×2048 pixels. Mosaic type in the System parameters is 'Frame Sequence with Stage'. For programming the sample stage please see 'Motorised Sample Stages', page 114.

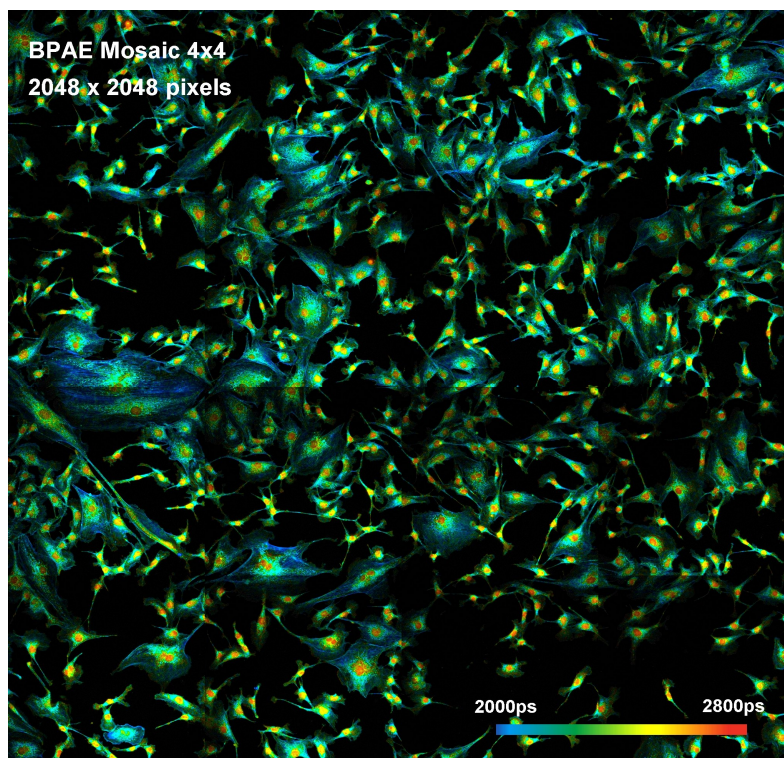


Fig. 153: X-Y mosaic recorded with a sample stage

Time-Series Mosaic

A time-series of images recorded by Mosaic FLIM is shown in Fig. 154. Mosaic Type in the System Parameters is 'Sequence of Frames'. For details on triggered accumulation of time series please see 'Time-Series FLIM by Mosaic Imaging Function' page 151.

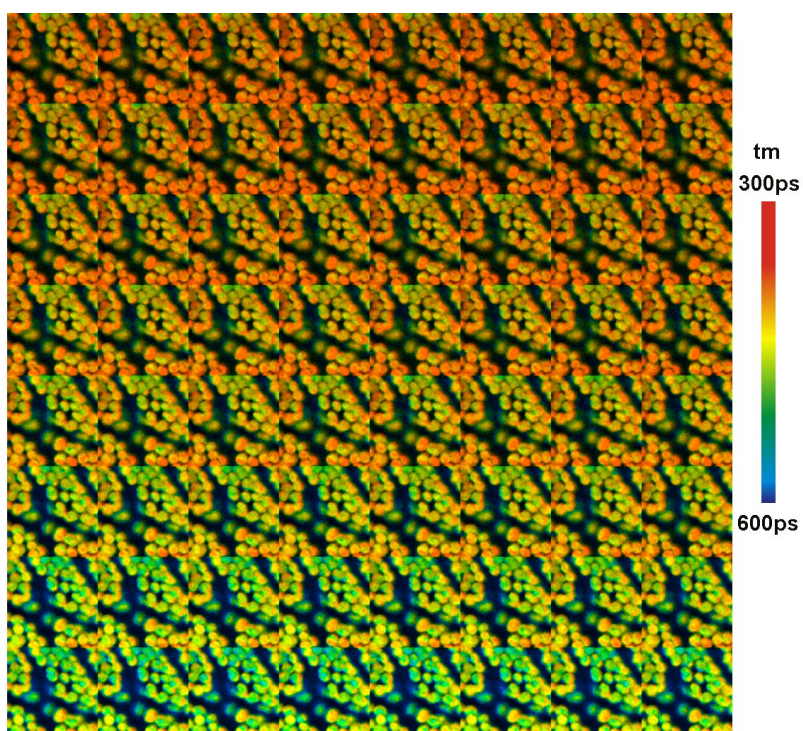


Fig. 154: Time series mosaic. Change of the lifetime of chloroplasts in a moss leaf. 64 elements, acquisition time 1 second per element.

Z Stack Mosaic

Also Z stacks of FLIM images can be recorded and displayed via the Mosaic imaging function of SPCM. Please see 'Z Stack Recording by Mosaic FLIM', page 158. An example is shown in Fig. 155.

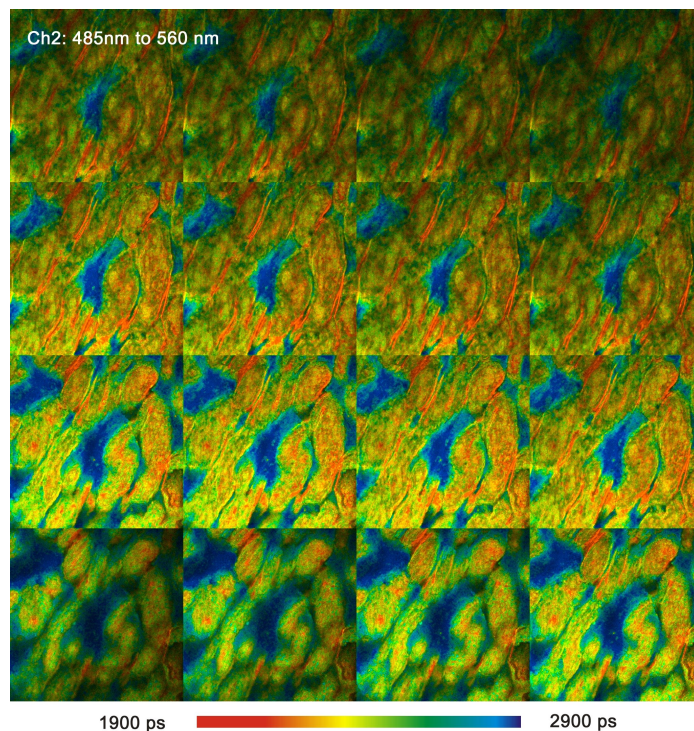


Fig. 155: Z Stack mosaic of a pig skin sample. 16 Z planes recorded into one 4x4 element mosaic

Simultaneous FLIM / PLIM Data

Simultaneous FLIM/PLIM is based on on-off modulation of a high-frequency pulsed excitation laser. FLIM is recorded in the laser-on phases by the normal FIFO Imaging procedure. PLIM is recorded in the laser-off phases by 'MCS' recording, for details please see 'Simultaneous FLIM / PLIM', page 172. On-Off modulation is controlled by the GVD-120 or GVD-140 scan controller. To better separate the photons from the laser-on and laser-off phases a routing signal from the laser modulation is sent into the SPC module. Laser-on photons are stored into routing channel 1, laser-off photons into routing channel 2.

Fig. 156 shows the trace and window parameter setup. The 3D Trace parameters define four display windows. The first one is the FLIM window. It displays the photons from the laser-on phases, recorded in routing channel 1. The second one is a pure PLIM window. It contains the photons from the Laser-off phases, recorded in routing channel 2. The third and the fourth window show the total luminescence. Data type for the first window is ps FLIM, for the other windows it is MCS imaging.

The window intervals define three routing windows. The first one contains the photons from the laser-on phases, the second one the photons from the laser-off phases. The third routing window contains the photons from both phases.

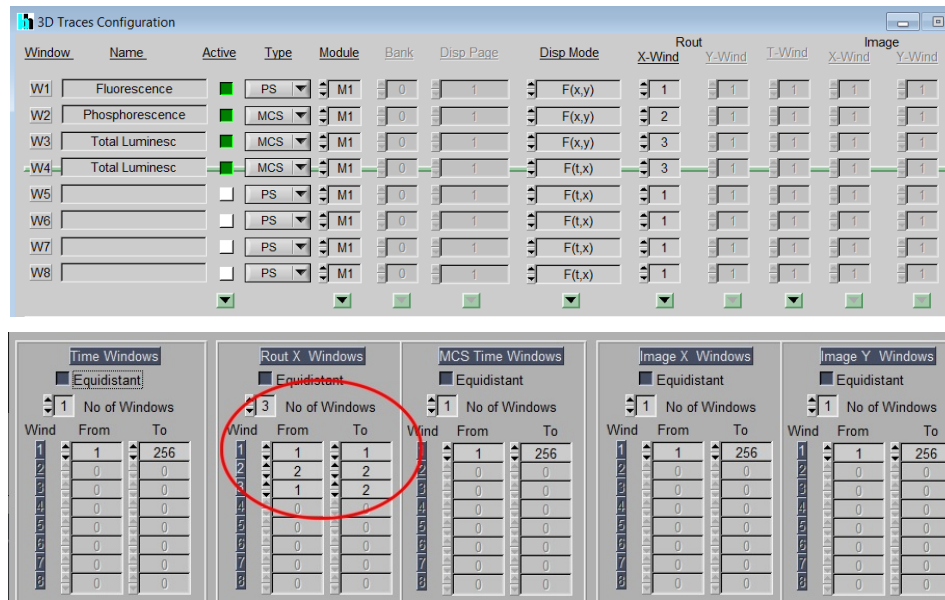


Fig. 156: 3D Trace parameters and window parameters for simultaneous FLIM/PLIM. There are three routing windows, one for fluorescence, one for total luminescence, an one for phosphorescence

The display parameters for the four windows are shown in Fig. 157. The parameters for W1 display an intensity image for the FLIM data. The data are from window 1, i.e. from the ‘laser on’ phases. Colour goes from black over green to white, autoscale is set, intensity scale is linear.

The display parameters for W2 display an intensity image of the phosphorescence in the laser-off phases. Routing window is 2 (laser off), the colour goes from black over red to white.

Window W3 displays an image of the total luminescence, i.e. entire signal in both phases of the laser modulation. Therefore ‘Routing window’ is 3.

In contrast to fluorescence lifetimes phosphorescence lifetimes can vary over an extremely wide range. It is therefore useful to have online information whether the PLIM time range has been selected appropriately. Therefore, W4 displays the phosphorescence data as a series of decay curves over the horizontal axis of the image. Display mode is ‘3D Curves’ and $F(t,x)$, the scale is logarithmic.

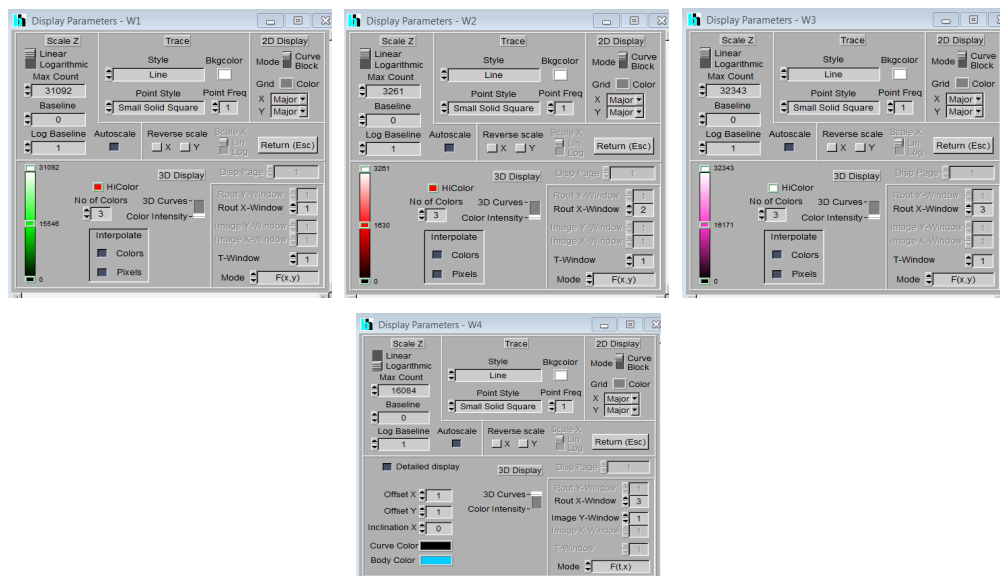


Fig. 157: Display parameters for fluorescence image, phosphorescence image, total luminescence, and phosphorescence decay curves

The SPCM main panel with these setup parameters is shown in Fig. 158. The fluorescence image is displayed on the left, the phosphorescence image in the middle, and the image of the total luminescence on the right. The decay curves of the total luminescence are displayed middle left.

Several modifications are possible to this setup. Often the fluorescence and the phosphorescence are recorded at different wavelength, and by different SPC modules. This can be accounted for by changing the Module from M1 to M2 in the trace parameters. It is also possible to activate the run-time lifetime display for the fluorescence and for the phosphorescence. Just change to 'Lifetime' in the trace parameters, and set an appropriate lifetime range and IRF moment in the display parameters.

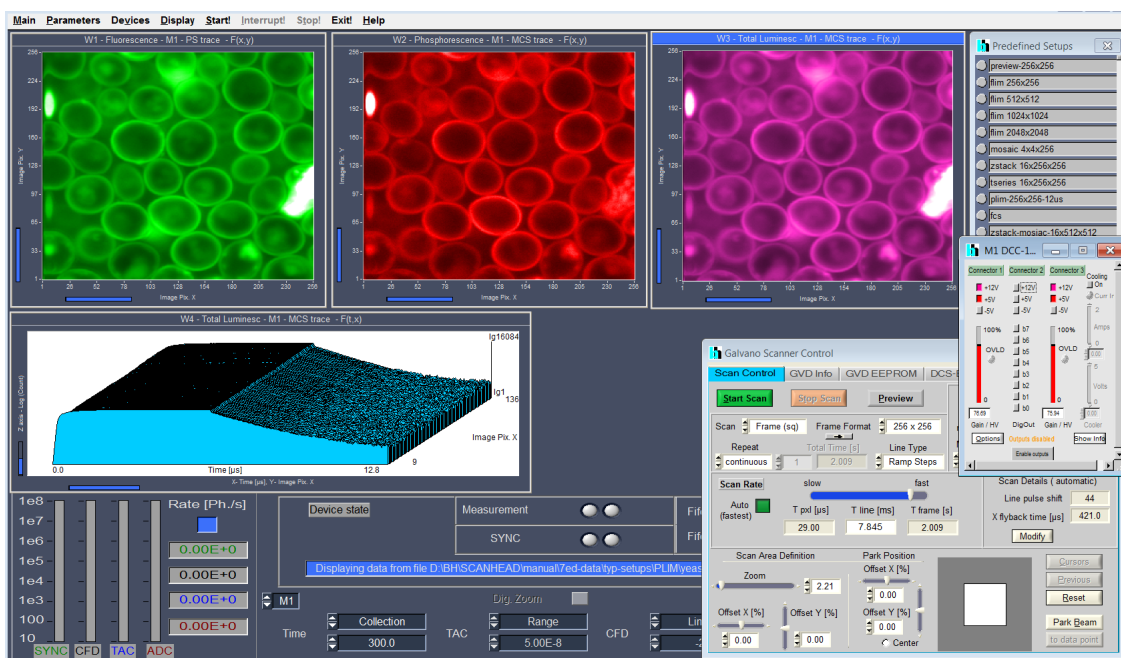


Fig. 158: SPCM main panel for simultaneous FLIM/PLIM

Cursors in the FLIM Images

Cursors in the display windows of the main panel are enabled by a right-click into the display window. This opens a small panel in which the cursors functions can be enabled or disabled, see Fig. 159, left. The display window with the cursors enabled is shown in Fig. 159, middle. Two cursors and a 'data point' are available.

A right-mouse click opens a window with the cursor settings, see Fig. 159, right. The cursor settings window can be placed anywhere in the screen area.

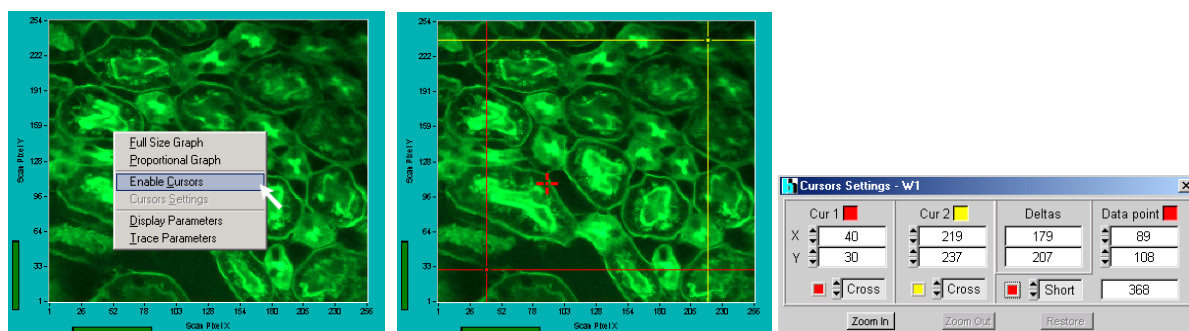


Fig. 159: Cursors in the display windows of the main panel

The cursors and the data point can be shifted by the mouse cursor, or by changing the cursor positions, X and Y, in the cursor settings window.

When the cursors are enabled you can zoom into the selected area by clicking the 'Zoom in' button in the cursor-settings window. Please see Fig. 160.

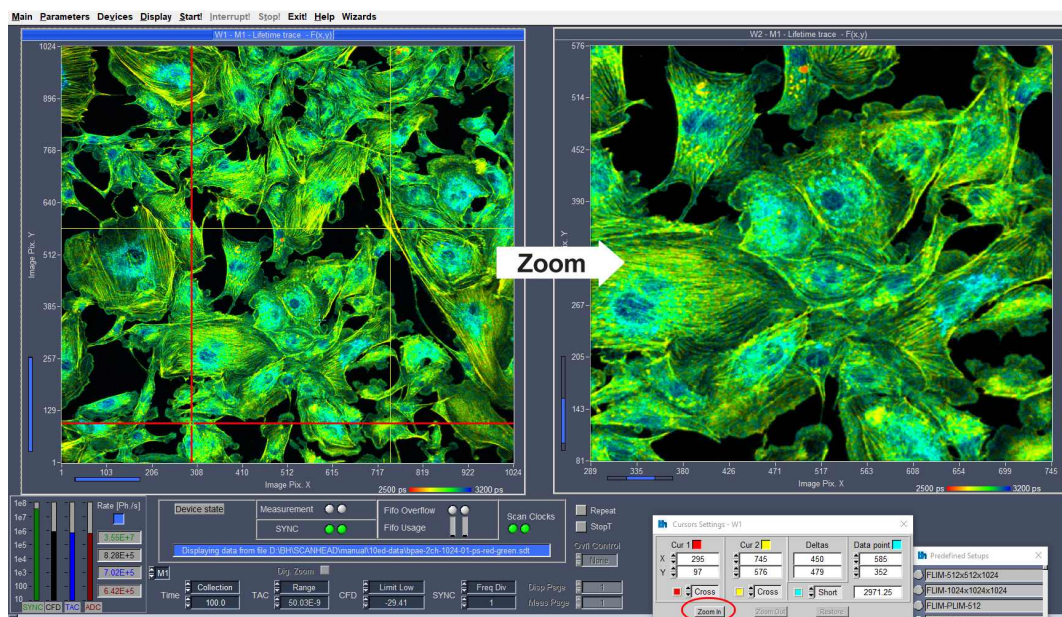


Fig. 160: Zoom function in the Display windows of the Main panel

Displaying the Photon Number in a Selected Pixel

You can use the 'data point' to display the photon number in a selected pixel. The photon number at the data point position is displayed in the lower right of the cursor-settings panel, see Fig. 161.

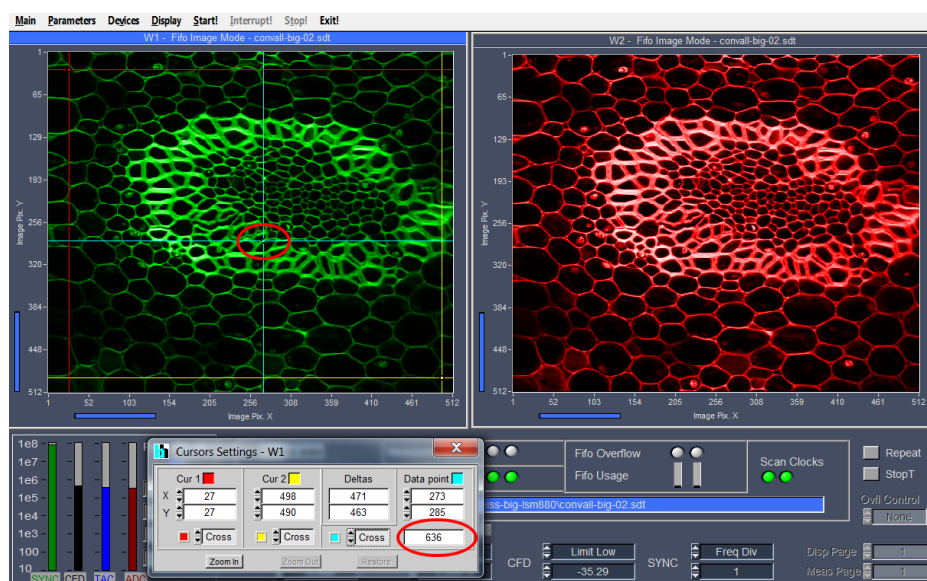


Fig. 161: Displaying the photon number in the data-point location

Fig. 162: Displaying the photon number in the brightest pixel. Open the display parameter panel and select the display window you are interested in.

Decay Curves from Regions of Interest

Decay curves from FLIM data can be displayed for a region of interest, a point of interest, or for the entire image. A right-mouse click into one of the images in the SPCM main panel opens a small menu from where you can access the image cursors, the display parameters, trace parameters, window parameters, etc. A click into 'Show Decays' opens the decay-curve window as shown in Fig. 163, right.

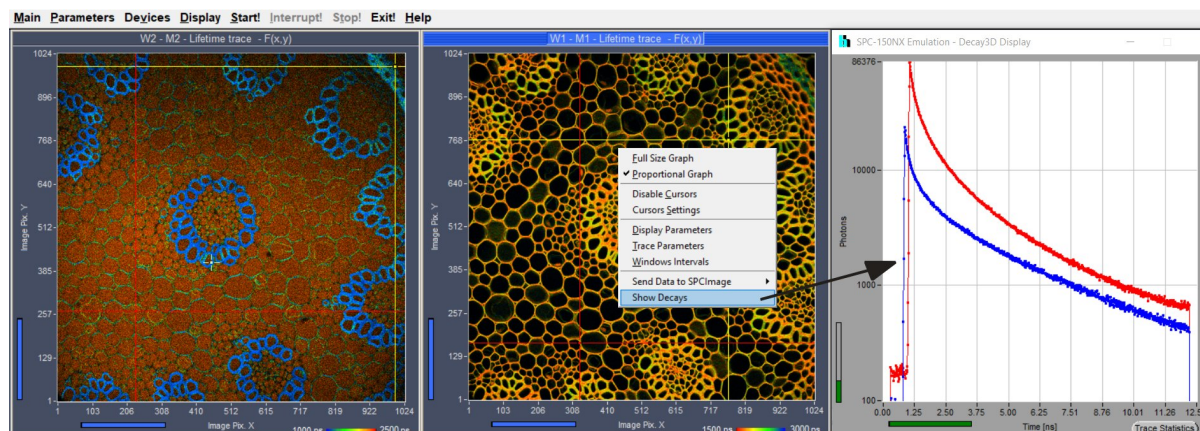


Fig. 163: A right mouse click into an image and a click on 'Show Decays' opens the decay curve panel shown on the right

Decay curves can be displayed for all active image windows. A right mouse click into the decay window opens a panel with the 'Trace Attributes' of the individual curves, see Fig. 164. From left to right, you can define the colours of the curves, the 'Type' (ROI, POI, or ALL) for which they are calculated, the image window from which they are derived, and the size and position of the region or point of interest. The size and position parameters can be linked to the cursors in the image windows, see Fig. 164, right. With 'Manage Traces' you can add or remove curves from the decay-curve display.

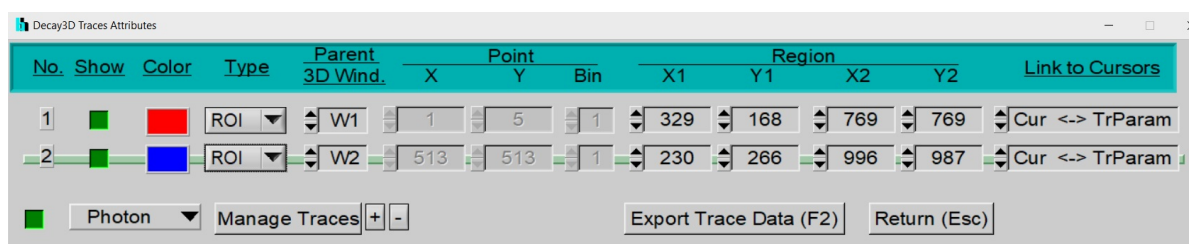


Fig. 164: SPCM Main Panel with lifetime images and decay curve window

The decay-curve display works for all images that contain decay data in their pixels. These can be intensity images, time-gated intensity images, lifetime images, combined images from several TCSPC modules, or images from different routing channels of a TCSPC module.

The display of the decay curves themselves is controlled by the 'Display Parameters'. The display scale can be linear or logarithmic, an autoscale function is available, and the curves can be displayed as individual data points, lines, or data points connected by lines. For details please see SPCM software description in [77].

Lifetime Histogram

When in the 'Lifetime' display mode is active SPCM can display a Lifetime histogram. Click into the image of interest, and select 'Lifetime Histogram'. This opens a window showing how often a lifetime value appears in the image. The lifetime is calculated from the first moment of the decay curves. The histogram applies to the active window. An example is shown in Fig. 165.

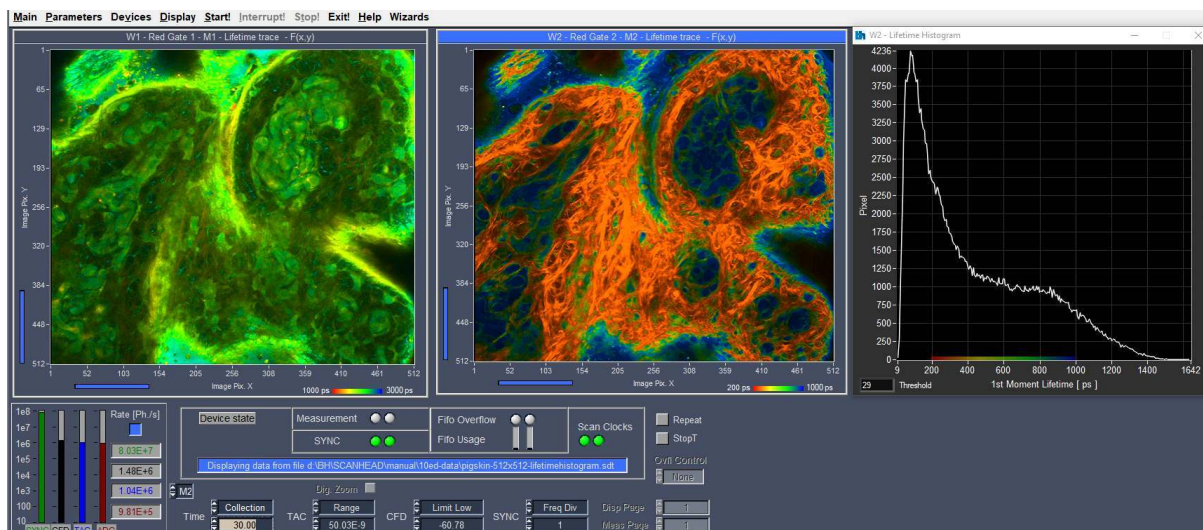


Fig. 165: Display of a lifetime histogram by SPCM. The histogram applies to the active (right) image window.

Multi-File View

The results of a series of measurements, such as a time series or a Z stack can be inspected by the Multi-File View functions of SPCM. The function is accessed via the 'Main' pull-down menu of the SPCM software, see Fig. 166.

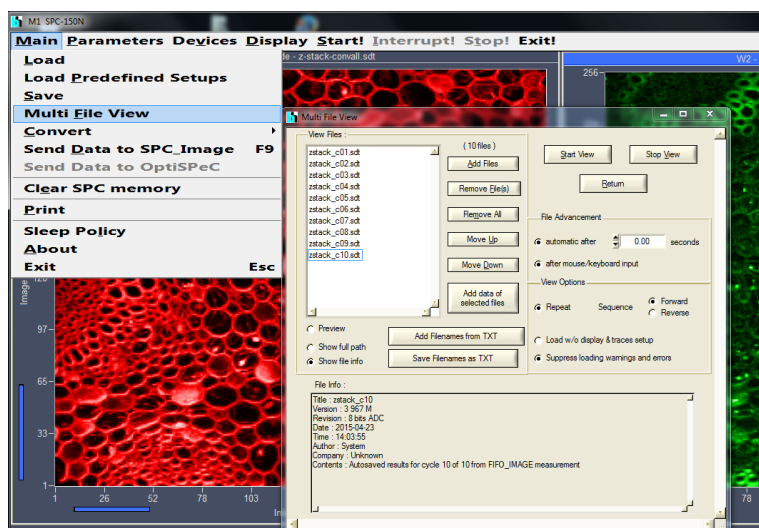


Fig. 166: Multi-File View function of SPCM

The Multi-File View allows you to define a list of data files, and select either single files from this list or show the individual files sequentially like a movie. Please see SPCM software description in [77] for details.

Display of Decay Curves from 'Single' mode

When working in the 'Single' or 'Oscilloscope' mode the SPC modules record decay curves or other optical waveforms and displays them in a 2-dimensional curve window. The SPCM

software can display up to 16 decay curves or other optical waveforms simultaneously. The curves on the screen are referred to as 'Traces'. The '2D Trace Parameters' define which information the traces contain and in which style and colour they are displayed. An example is shown in Fig. 167.

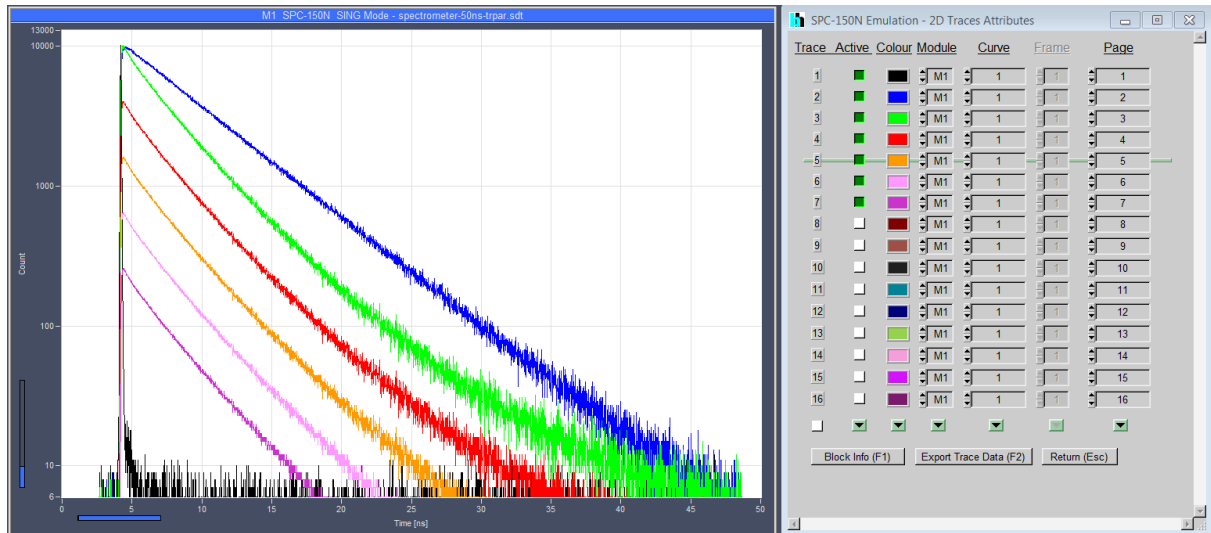


Fig. 167: Left: Display of several decay curves recorded by a single SPC module. Right: 2D trace parameters

The 2D trace parameter panel contains the definitions for up to 16 traces. For each trace the display can be switched on and off by the 'Active' button, and different colours can be defined.

'Module' is the TCSPC module from which the displayed trace comes from, 'Curve' is the routing channel, and 'Page' is one of several memory pages in the internal memory of the TCSPC module. 'Page' can also be the step of a page stepping sequence, see 'Measurement Control Parameters' in the 'System Parameters' section. Please see also SPCM software description in [77].

Scanner and Laser Control

Overview

The Scanner and Laser Control panel allows you to start and stop the scanning, define the scan format, the scan area, the scan speed, the laser power, the active laser, or to define if and how lasers are multiplexed.

DCS-120 systems uses two different Scanner / Laser control modules. The GVD-120 module controls two lasers, the GVD-140 four. The corresponding control panels are shown in Fig. 168 and Fig. 169. The correct panel opens automatically when SPCM is started ('Application Options', page 109). If the panel has been closed unintentionally, open it by a click into 'Parameters' and 'B&H Galvano Scanner'.

In the upper left part the Scanner / laser control panel allows you to start and stop a regular scan, run a fast preview scan, define the type of the scan and the frame format. An automatic start of the measurement with the scan can be (and normally is) defined in the application options of the SPCM software, see 'Application Options', page 109. The standard scan type is 'Frame Square' and 'continuous'. There are other scan types, such as rectangular frames, line scans and 'point' scans. These are not used in basic FLIM experiments. The standard frame size is 512 x 512 pixels. Larger and smaller frame sizes can be defined. Definition of frame sizes requires that the same numbers are used in the page control section of the SPCM system parameters. This is achieved automatically (and permanently) by activating the little button next to the frame size field or by clicking on the arrow symbol.

'Scan Details' opens a panel that defines technical details of the scanner control, such as minimum and maximum line time, speed of the beam flyback, and compensation of the image shift with the scan speed. These settings need not normally be changed for standard DCS applications.

The scan rate is defined in the left, middle part of the scanner panel. Recommended selection is 'Auto', which automatically selects the fastest possible scan rate for the selected frame format and zoom factor.

The scan area is defined lower left. The image size is defined by the 'Zoom' factor, the offset of the scan area within the entire field of view is defined by offset. A 'Park Beam' mode is available for single-point measurements, FCS, or single-molecule experiments.

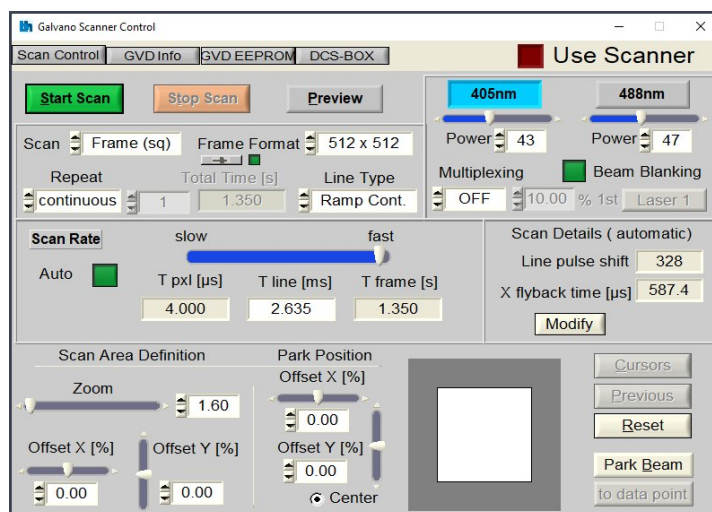


Fig. 168: Scanner control panel of the SPCM software. GVD-120, control of two lasers.

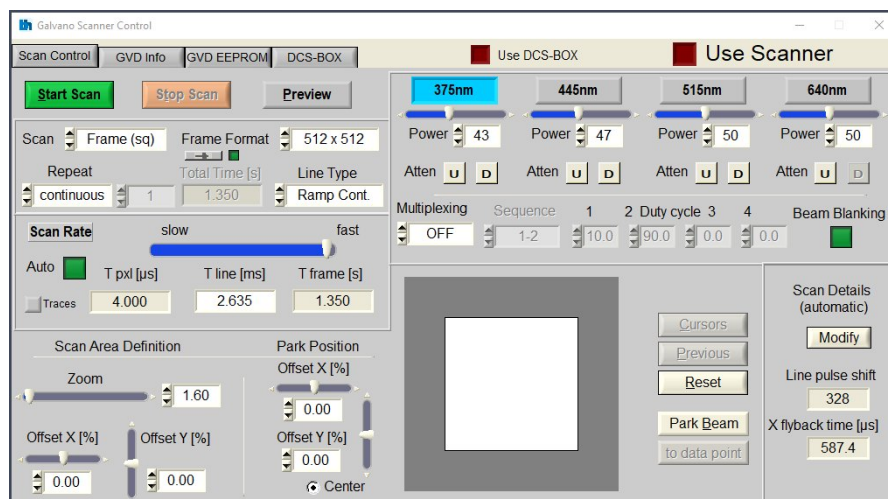


Fig. 169: Scanner control panel of the SPCM software. GVD-140, control of four lasers.

Lasers are activated in the upper right of the panel. In the basic modes of the DCS-120 only one laser is active at a time. 'Beam Blanking' means that the laser is turned off when the system is not scanning, and during the x and y flyback of the scanner.

Lasers can be multiplexed or turned on only for a part of the pixel time. These functions are controlled under 'Multiplexing'. They are used for advanced applications, see Excitation Wavelength Multiplexing, page 137 and 'Simultaneous FLIM / PLIM', page 172.

Scan Parameters

Frame Size

The frame size can be defined from 16 x 16 pixels up to 2048 x 2048 pixels. Please note that the 'Scan Pixels X' and 'Scan Pixels Y' in the TCSPC System Parameters should correspond to the selected image size. To achieve this automatically, activate the little button next to the frame format field. You can also click on the  symbol to transfer a new pixel number into the SPCM system parameters.

For normal FLIM acquisition the scan format is 'Frame'. Line scans can be performed by setting 'Scan' to 'Line'. After being started, scanning is normally continuously repeated (Repeat = 'continuous'). If necessary, the scan can be stopped after a defined number of frames.

'Total Time' is the time to scan one frame. It depends on the used frame size and scan rate. The times of the line and frame flyback are included in the total time.

Scan Rate

The scan rate is defined either by changing the line time or by moving the scan rate slider. Please note that the applicable scan rate depends on the image size and the zoom state. Therefore the scan rate may change if you change one of these parameters.

If you want to run a fast scan without taking care of these relationships you may activate the 'Auto' button. The software then automatically selects the fastest scan rate that can be used with the selected image size and zoom without compromising image linearity. Thus, the DCS-120 will scan fast, but not necessarily at the fastest rate possible. To obtain the absolutely fastest rate, turn off 'Auto', shift the rate slider to the right end, or type in a small line time. The shortest pixel times and frame times for different images sizes and zoom settings are shown in Table 1 and Table 2.

Image Size	Zoom = 1	Zoom = 2	Zoom = 4	Zoom = 8
128 x 128	12.8 μ s	6.4 μ s	4.8 μ s	3.2 μ s
256 x 256	6.4 μ s	3.2 μ s	2.4 μ s	1.6 μ s
512 x 512	3.2 μ s	1.6 μ s	1.2 μ s	0.8 μ s
1024 x 1024	1.6 μ s	1.2 μ s	0.8 μ s	0.6 μ s

Table 1: Minimum pixel time for different image size and zoom

Image Size	Zoom = 1	Zoom = 2	Zoom = 4	Zoom = 8
128 x 128	0.367 s	0.184 s	0.120 s	0.074 s
256 x 256	0.640 s	0.643 s	0.259 s	0.173 s
512 x 512	1.24 s	0.636 s	0.480 s	0.320 s
1024 x 1024	2.6 s	2.0 s	1.2 s	1.0 s

Table 2: Minimum frame time for different image size and zoom

Laser Control

Two (GVD-120) or four (GVD-140) bh BDS-SM picosecond diode lasers can be controlled from the scan control panel. The lasers can be switched on and off via the ‘Laser 1’ and Laser 2’ buttons. The power can be controlled by the ‘Power’ sliders. Important: The power sliders control the electrical driving power of the laser diode. Therefore also the shape of the laser pulse changes. The power sliders should therefore not be changed within a series of comparable measurements.

For FLIM operation the bh diode lasers can (and should be) used with beam blanking. That means, the lasers are turned off when the system is not scanning and during the line and frame flyback. Beam blanking thus helps reduce photobleaching of the sample. Please note that beam blanking has to be turned off for measurements in the ‘Park Beam’ mode.

If two lasers are operated simultaneously they can be multiplexed on a pixel-by-pixel, line-by-line, or frame-by-frame basis. If only one laser is used ‘multiplexing’ must be ‘off’.

Preview

Clicking on the ‘Preview’ button starts a fast scan at a resolution of 256 x 256 pixels. Simultaneously a measurement is started, and the results are displayed in one-second intervals. The measurement runs with only one time channel, i.e. without time resolution. The ‘Preview’ mode is used to adjust the focal plane, find the right spot in the sample, identify objects for further investigation, and adjust the zoom and the scan area. The scanner panel in the preview mode is shown in Fig. 170, left, a typical preview image in Fig. 170, right.

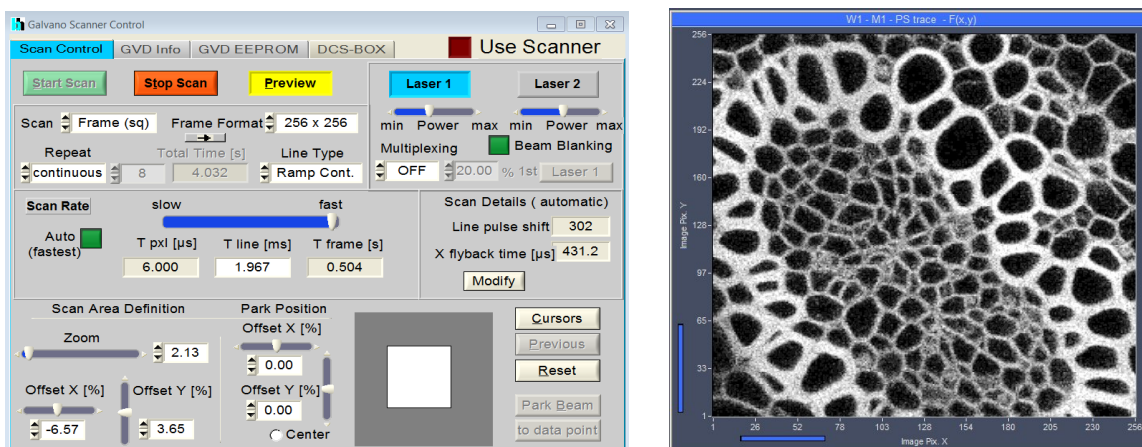


Fig. 170: Scanner panel in the ‘Preview’ mode

The preview is stopped by clicking the 'Preview' button a second time. The system then returns to the configuration it was in before the preview was started.

Scan Area

The scan area can be changed by the parameters under 'Scan Area Definition'. 'Zoom' determines the size of the scan, 'Offset' the centre position, see Fig. 171. The approximate location of the selected area within the maximum scan area is shown in a graph on the right. You can shift the area with the mouse cursor. 'Reset' resets the scanner to the maximum scan area.

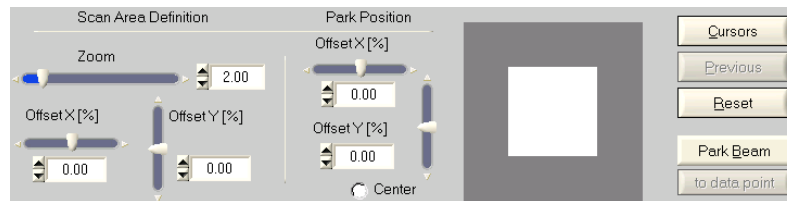


Fig. 171: Selecting a scan area in the scan control panel

After a preliminary image has been recorded the scan area can be selected from the display window, see Fig. 172. A right mouse click into a display window opens the select panel shown in Fig. 172, left. Select 'Enable Cursors' and select an area of interest (Fig. 172, middle). Then click on 'Cursors' in the area definition part of the scan control panel (Fig. 172, right). The software then selects the closest possible scan area around the image area selected by the cursors.

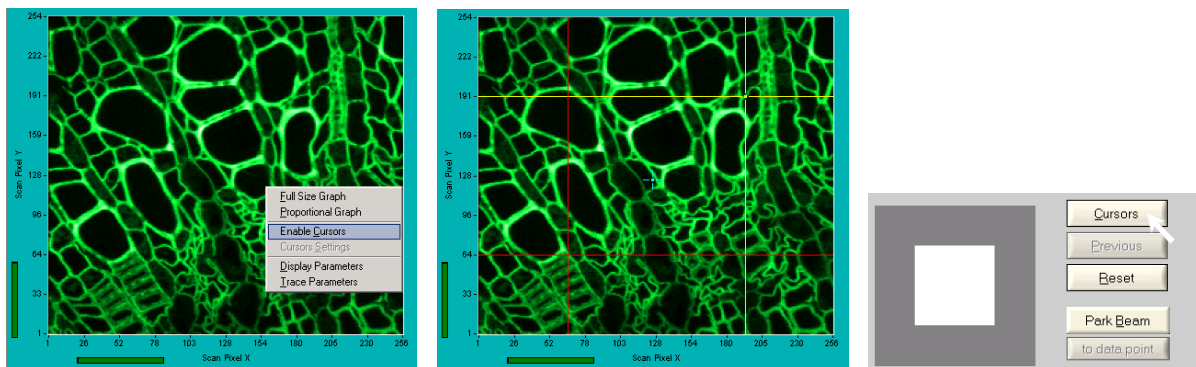


Fig. 172: Defining a scan area from the display window

Park Beam Function

'Park Beam' places the laser beam in a defined spot of the scan area. Please note that the beam blanking of the laser must be switched off to run a measurement in the beam park mode. In the scanner panel, the location can be set either by changing the Offset X and Offset Y of the park position, or by moving the yellow spot in the image area by the mouse cursor. Please see Fig. 173.

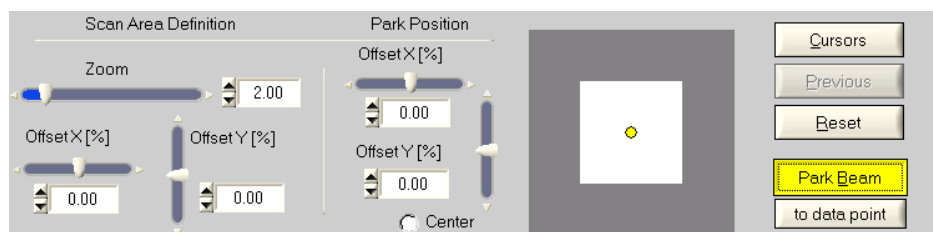


Fig. 173: Defining the beam position in the Beam Park mode

The beam-park position can also be selected in an image recorded before, see Fig. 174. Activate the cursors by a right mouse-click into the display window (Fig. 174, left). Set the 'Data Point' to the desired beam-park position (Fig. 174, middle). Then click on 'To Data Point' in the area-select part of the scanner panel, see Fig. 174, right.

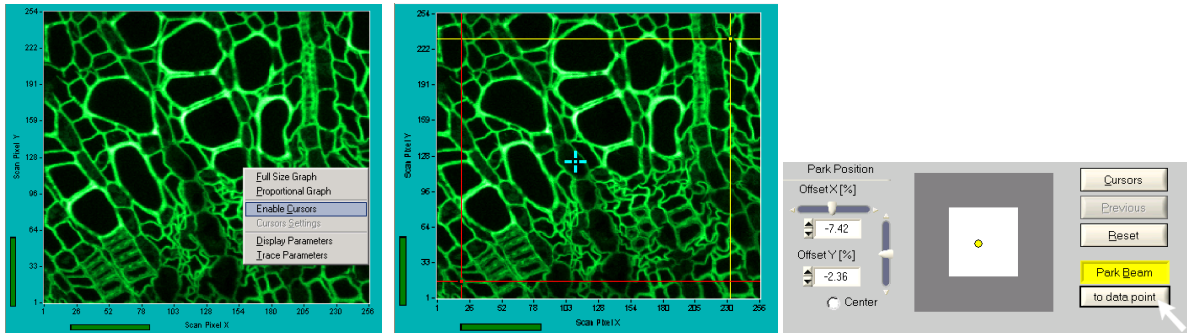


Fig. 174: Selecting the beam-park position in the display window

Scan Details

'Scan Details' define technical details of the scanner control, such as minimum and maximum line time, speed of the beam flyback, and compensation of the image shift with the scan speed. These settings need not normally be changed for standard DCS applications. The corresponding panel opens via the 'Modify' button. The panel is shown in Fig. 175.

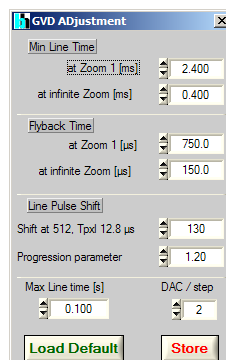


Fig. 175: Panel for adjusting the details of the scanner control

There is 'Minimum Line time' and 'Flyback Time' for zoom 1 and for infinite zoom, the shift of the line pulse, and its variation with the zoom and the scan speed. The parameters are used by SPCM to calculate the maximum forward and flyback speed of the scanner in dependence of zoom and line time and set the internal register values of the GVD accordingly. The parameters are mainly used to adapt the GVD-120 and GVD-140 scan controller to different scanners. For standard DCS-120 systems we discourage to change these parameters.

Assigning Names to the Laser Buttons

The laser buttons can be assigned user-defined names. The names are defined under 'GVD EEPROM', see Fig. 176, left. The appearance in the scanner control panel is shown in Fig. 176, right.

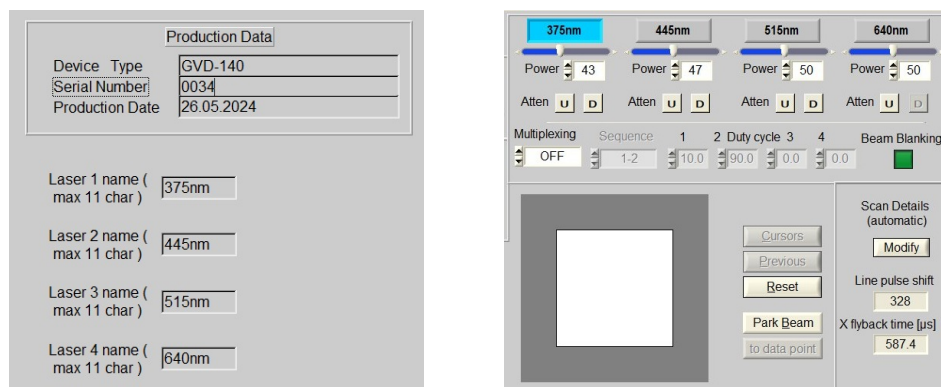


Fig. 176: Assigning user-defined names to the Laser buttons. Left: Definition of names. Right: Appearance in Scanner / Laser panel. Right part shown only.

System Parameters

The 'System Parameters' define the internal functions of the SPC modules, the operation mode, the synchronisation of the FLIM recording with the FLIM scanner, the data transfer between the hardware and the software, the control of sequential measurements, routing parameters, and accumulation and autosave functions. These parameters are similar for SPC and SPC-QC series modules.

The system parameters also define the discriminator settings for the synchronisation and detector inputs and the timing parameters for the measurement of the photon times. These parameters are different for SPC and SPC-QC modules.

SPC Series

The typical system parameter panel for SPC series modules is shown Fig. 177. The panel is shown in a configuration as it is used for basic FLIM measurements.

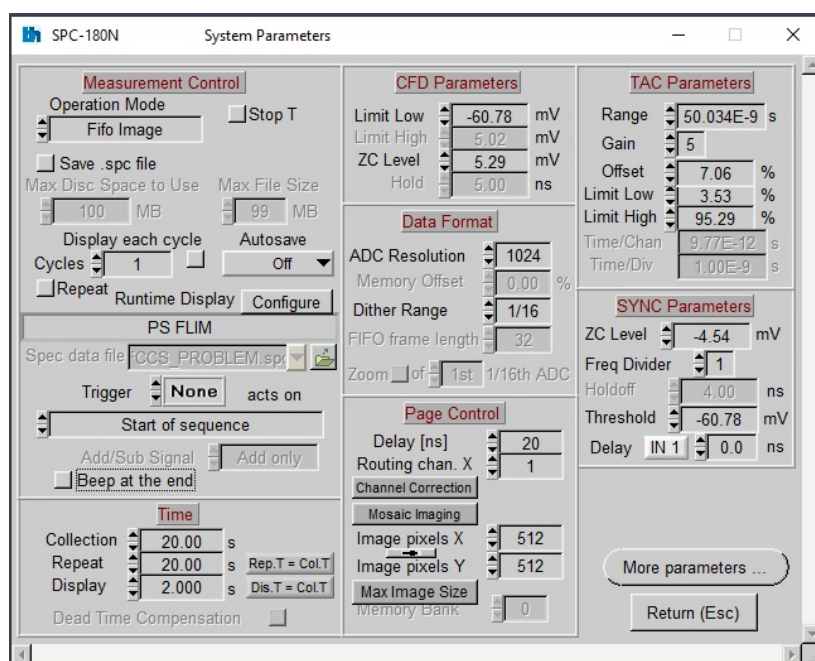


Fig. 177: System parameter panel, SPC-180N

The panel contains a measurement control section to set the operation mode, to configure the measurement procedure by defining page stepping, cycle, accumulate and autosave functions, and to define the action of the experiment trigger. Operation mode for FLIM is 'FIFO Imaging', saving of time-tagged single-photon data, autosave and Experiment Trigger are disabled, and only one measurement 'Cycle' is performed for each measurement.

The collection time, repeat time, and display time are defined in the 'Time' section below the measurement control parameters.

Under 'Data Format' and 'Page Control' the ADC resolution (number of time bins per pixel), the ADC error correction parameter, the number of detector channels, the number of the pixels of the scan, and the memory page in which a measurement is started are defined.

The CFD, SYNC, and TAC parameters are arranged in the upper right. The CFD and SYNC parameters define the discriminator thresholds and zero-cross levels for the detector and the synchronisation input. The values shown are for HPM-100 hybrid detectors and BDS-SM picosecond diode lasers.

'More Parameters' opens an additional panel that contains hardware parameters which are specific for the particular operation mode. In the FLIM mode this part defines the synchronisation with the FLIM scanner.

SPC QC Series

The SPC-QC series modules differ from the SPC series in that there are four input channels, and the time-conversion is performed by a TDC. Hence there are four parameter sets for the input discriminators, and different timing-control parameters. The SPC-QC is described in a separate manual, please see [79]. An SPC-QC-104 system parameter setup as it is used for the DCS-120 FLIM systems is shown in Fig. 178.

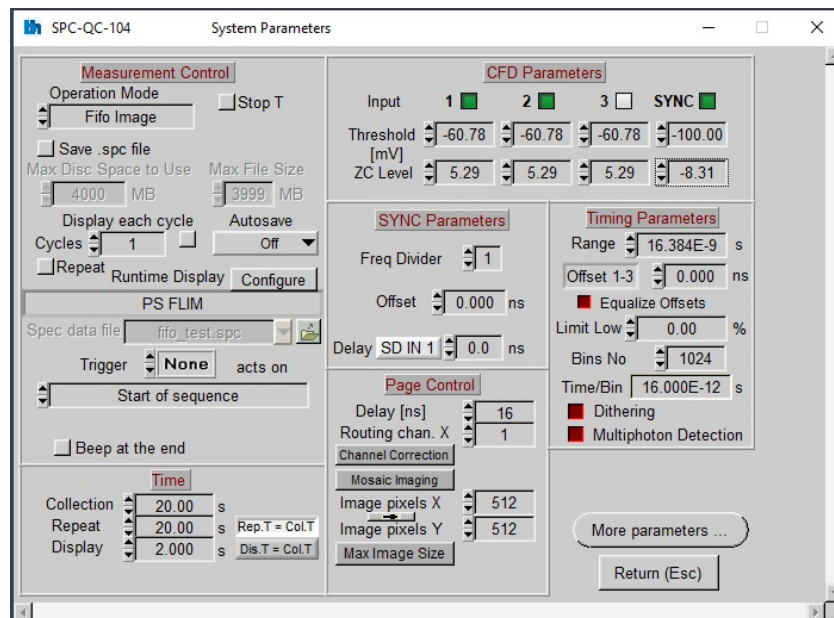


Fig. 178: System Parameter panel, SPC-QC-104

The measurement control section is the same as for SPC series modules. The CFD and SYNC parameters define the discriminator thresholds for the detector and synchronisation inputs of the module. There are three inputs for detectors and one input for synchronisation with the laser. The inputs can be enabled or disabled by the buttons on top of the CFD-parameters section. Inputs which are not used should be disabled to avoid unnecessary data transfer from the SPC-QC to the software. The settings shown are for typical DCS systems with two HPM-100 detectors and BDS-SM ps diode lasers.

The Sync parameters also contain a frequency divider to record decay data over several laser pulse periods, and a timing offset to compensate for optical and electrical delay in the detection path.

The timing parameters (middle right) define the width of the recording-time interval and its location within the laser pulse period (Range and Offset), and the number and the width of the time bins into which decay data are recorded. 'Dithering' smoothes out non-uniformities of the time conversion characteristics, 'Multiphoton Detection' allows for recording of several photons within one laser pulse period. Please note that multiphoton detection has no effect for observation-time intervals shorter than the dead time of the SPC-QC module.

Operation Modes

The operation mode selection panels of the bh TCSPC modules are shown in Fig. 179. The mode used for FLIM recording is 'FIFO Imaging'. Other modes may be used for special application of a FLIM system.



Fig. 179: Operation modes. Left: SPC-180N. Right: SPC-QC-104

The '*Single*' mode records one decay curve for each of the detectors connected to the SPC module(s). It can be used for fluorescence decay measurement with the laser beam being parked in a pixel of interest, or in combination with scanning. If used in combination with scanning it delivers an average decay curve over the complete scan area.

The '*Oscilloscope*' mode performs a repetitive measurement of decay curves and displays the results like an oscilloscope. The mode is an excellent tool for setup, maintenance and alignment purpose.

The $F(t,T)$ mode runs a time-controlled sequence of '*Single*' measurements. It is useful for single-point photobleaching experiments, experiments of photodynamic therapy, and for recording chlorophyll transients.

The $F(t,EXT)$ mode is implemented for recording sequences of curves in connection with external experiment control. The $Fi(T)$ and $Fi(EXT)$ modes record time-gated intensity curves.

The '*Scan Sync In*' mode is one of the two FLIM modes used in combination with laser scanning microscopes. The SPC module records a photon distribution over the time in the laser period and over the coordinates of the scan area. The photon distribution is built up in the memory of the SPC module. The computer is not involved in the data acquisition process. Thus, the acquisition speed is not limited by the bus transfer rate or any software response times. This limitation has become less and less significant for modern computers. Presently, the Scan Sync In mode is used for special applications only.

The '*Continuous Flow*' mode combines the Scan Sync In mode or the measurement of decay curves with continuous sequential operation. In this mode the memory is split in two banks. While the module is recording in one bank the other bank is read. Then the banks are swapped and the measurement continues. Thus, fast sequences of images or curves can be recorded without spending time on the readout on the data. The mode is used in NIRS and fNIRS applications.

'*Scan Sync Out*' is an imaging mode that actively controls a scanner. It was implemented mainly for scanning with piezo-driven scan stages. The mode is not used for FLIM acquisition with galvanometer scanners.

The '*FIFO*' mode differs from the other modes in that the SPC module does not build up a photon distribution in its memory. Instead, the FIFO mode delivers information about each

individual photon and transfers it into the computer. FIFO-mode data are also called ‘time-tagged’ or ‘parameter-tagged’ data. Parameter tagged data contain the time of the photon in the laser period (micro time), the time since the start of the experiment (macro time), and, if several detectors are used, the number of the detector that detected the photon. The memory of the SPC module works as a first-in-first-out (‘FIFO’) buffer. The photon data are buffered and continuously transferred into the computer. The computer either processes the data online or saves them into single-photon (.spc) files. The FIFO mode is key to single-molecule techniques. It is also used to record FCS curves [470] in combination with fluorescence decay curves, photon counting histograms, or BIFL (burst-integrated fluorescence lifetime) data [67, 68, 211, 457]. The FIFO mode is normally used in combination with the ‘Park Beam’ function of the scanner.

‘FIFO Imaging’ is the standard FLIM mode for the DCS-120 FLIM systems. Different that the ‘FIFO’ mode it not only records parameter-tagged single photon data but also the pixel, line, and frame synchronisation pulses from the scanner. The data are buffered in the FIFO and read out by the computer. The SPCM software analyses the incoming data and builds up FLIM images. Because the FLIM images are built up in the main memory of the computer large pixel numbers and large numbers of time channels can be used [36]. With the introduction of 64-bit SPCM software the available memory space has become virtually unlimited [47, 563]. As a result, not only images of megapixel size but also complex photon distributions like multi-wavelength FLIM or spatial and temporal mosaic FLIM can be recorded.

The FIFO Imaging mode records photon times not only with reference to the laser pulses but also to the scan clock pulses or other external events. This allows photon distributions to be built up over two different time coordinates simultaneously. This ‘dual-time base’ operation is used to simultaneously record fluorescence and phosphorescence lifetime images. Please see ‘Simultaneous FLIM / PLIM’, page 172.

Measurement Control Parameters

Step, Cycle and Accumulate Function

The memory of the SPC modules provides memory space for a large number of decay curves. The memory may even hold data of several measurement blocks. Each block can contain a large number of decay curves. There may even be enough space to store the data of several images of moderate numbers of pixels and time channels. The individual memory blocks are termed ‘pages’. By defining a number of ‘steps’ greater than one a sequence of recordings can be defined that automatically steps through subsequent pages. A measurement sequence may also be repeated for several measurement ‘cycles’. The results of the cycles can be accumulated, written into subsequent memory pages, or saved into subsequent files.

Autosave Function

The result of a measurement or the results of the individual cycles can be automatically saved into subsequent data files (‘autosave’). The results of the cycles can also be accumulated (‘accumulate’ button).

Repeat Function

By activating the ‘repeat’ button the complete measurement cycle is repeated until it is stopped by user interaction.

Trigger

The start of a measurement, the steps of a page stepping sequence, or the cycles of a measurement sequence can be triggered by an external ‘experiment trigger’ [77]. The trigger function is used to record Z stacks or microscope-controlled time series in combination with laser

scanning systems of other manufacturers, e.g. with the Zeiss LSM series laser scanning microscopes.

Collection Time

Collection time is the acquisition time for the measurement. For measurements running only for one cycle or step it is the time for the complete measurement. If cycles or steps are defined it is the time for the individual cycles or steps. Please note that ‘Collection Time’ stops the measurement only if the ‘Stop T’ button is activated.

The measurement can be stopped at any time by an operator stop command. You may therefore run the measurement simply until you are satisfied by the signal-to-noise ratio of the images displayed.

Important: After a stop by the collection timer or an operator stop command the internal scanning machine completes the current frame. Artefacts by recording incomplete frames are thus avoided.

CFD, SYNC and TAC / TDC Parameters

These parameters control the constant fraction discriminators at the inputs of the detector and laser synchronisation signal, and the time conversion circuitry in the TAC or the TDC. CFD Limit Low and CFD Threshold and SYNC Threshold and Sync Zero Cross control the discriminators for the detector signal and for the synchronisation signal from the laser. TAC gain and TAC offset determine the time-conversion range of the measurement. We discourage to change these parameters unless you are familiar with the internal function of the TCSPC electronics. If you need changes of the parameters, please refer to the bh TCSPC handbook [77] or contact bh.

Parameter Setup for FLIM Operation

FIFO Imaging Mode

Configuration of different image data types

The types of images to be built up are defined in a panel ‘Select & Configure Histograms’, see Fig. 180, right. It opens by a click on the ‘Configure’ button in the measurement control part of the system parameter panel, see Fig. 180, left.

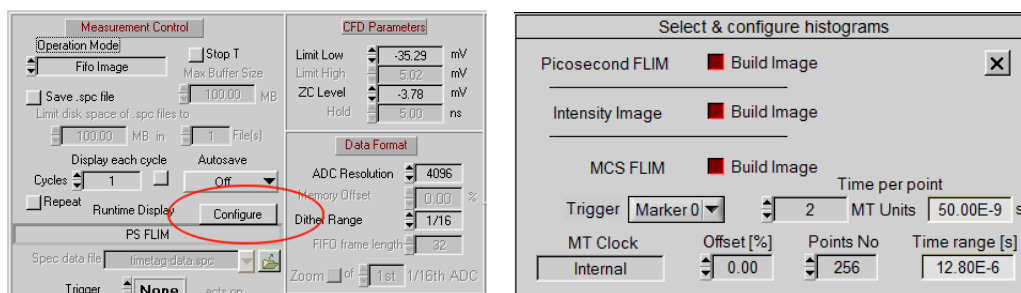


Fig. 180: Configuring the types of images to be built up in the FIFO Imaging mode.

The ‘Picosecond FLIM’ button selects normal FLIM. ‘Intensity Image’ selects the build-up of an intensity image from the parallel-counter channel of the SPC-180N, see [77]. The MCS FLIM button activates a multi-channel scaler (MCS) imaging mode. Instead of the photon times (micro-times) in the laser period it uses macro-time differences of the photons to a reference pulse at one of the marker inputs. In the DCS-120 system the MCS imaging mode is

used to record PLIM by the procedure described in section ‘Simultaneous FLIM / PLIM’, page 172. All three functions can be activated simultaneously, as shown in Fig. 180, right.

Display of different image types

Images of different data types can be recorded simultaneously and displayed simultaneously. Display windows for the images are defined in the ‘3D Trace Parameters’, please see ‘Examples for Display’, page 75.

Measurement Control

The basic parameter setup for FIFO imaging is shown in Fig. 181, left. The operation mode is ‘FIFO Imaging’, the cycle, repeat, and autosave functions are turned off. The .spc (parameter-tagged photon data) are not saved. Instead, the SPCM software analyses the FIFO data online. What the software does with the data is defined in the ‘Configure’ menu, as described above. Stop T is not set, the measurement is stopped by the operator when an appropriate image quality has been reached. The collection time, display time, and repeat time parameters have the same effects as in the other operation modes. The Display Time defines how often intermediate images are displayed. Typical measurement-control setups are shown in Fig. 181.

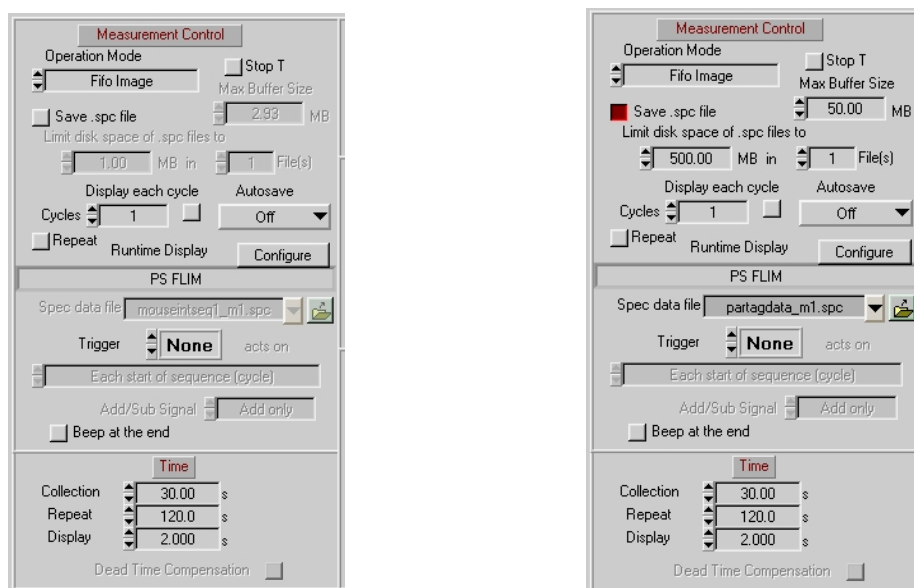


Fig. 181: Measurement control parameters for FIFO Imaging. Left without, right with saving of time-tag data.

For special data analysis procedures [57] it may be desirable to save not only the final FLIM images but also the parameter-tagged photon data. A measurement control parameter setup with saving of the time-tag data is shown in Fig. 181, right. Because time-tag data can be extremely voluminous the file size is limited by ‘Limit .SPC File Size’. When this file size is reached the recording stops. The name of the FIFO data file is specified in the ‘Spec Data File’ field. To change the file name, click on the disc symbol right of the name field. A list of previously used data file names is available by clicking on the ▼ symbol. *Important:* Before starting a new measurement define a new file name for the FIFO data. If you don’t do so you risk overwriting the FIFO data of an earlier measurement.

Before the data are written to the hard disk they are buffered in the main memory of the computer. The buffer size is defined by ‘Max Buffer Size’. The SPCM software checks whether enough memory is available; buffers sizes too large are rejected.

Both the start and the stop of a FIFO imaging measurement are synchronised with the frame clock. This avoids intensity steps in the images by recording incomplete frames. An unconditional stop of the measurement can be forced by a click on the stop-measurement button followed by a second click.

Image Format in FIFO Imaging Mode

The 64-bit version of the SPCM software achieves much larger image formats than the older 32-bit versions. The available image size depends on the amount of memory installed in the computer. Different combinations of pixels numbers, time-channel numbers, and numbers of routing channels are shown in Fig. 182. Images with up to 2048 x 2048 pixels can be recorded while maintaining an ADC resolution of at least 256 time channels.

ADC Resolution	Memory Offset	Dither Range	FIFO frame length	Zoom	Delay [ns]	Routing chan. X	Routing chan. Y	Image pixels X	Image pixels Y	Max Image Size	Memory Bank
4096	0.00 %	1/16	32	1st	0	1	1	512	512	0	0
1024	0.00 %	1/16	32	1st	0	1	1	1024	1024	0	0
256	0.00 %	1/16	32	1st	0	1	1	2048	2048	0	0
256	0.00 %	1/16	32	1st	0	16	1	512	512	0	0
64	0.00 %	1/16	32	1st	0	16	1	1024	1024	0	0

Fig. 182: Time-channel, pixel, and detector channel number of typical FLIM data formats. 64 bit SPCM software.

The amount of memory Windows allocates for SPCM can be changed manually by clicking on Max Image Size in the Data Format definition. This opens the panel shown in Fig. 183.

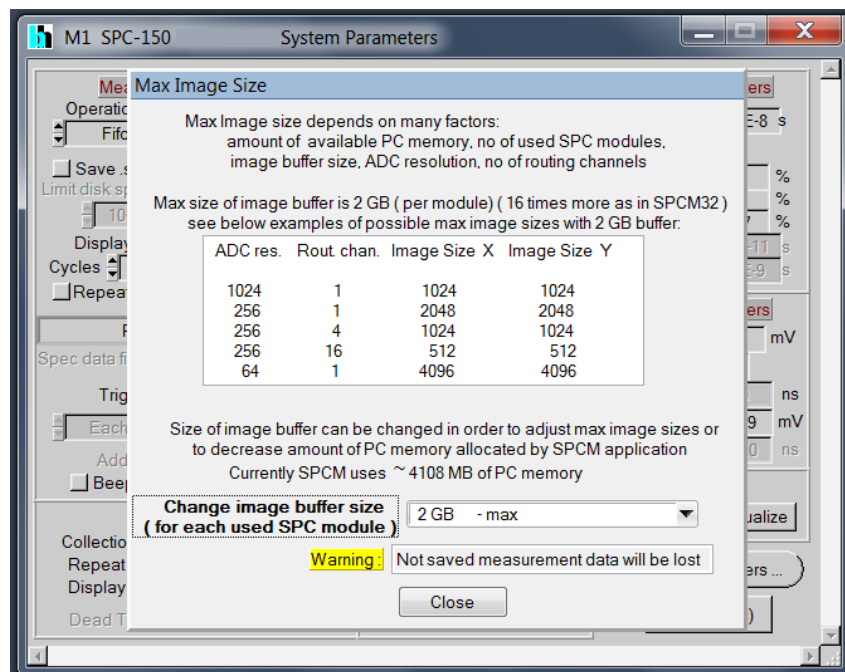


Fig. 183: Definition of maximum amount of memory reserved for image recording

Scan Synchronisation Parameters

FLIM recording in the SPC module is synchronised with the scanner via Frame Clock (Y Sync), Line Clock (X Sync) and Pixel Clock pulses. The hardware parameters controlling the synchronisation with the scanner are defined under 'More Parameters'. The More Parameters panel allows you to select the scan pulse polarity, a line and pixel predivider for pixel

binning, and a left boarder and upper boarder of the valid part of the scan. In combination with Pixel clock 'internal' or 'external' and Scan Type 'unidirectional' or 'bidirectional' these settings are used to adapt the bh FLIM systems to laser scanning microscopes of external manufacturers. The settings for the DCS-120 system are shown in Fig. 184. The polarity of all scan clock pulses is 'Positive', there are no line and pixel clock dividers, the pixel clock is external, and the scan type is unidirectional.

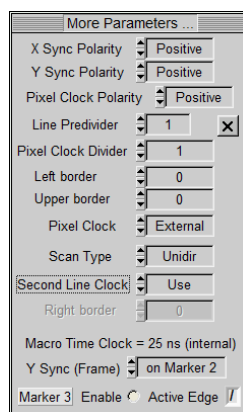


Fig. 184: Scan synchronisation parameters for the DCS-120 system

Event Markers

The scan clock pulses are detected via the 'Event Marker' inputs of the SPC SPC-QC modules. Normally, Marker 0, 1, and 2 are used for pixel, line, and frame clock, respectively. They are enabled automatically. The fourth marker input (Marker 3) is not normally used for FLIM with the DCS-120 and therefore not enabled, see Fig. 184. It can, however, be used for FLITS (see page 164). FLITS requires that the source of the frame clock be switched from the scanner to the stimulation source. This can be conveniently achieved by connecting the scanner frame clock and the FLITS trigger to Marker 2 and Marker 3, respectively, and selecting between both via the 'Y Sync (Frame)' source parameter in the scan synchronisation panel. Switching from FLIM to FLITS is then only a matter of loading the appropriate predefined setup.

Cycle, Repeat, and Autosave Functions

A FIFO or a FIFO Imaging measurement can be repeated for a defined number of cycles, and the results saved into individual data files. For using these functions, please set Stop T to stop the individual cycles after the defined collection time.

Image Pixels X and Y

Image Pixels X and Image Pixels Y define the pixel numbers at which FLIM data are recorded, see Fig. 185. To obtain correct images the numbers have to correspond with the numbers defined in the scanner panel, see 'Scan Parameters', page 94. The pixel numbers for the scanner can be synchronised with the pixel numbers of the recording by clicking the pointer sign next to the pixel numbers.

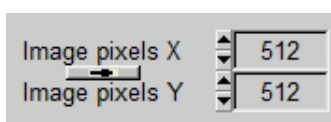


Fig. 185: Definition of the pixel numbers of the image recorded

Routing

Routing signals are used to direct photons into different memory blocks. Frequent applications are multi-wavelength detection and laser multiplexing. In the first case a multi-wavelength detector generates a routing signal that indicates in which wavelength channel of the detector the current photon was detected. In the second case, the scan controller indicates which of two multiplexed lasers is currently on. The 'Routing' information under 'Page Control' defines how many routing channels are present, and at which moment after the detection of a photon the routing information is read by the SPC module. Please see Fig. 186.

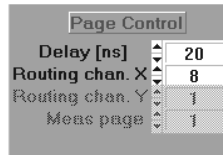


Fig. 186: Definition of the number of routing channels and the time at which the routing information is read after the photon detection.

Mosaic Imaging Function

SPCM 64 bit has a 'Mosaic Imaging' function to record arrays of lifetime images. The array can represent a spatial array of images scanned with the 'Tile Imaging' function of a laser scanning microscope, images in different depth of a sample, a time-series of images, or images in different wavelength channels of a multi-wavelength detector. Please see 'X-Y Mosaic FLIM', page 162 and following for details. The definition of the mosaic FLIM parameters is shown in Fig. 187. Every element of the mosaic has the format defined in the Data Format section, see Fig. 182. With the parameters shown in Fig. 187 an array of 64 images of 256x256 pixels would be recorded, see Fig. 187, right. Mosaic data can be accumulated to record fast time series for repeatable processes. The restart of the mosaic recording can be triggered by a signal at the trigger input of the TCSPC module or by a pulse edge at the Marker 3 input.

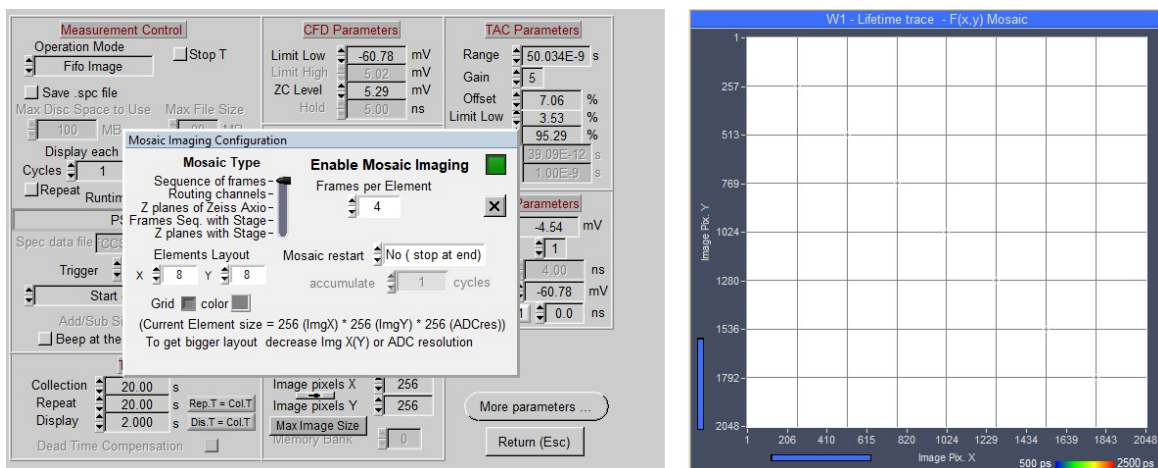


Fig. 187: Definition of a FLIM mosaic

Microsecond Lifetime Imaging: MCS FLIM

The SPCM software has a 'Triggered Accumulation MCS' option in the FIFO or FIFO Imaging mode. The MCS mode uses the macro times of the photons to record decay data at the microsecond or millisecond time scale. The timing reference for the photons is a pulse at one

of the ‘Marker’ inputs of the SPC card. The triggered MCS procedure can also be used to record microsecond or millisecond lifetime images in the FIFO Imaging mode. ‘MCS FLIM’ is defined in the lower part of the runtime definition panel, see Fig. 188.

‘Trigger’ defines which of the marker inputs is used as a timing reference. It is usually Marker 3 because Markers 0, 1, and 2 are used for the scan clocks. In PLIM experiments, the effective excitation pulses can be synchronous with the pixels. ‘Trigger’ can then be identical with the pixel clock, i.e. Marker 0. Please make sure that the marker input used for trigger is enabled in the ‘More Parameters’ panel of the System Parameters, see Fig. 188, right.

The time channel width (Time per point) can be any multiple of the macro time clock period. It is defined by a number of Macro Time units. The number of points of the curves is defined by ‘Points No.’. The time range of the curves recorded is then given by the product of Time per Point and Points No. It is displayed under ‘Time range’. The recording can be shifted in time by applying an ‘Offset’ to the photon times. Both positive and negative offsets are possible.

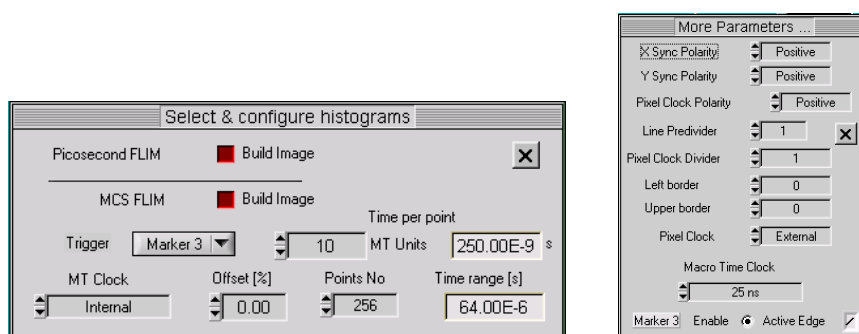


Fig. 188: Left: Definition of MCS FLIM parameters. Right: Definition of the macro time clock source and the marker clock edge

MCS FLIM works in combination with normal FLIM recording, see option ‘ps FLIM’ in the configuration panel. The combination of both requires that a high-frequency pulsed laser is turned on and off at a period in μs or ms range. Please see ‘Simultaneous FLIM / PLIM’, page 172. MCS FLIM works also in combination with routing.

Switching Between Different Instrument Configurations

The entire system parameter set, including the user-interface configuration, is restored when the corresponding measurement or setup data are loaded. To simplify switching between different configurations the SPCM software has a ‘predefined setup’ panel, see Fig. 189. Setups of frequently used system configurations are stored in this panel, and then recalled by a single mouse click, see Fig. 190. We recommend to keep the predefined setup panel constantly open, see Application Options, page 109. For definition of predefined setups, please see page 121.

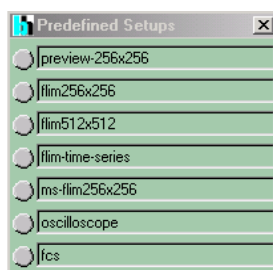


Fig. 189: Predefined-Setup panel. You can change between different instrument configurations by a single mouse click, see figure below.

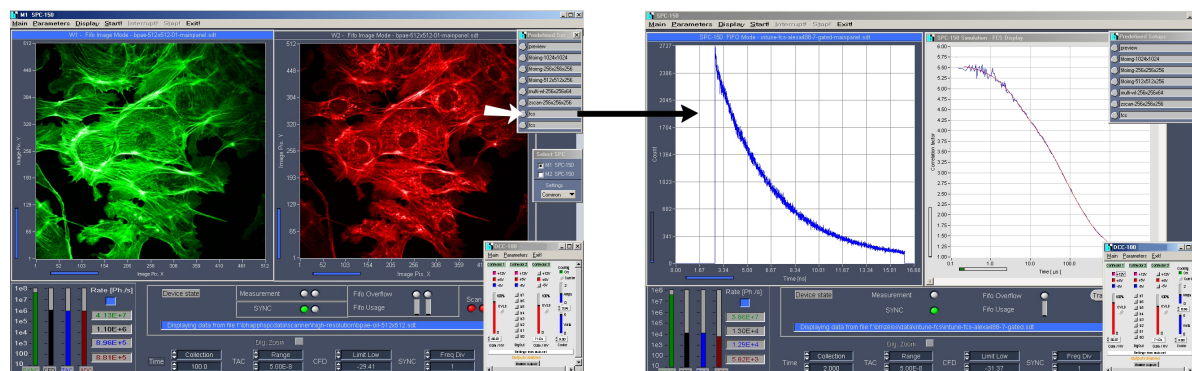


Fig. 190: Switching the instrument configuration via the 'Predefined Setup' panel

Application Options

Additional software configuration data can be defined in the 'Application Options' panel, see Fig. 191. These parameters are not stored in the SPCM data or setup files but in the registry of Windows. They are thus specific of your FLIM system, not of the data recorded.

'Autostart' specifies which control panels open automatically when SPCM is started. For the DCS-120 we recommend to open 'Detector Control', 'Scanner Control' and 'Predefined Setups'.

'Main Panel' enables or disabled the display of the TAC, CFD, and SYNC parameters in the main window.

The 'Scanner Control' defines features of the scanner control in the DCS-120 and other systems using the bh GVD-120 or -140 scan controller. The parameters have no influence on FLIM systems for microscopes that have their own scanner control, such as the FLIM systems for the Zeiss LSM 710/780/880/980 microscopes [34, 35].

The load options (upper right) allow you to maintain the current scan parameters, FCS fit parameters, display window configurations, and DCC or DCU settings when you load new data or setup files.

'Saving Behaviour' defines features of the data save procedure. Depending on these settings, the save procedure suggests the file name of the previous save operation, the current file name extended by a number, or the file name of a previous load operation. Similar options are available for the file description text in the file information field. Please handle these options with care, otherwise there is risk of overwriting previous data or file descriptions.

'Data Compression' contains options for the compression of FLIM data when they are saved. Without anything enabled data are compressed by a flexible loss-free algorithm that results in minimum data size and minimum compression time. With 'Legacy Compression' you can switch to a compression algorithm which was used in older SPCM versions. It is less efficient than the new one, and is much slower in terms of processing time. For communication with external software packages you can turn off data compression altogether. However, be aware that this results in extremely large data files.

'SPCImage Data Transfer' defines options for sending data to SPCImage NG. You can send data from all display windows, or data from the active display window only. You can also send data automatically at the end of a measurement. Please handle these options with care: Not every FLIM measurement delivers the desired result. Running a FLIM analysis automatically after *any* FLIM measurement may therefore not always be appropriate.

'Theme' allows you to switch from the 'light' version of SPCM to the 'dark' one and back. This option takes effect only after restart of SPCM.

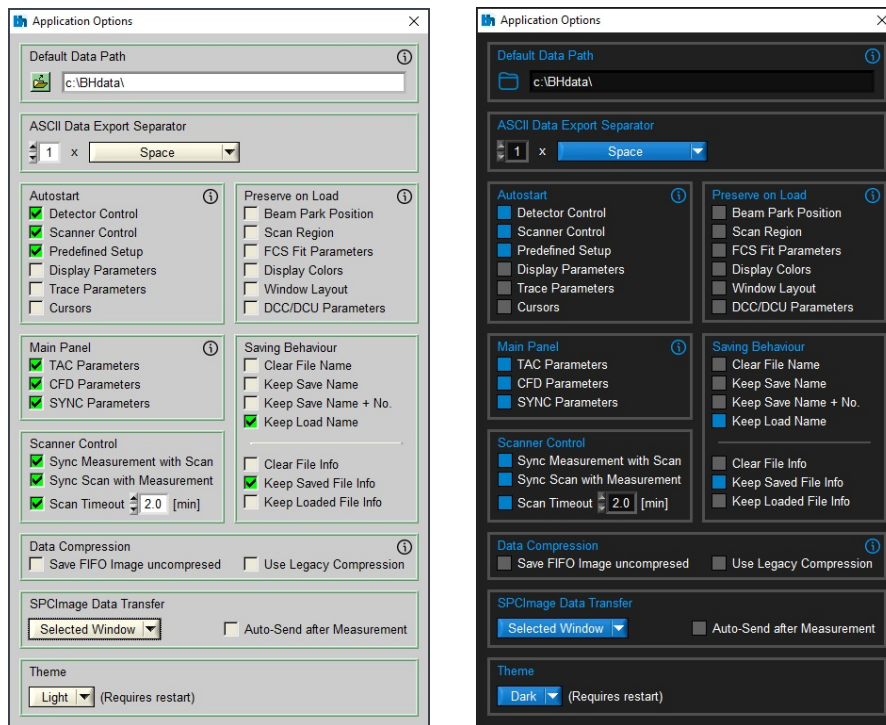


Fig. 191: Application Options. Left: 'Light' mode of SPCM software. Right: 'Dark' mode of SPCM

Link to SPCImage Data Analysis

The SPCM data acquisition software has a direct link to the SPCImage data analysis. There are several ways to initiate the data transfer. The first one is a click into 'Main', 'Send Data to SPCImage', see Fig. 192. Depending on what is defined in the 'Application Options' (see page 109) the functions sends either the data of the active window or the data of all windows.

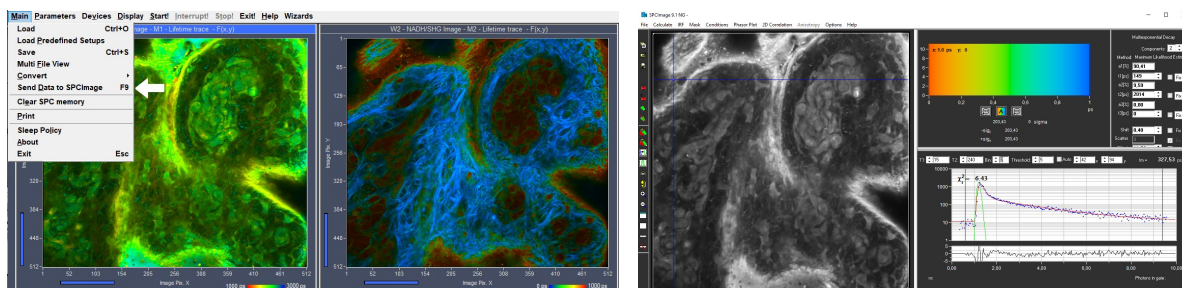


Fig. 192: Sending data to the data analysis from the Main menu. Click into 'Main', and 'Send Data to SPCImage' (left). SPCImage opens with the data sent to it (right). Data are not analysed yet, therefore the image is shown in grey scale.

The second way is a right-click into one of the image windows. This opens a selection panel from which you can select 'Send Data to SPCImage' from the 'Current Window' or from 'All Windows', see Fig. 193.

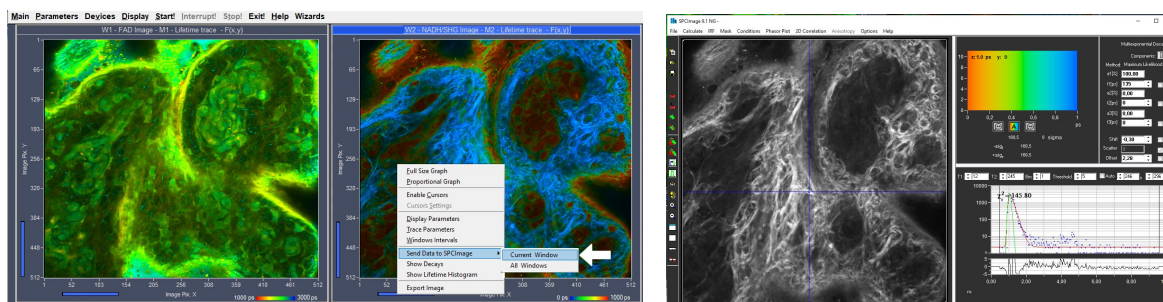


Fig. 193: Sending data to the data analysis by right mouse click. Right: Click into the image you want to send and select 'Current Window' or 'All Windows'. Right: SPCImage opens with the data sent to it (right).

A third way to transfer data to SPCImage is by automatically sending them at the end of every measurement. Please see 'Application Options', page 109. This function is not recommended for general use: Not every measurement produces useful data, and running through a full data analysis procedure every time may not be the most efficient way of using your time.

Trace Statistics

Clicking on the 'Trace Statistics' button opens a window which displays information about the data shown in the curve windows. For decay curves or other waveforms the FWHM values, the peak counts, the total counts and the first moment of the photon distribution are displayed, see Fig. 194, left. For an FCS window the trace statistics window displays the results of the FCS fit, see Fig. 194, right.

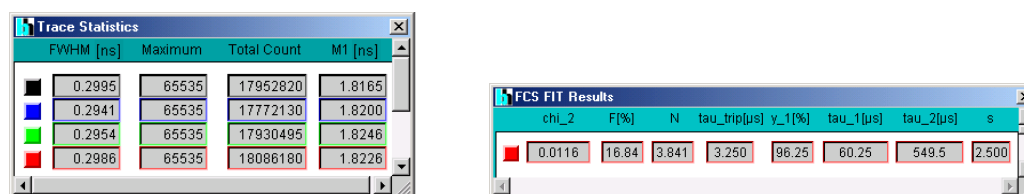


Fig. 194: Trace statistics panel. Left for a waveform window, right for FCS window

The window can be placed anywhere in the screen area. Please note that the trace statistics window works also in the oscilloscope mode. The window is thus an efficient tool to adjust the system for best time resolution, correct signal transit time, counting efficiency, or IRF stability.

Starting and Stopping a Measurement

Manual Start-Stop

A measurement can be started and stopped by the start and stop buttons in the command bar on top of the SPCM main panel or by the 'Start Scan' and 'Stop Scan' buttons in the scanner control panel.

'Stop on T' Option

The SPCM software can stop the measurement also after a defined acquisition time. To use this function define the desired 'Collection Time' and set the 'Stop T' button, see lower right part of the SPCM main panel.

Start-Stop Synchronisation with the Scanning

The start and the stop of a FLIM acquisition is automatically synchronised with the scanning. No matter whether you start the FLIM system first or the scanning: The measurement will not start until the TCSPC system receives the first frame clock pulse from the scanner. When you

stop the measurement the FLIM acquisition continues until the scanner has finished the current frame. Consequently, the FLIM data always contain a number of complete frames. Horizontal steps in brightness caused by recording incomplete frames result are thus avoided.

Automatic Actions at the End of the Measurement

Auto-Saving the FLIM Data

When a measurement has been completed the SPCM software can save the data automatically into a file. The file name is defined in the Measurement Control section if the SPCM System Parameters. Define Autosave 'End of Measurement' and specify a file name. The file name will be extended by a number as more files are saved.

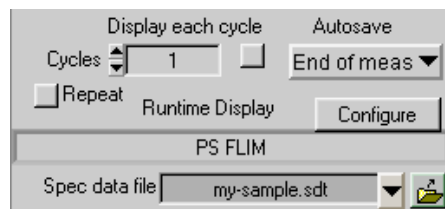


Fig. 195: Auto-saving FLIM data at the end of the measurement

Auto saving may look convenient but has also a few disadvantages. The first one is that not every FLIM measurement yields good data. The sample may have moved during the acquisition time, the focal plane may have changed, or the image got corrupted otherwise. The autosave function will save all these 'junk data', and you have to seek out the good images from the junk later. (Please note that the SPMC software has a 'Multi-File View' function for that.) Moreover, the autosave function does not let you type in sample information in the file info field. Written information about the individual measurements is thus not included in the files.

Automatic Transfer to SPCImage

The FLIM data can be transferred to the SPCImage data analysis at the end of the measurement. Please see 'Application Options', page 109. However, the automatic transfer does not decide whether the data you recorded are good images or not. It will also transfer the 'junk' images to the data analysis, and possibly drive you crazy with stubborn attempts to analyse them. Moreover, please note that the raw data are not automatically saved before they are transferred to SPCImage. We therefore suggest to use the Automatic Data Transfer function in combination with the Autosave function.

Module Select (Multi-SPC Systems)

The Multi SPC Software is able to control up to four TCSPC modules. The information shown in the status window of the SPCM software belongs to the module in the 'Select SPC' panel, see figure right. The 'Select SPC' panel can be placed anywhere in the screen area.

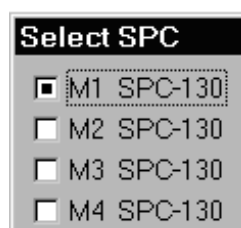


Fig. 196: Module-select panel. Parameters, count rates, and status information are displayed for the selected module

Ti:Sa Laser and AOM Control

In multiphoton systems with a Ti:Sa laser and an AOM the SPCM software is able to control the Laser and the AOM directly. The control panel opens by a click into ‘Devices’, ‘Ti:Sa laser & AOM Control’. The panel is shown in Fig. 197, the laser and the AOM in Fig. 198.

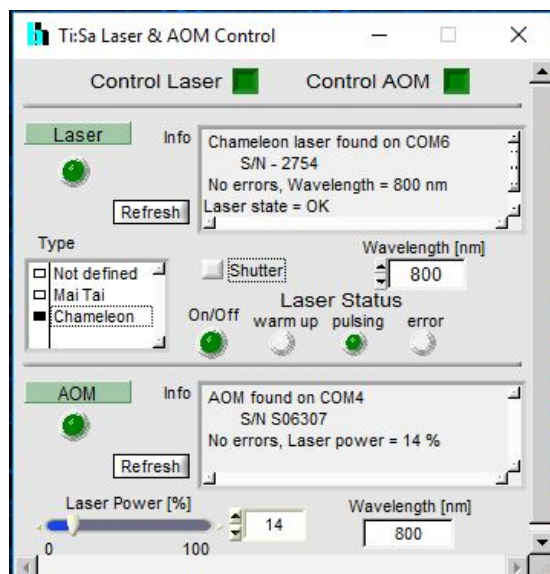


Fig. 197: Ti:Sa laser and AOM control panel.



Fig. 198: Coherent Chameleon laser and bh AOM

The laser is controlled in the upper part of the panel. The type of Ti:Sa laser must be selected under ‘Laser’, ‘Type’. The system computer connects to the laser via a USB interface. If it does not connect automatically you can force the connection by clicking on ‘Refresh’. The laser panel controls the shutter of the laser and the wavelength, and displays the status of the laser.

The lower part of the panel controls the bh AOM. Since an AOM is wavelength-dependent the AOM controller has to know the wavelength selected for the laser. If both the laser and the AOM are controlled from the panel shown in Fig. 197 the wavelengths for the laser and the AOM are automatically coupled.

The reason why an AOM needs to be tuned to the wavelength of the laser is shown in Fig. 199. An acoustic wave is excited in the AOM crystal electrically by a high-frequency signal. The wave acts like a grating, and diffracts away a part of the laser light. The amount of diffracted light depends on the amplitude of the high-frequency signal. The angle at which the light is diffracted depends on the frequency and on the wavelength of the light. To obtain a constant diffraction angle the frequency of the electrical signal must be adjusted to the wavelength to maintain a constant angle of the output beam.

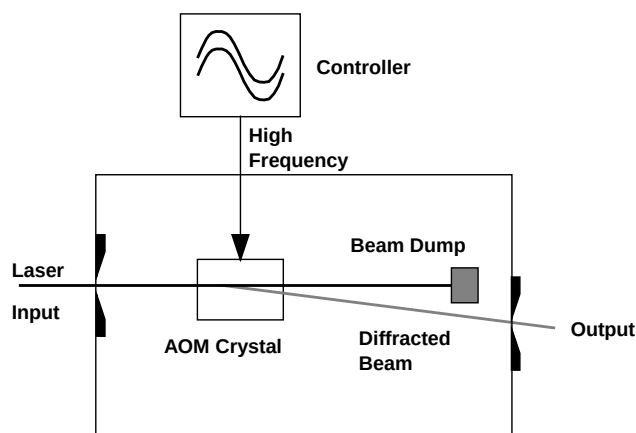


Fig. 199: Principle of the AOM

Maintaining a constant output beam angle is important. If the angle is not constant the laser beam moves out of the optical axis of the scanner and the microscope lens. The result is loss in optical resolution, excitation efficiency, or even failure to record an image at all. Therefore, if you use an AOM with a laser that is not controlled by SPCM software, you have to type the correct laser wavelength into the AOM control panel.

Motorised Sample Stages

Starting from version 9.76, SPCM is able to control a motorised sample stage. So far, the ES 111 motor stages with the ES 11 and Pro Scan III controllers of Prior are integrated but other will possibly follow. The stage can be used to control the position of a sample manually or to record mosaics of images in cooperation with the GVD-120 scan controller of the bh DCS-120 system. A photo of a DCS-120 MACRO system with the motor stage is shown in Fig. 200.

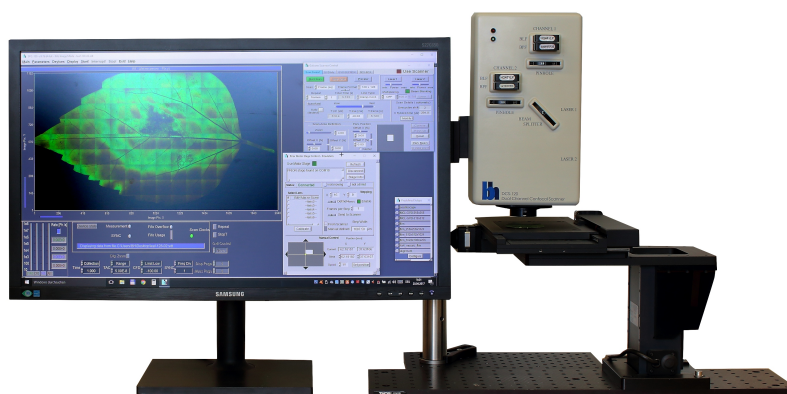


Fig. 200: DCS-120 MACRO scanner with motor stage

The motor stage control panel is shown in Fig. 201. The panel shown left has Tile Imaging disabled, i.e. the stage can be controlled manually but does not interact with the DCS-120 scanner. When a DCS scan is started the system records a normal FLIM image in the selected place of the sample. The panel on the right has Tile Imaging enabled. The motor stage interacts with the scanner, and, in cooperation with the DCS scanner, a spatial mosaic of images is recorded.

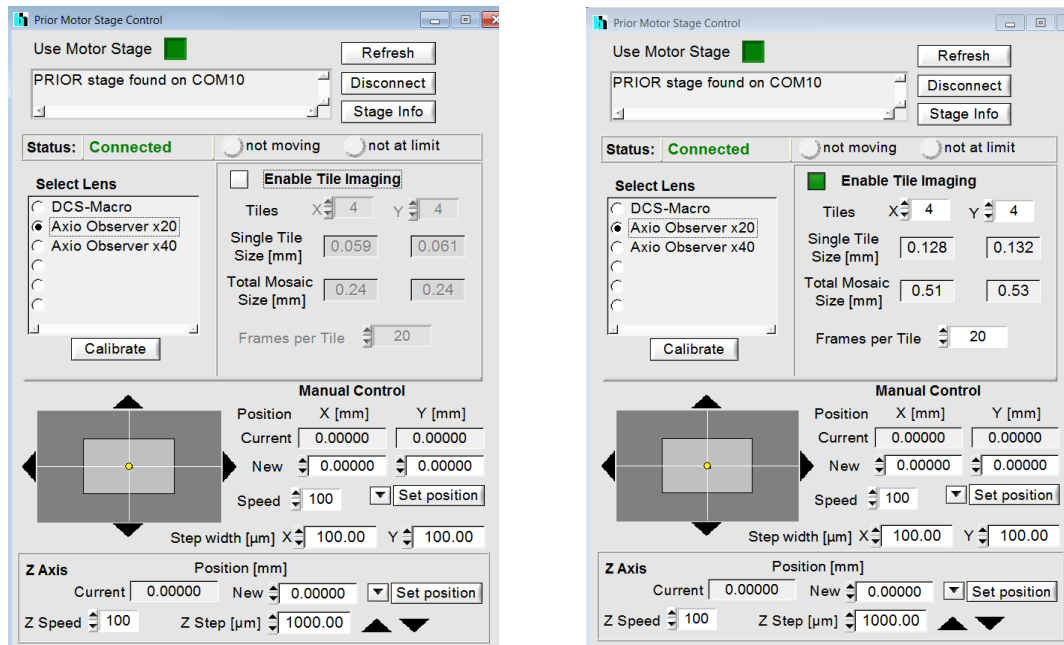


Fig. 201: Control panel for motor stage. Left: Tile imaging disabled. Normal scanning, manual control of motor stage. Right: Tile imaging enabled. Spatial mosaic scanning and manual control of stage.

Fig. 202 shows how the optical scanner interacts with the motor stage. When Tile Imaging is enabled the step width of the motor stage automatically adjusts to the scan area (Zoom factor) selected in the DCS scanner panel. When the measurement is started the SPC system records a mosaic of FLIM images the elements of which have the same x and y size as the step width of the motor stage. The result is a large image consisting of individual scans offset by the motor-stage step width. The data of the individual elements of the mosaic (or tiles) can be accumulated over a selectable number of frames (20 in Fig. 202). The number of frames per tile can be selected both in the motor stage panel and in the scanner panel. It is automatically adjusted to the same value.

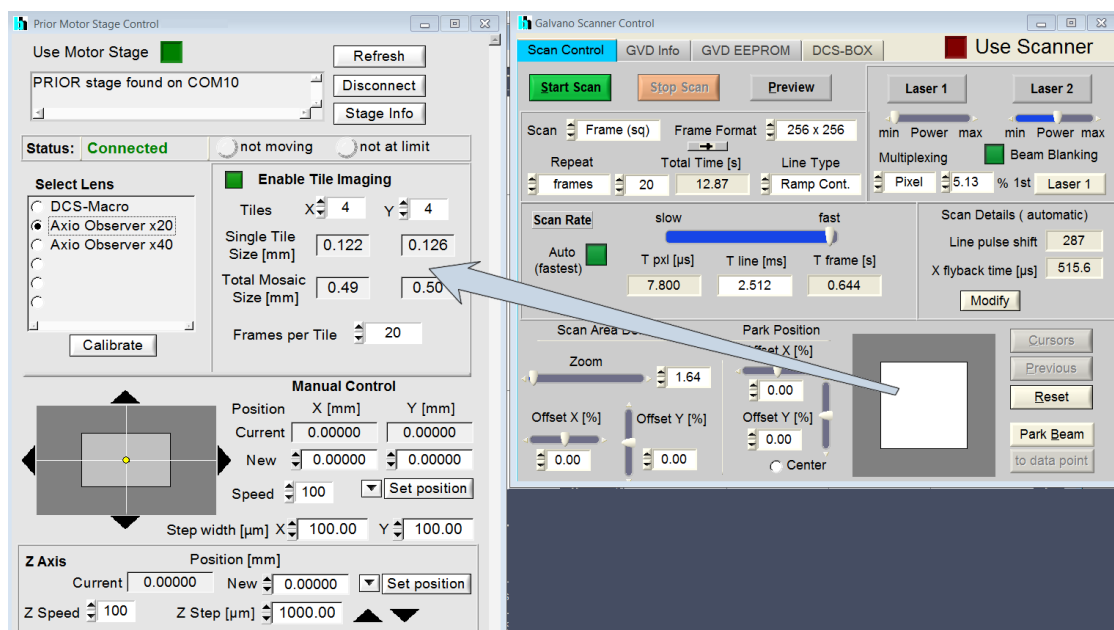


Fig. 202: Interaction of the motor stage with the DCS-120 scanner

The size of the DCS scan area differs for different optical systems and for different microscope lenses. To guarantee that the individual tiles fit together seamlessly the step width of the motor stage can be calibrated. The calibration panel is shown in Fig. 203. The calibration table contains the size, X and Y, of the field of view (scan area) of the optical scanner for Zoom = 1. The calibration factors can (but need not) be made different in x and y to account for possible tolerances in the drivers of the galvanometer mirrors. Up to six calibration values can be defined for different microscope configurations or objective lenses. The calibration values are stored in a file. They are local to the particular TCSPC system, and not stored in the sdt files of the measurement data.

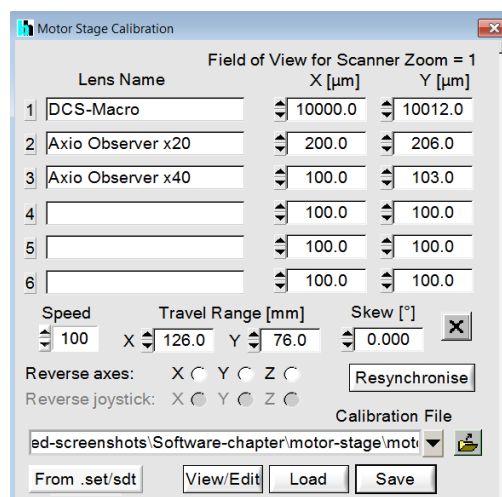


Fig. 203: Calibration of the motor stage

Detector Control

The detectors of the DCS-120 scanner are controlled via the DCC-100 detector controller card or the DCU-400 detector controller [28]. The DCC-100 controls one or two HPM-100 hybrid detectors or one or two MW-FLIM multi-wavelength detectors. The software panel of the DCC-100 is shown in Fig. 204. The DCU-400 panel is similar to that of the DCC-100 except that it controls four detectors, see Fig. 205.

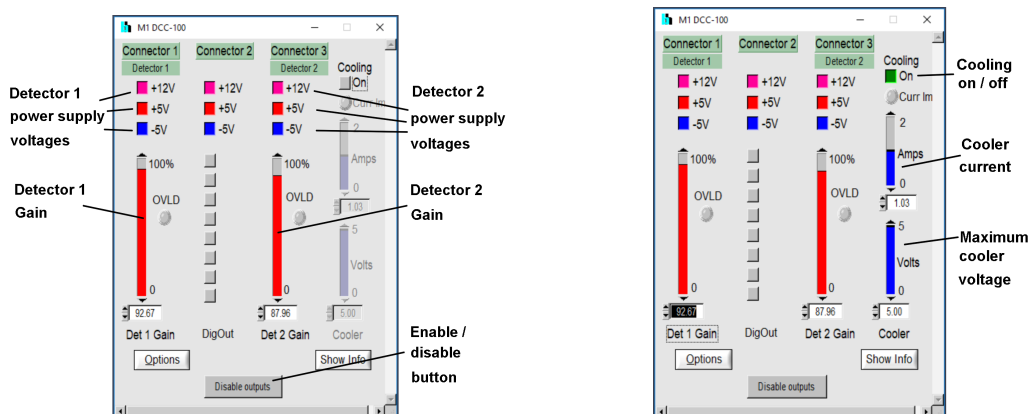


Fig. 204: DCC-100 panel, control of two detectors. Left: Standard HPM-100 detectors. Right: HPM Detectors with coolers

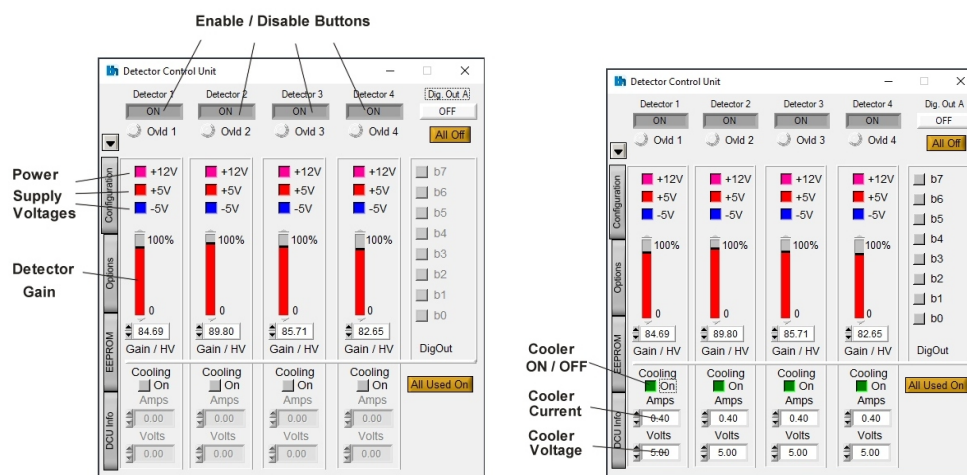


Fig. 205: DCU-400 panel, control of four detectors. Left: Standard HPM-100 detectors. Right: HPM Detectors with coolers

Enable Outputs Button

After the start of the DCC-100 software the outputs of the DCC-100 or DCU-400 are disabled. This is a safety function. It avoids unintentionally switching on a laser, the output voltage of a high-voltage power supply, or the power supply of highly sensitive detectors. Please note: The DCC software can be configured to turn on the outputs automatically (Option ‘Enable Outputs on Startup’). This option is *not* intended for operation of PMTs and lasers, and should *not* be used in conjunction with standard bh FLIM systems.

Power supply of detectors

The power supply of the detectors can be turned on and off. Turning off the power supply may be required when a detector is replaced, or when a filter on front of a detector has to be replaced.

Power for coolers

Thermoelectric coolers of HPM-100-C or of PMC-100 detectors are driven by the DCC-100 as well. The DCC-100 panel with the cooling driver turned on is shown in Fig. 204., right. The cooler current and the maximum cooler voltage can be set independently. The values act on the coolers of both detectors. For detectors without coolers the ‘Cooling’ settings have no effect.

Gain of Detectors

The gain of the detectors is set by the sliders under ‘Connector 1’ and Connector 2’. When changing the detector gain, please remember that the detectors work in the photon counting mode. In other words, the detector delivers a pulse for every photon detected. The data acquisition system counts these single-photon pulses. The light intensity is proportional to the number of pulses per time interval. A change in detector gain changes the *amplitude* of the single-photon pulses, not their frequency. The detector gain has to be adjusted to obtain a single-photon pulse amplitude well above the threshold of the input discriminator of the TCSPC module (the CFD threshold). With the right combination of CFD threshold and detector gain the count rate (and thus the intensity of the image recorded) becomes almost independent of the detector gain. The setup of the detector gain and the CFD threshold is described in [27] and [77]. Gain setting for the HPM-100-40 is described in [40].

Please note: The detector gain should *not* be used to control the intensity of the recording, or to avoid overload shutdown at high intensity. Reducing the detector gain results in loss of

photons, i.e. a decrease of the signal-to-noise ratio of the lifetime images. It is *not* equivalent to a reduction in the detector gain of a conventional laser scanning microscope.

Overload Shutdown

If the light intensity at one or more detectors becomes too high the DCC-100 shuts down the gain and the +12 V supply voltage, and closes shutters that may be controlled via connector 2. In extreme cases shutdown may happen at a gain far below the single-photon detection level, i.e. before the SPC module displays a CFD count rate. The DCC-100 panel after an overload shutdown is shown in Fig. 206.

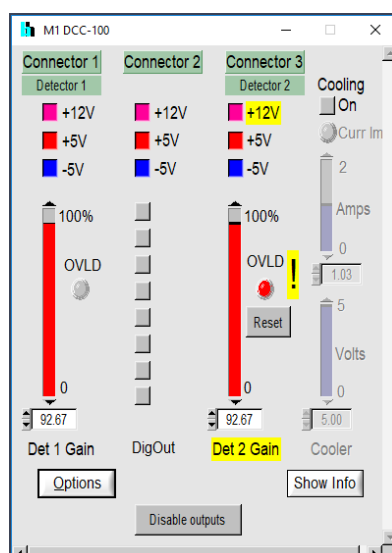


Fig. 206: DCC-100 panel after an overload shutdown

If an overload shutdown has occurred, find and remove the source of the overload. Then click on the 'Reset' button. The detector then resumes normal operation. Please do *not* attempt to avoid overload by using an extremely low detector gain. This may result in poor counting efficiency and, in a multi-wavelength system, poor channel uniformity [27].

Lock Detector Setup Parameters

Once the correct detector gain has been determined, we recommend to lock the detector setup, see Fig. 207. This avoids unintentional changes of the detector gain.

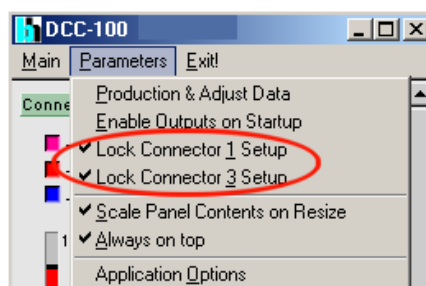


Fig. 207: Locking the detector setup

Shutters

The DCC-100 detector controller is able to control one or several shutters. In the DCS-120 system, a shutter is used only in the non-descanned detection (NDD) light path. Please see 'DCS-120 Multiphoton-Excitation System', page 199.

Saving and Loading Setup and Measurement Data

Save

The ‘Save’ panel is shown in Fig. 208. It contains fields to select different file types, to select or specify a file, to display information about existing file, and to select between different save options.

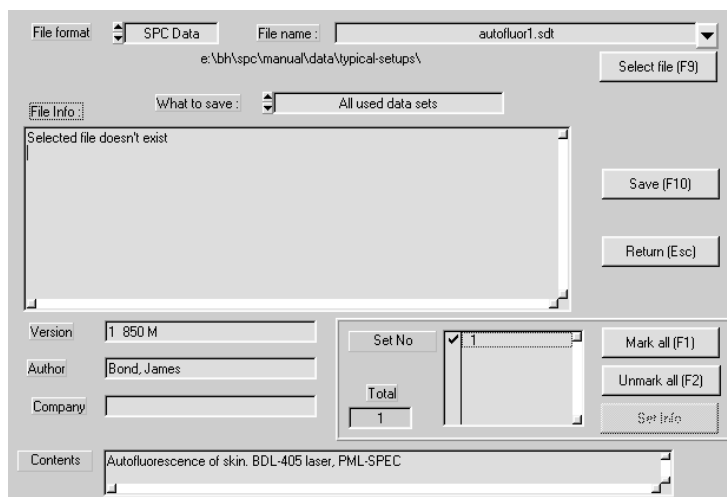


Fig. 208: Save panel

File Format

You can choose between ‘SPC Data’ and ‘SPC Setup’. The selection refers to different file types. With ‘SPC Data’ files are created which contain both measurement data and system parameters. When this file is loaded not only the measurement data are restored but also the complete system setup. With ‘SPC Setup’ files are created that contain the system parameters only. When such files are loaded the system setup is restored, but no data are loaded. Files created by ‘SPC Data’ have the extension ‘.sdt’, files created by ‘SPC Setup’ have the extension ‘.set’.

File Name / Select File

A file name can be written into the ‘File Name’ field. ‘Select File’ opens a dialog box that allows you to change or create directories. Moreover, it shows the names of existing files. These are ‘.sdt’ files or ‘.set’ files, depending on the selected file format. If you want to overwrite an existing file you can select it in the ‘File Name’ field. A history of previously saved files is available by clicking on the  button.

File Info

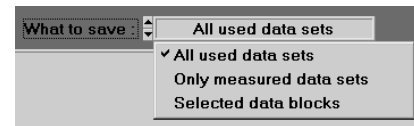
After selecting the file text can be written into the ‘Author’, ‘Company’ and ‘Contents’ fields. Both for ‘SPC data’ and ‘SPC setup’ the file information is saved in the file. The file information helps considerably to later identify a particular measurement among a large number of data files. We therefore strongly recommend to spend a few seconds on typing in a reasonable file information.

If you have selected an existing file the file information contained in it is displayed in the ‘File info window’. If you want to overwrite this file you can edit the existing file information.

Selecting the data to be saved

Under ‘What to Save’ the options ‘All used data sets’, ‘Only measured data sets’ or ‘Selected data blocks’ are available. The default setting is ‘All used data sets’, which saves all valid data available in the memory of the SPC modules.

These can be measured data, calculated data or data loaded from another file. *Except for special cases (see[77]) we recommend to use the ‘All used data sets’ option.*



Load

The ‘Load’ menu is shown in Fig. 209. It contains fields to select different file types, to specify a file, to display information about the file selected, and to select different load options.

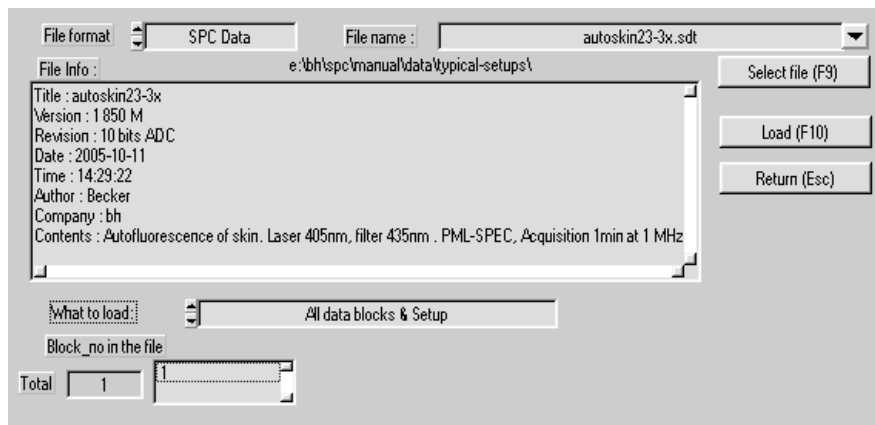


Fig. 209: Load panel

File Format

You can choose between ‘SPC Data’ and ‘SPC Setup’. The selection refers to different file types. With ‘SPC Data’, .sdt files are loaded. These files contain both measurement data and system parameters. Thus the load operation restores the complete system state as it was in the moment when the file was saved.

If you chose ‘SPC Setup’, .set files are loaded. These files contain the system parameters only. The load operation sets the system parameters, but the actual measurement data are not influenced.

Note: Measurements in the ‘FIFO’ (time tag) mode deliver an .spc file that contains the micro time, the macro time, and the detector channel for each individual photon. These files are loaded by using the ‘Convert’ routines, see [77].

File Name / Select File

The file to be loaded can be selected in ‘File Name’ field. ‘Select File’ opens a dialog box that displays the available files. These are ‘.sdt’ files or ‘.set’ files depending on the selected file format. A history of previously loaded files is available by clicking on the ▾ button.

File Info

The file info window displays information about the file selected. The first three lines of the file info were inserted automatically when a file was saved. The last three items were typed in by the operator, see Fig. 208 and Fig. 209.

Block Info

Activating a data block in the 'Block Number in File' field enables a 'Block Info Button'. Clicking on this button opens a list that contains the device number of the SPC modules by which the data were recorded, the time and data of the recording, and all system parameters, see Fig. 210. At the end of the block information the minimum and maximum count rates of the corresponding measurement are shown (see Fig. 210, right). The block info often helps recover the exact recording conditions of an older measurement.

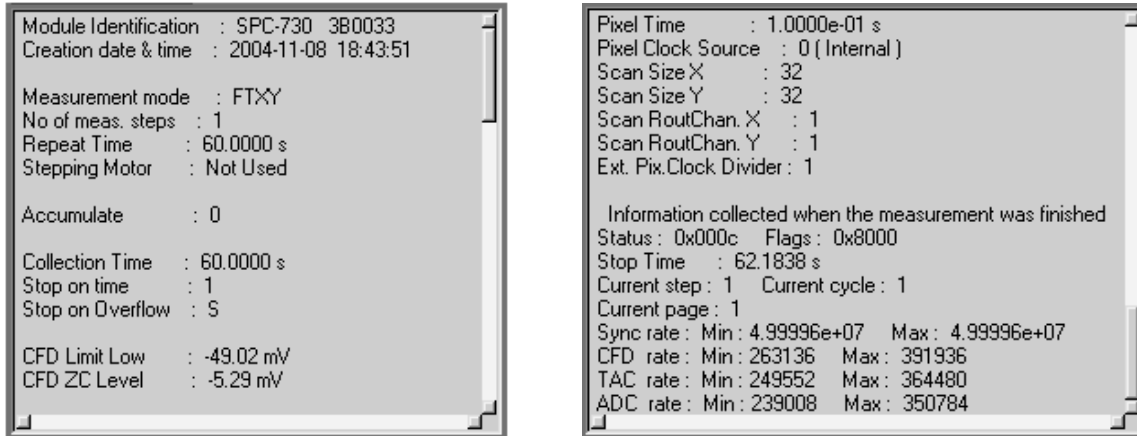


Fig. 210: Block info window of the load panel

Load Options

Under 'What to Load' the options 'All data blocks & setup', 'Selected data blocks without setup' or 'Setup only' are available. The default setting is 'All data blocks & setup', which loads the complete information from a previously saved data file. *Except for special cases (see below) we recommend to use the 'All data blocks & setup' option.*

Loading Files from older Software Versions

Older software versions may contain less system parameters than newer ones. Therefore, loading older files into a newer software (or vice versa) can cause warnings of missing or unknown parameters. To load the file, click on the 'Continue' button until the file is loaded. Unknown parameters are ignored, and missing parameters are replaced with default values. To avoid further problems with such a file, we recommend to save it in the current software version (Use option 'All used data blocks', see 'Saving and Loading Setup and Measurement Data').

Creating Predefined Setups

Setups of frequently used system configurations can be added to a list of 'predefined setups'. Changing between these setups then requires only a mouse click. To use the predefined setup option, click on 'Main', 'Load Predefined Setups'. This opens the panel shown in Fig. 211, left. A setup is loaded by clicking on the button left of the name of the setup.

To add or delete setups to or from the list, or to change the names of the setups, click into one of the name fields with the right mouse key. This opens the panel shown in Fig. 211, middle.

To add a setup, click on the disc symbol right of the 'File Name' field and select a '.set' file or a '.sdt' file. Select the files you want to put into the list of predefined setups, and click on the 'Add' button. In principle, you can select any .sdt or .set file in any directory of the computer. We do, however, discourage to use files in such locations for the simple reason that they can be overwritten. To avoid unintentional overwriting of files used for the setup panel

the SPCM software has a directory 'Default Setups', see Fig. 211, right. Files that are to be used as predefined setups should be saved or copied into this directory. If a file in the Default Setups directory has to be replaced, either copy it from another directory by using the Windows Explorer or delete the old file before you save the new one by the SPCM software.

Every setup has a user-defined 'nickname'. The default nickname is the file name of the file. To change the nickname, click into the nickname field and edit the name. Then click on 'Replace'.

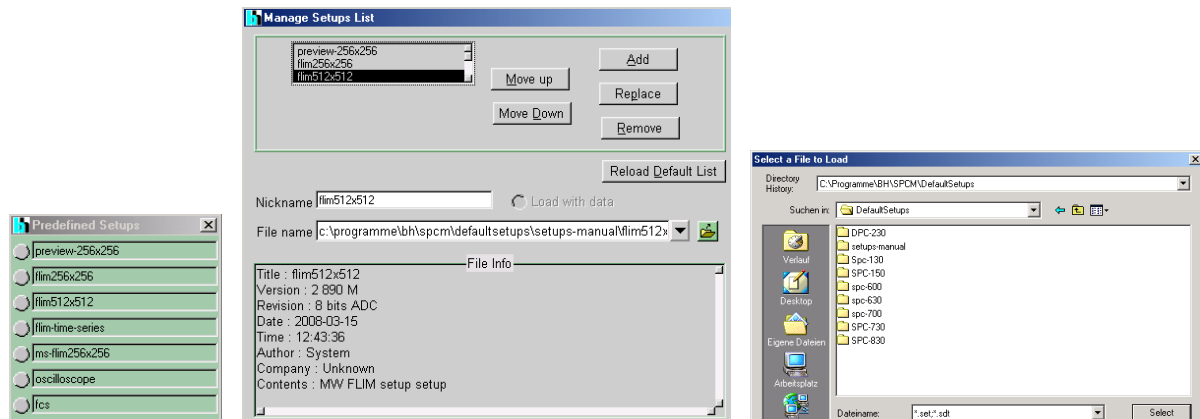


Fig. 211: Editing the list of predefined setups

You can add both '.set' files and '.sdt' files to the setup list. A '.set' file contains only setup parameters, a '.sdt' file contains both setup parameters and measurement data. You can define whether a '.sdt' file is loaded with or without the data by the 'load with data' button.

Parameter Setup for Basic FLIM Experiments

The following paragraphs describe the parameter setup for the basic measurement modes of the DCS-120 system. Setups for these modes have been (or will be) created during the setup of your system and placed in the Predefined Setup panel. Therefore you do not need to create the setups - just load them from the predefined setup panel. However, as you get familiar with the system you may want to create parameter sets for more complex measurement procedures or more advanced FLIM techniques. In these cases, you can start from the basic setups and add or modify parameters to exploit the full range of capabilities of the DCS-120 systems, see chapter ‘Advanced Techniques and Procedures’, page 137.

Basic Dual-Channel FLIM

Main Panel

The Main panel of the SPCM software for a dual-channel SPC-180NX DCS-120 FLIM system is shown in Fig. 212. Images for both detector channels are displayed, the display mode is 'Lifetime'. The predefined setups, the detector control panel, and the scanner control panel are open. There is also a ‘Select SPC’ panel. It allows you to switch the count rate and status display between the two TCSPC channels.

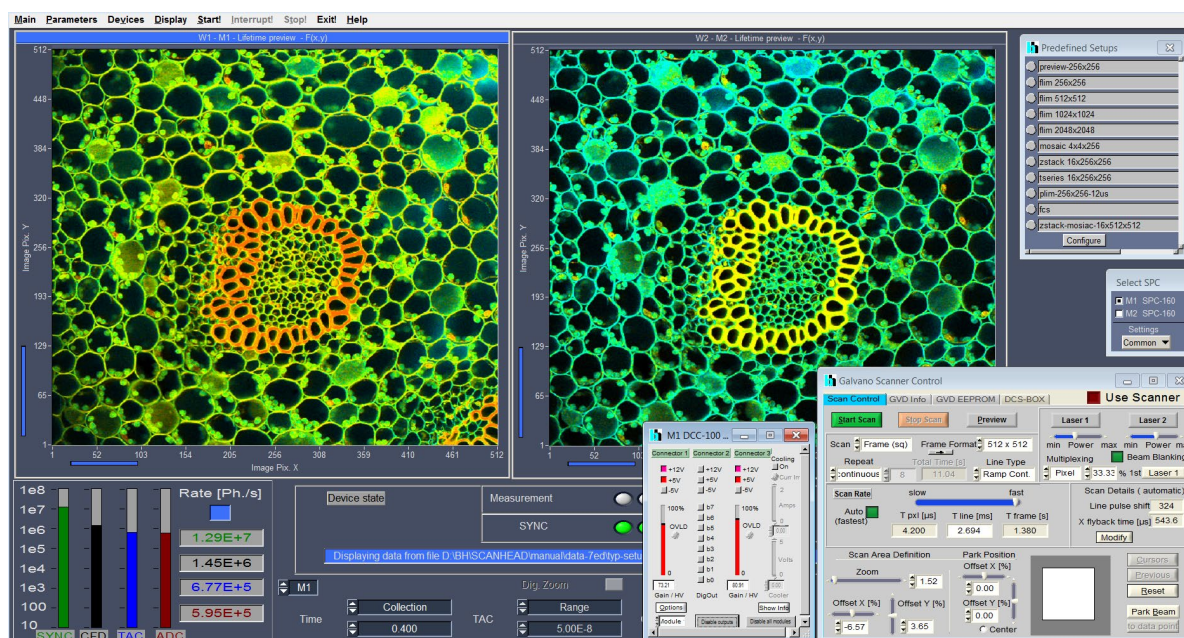


Fig. 212: Main panel for a dual-channel TCSPC system. Lifetime display, detector control, scanner/laser control and Predefined Setup panels are open.

System Parameters

The System Parameter panel is shown in Fig. 213, left. Operation Mode is ‘FIFO Imaging’. STOP T is *not* set, the acquisition runs until it is stopped by the operator. The size of the recorded image is 512 x 512 pixels, 256 time channels. The equivalent main panel for the SPC-QC-104 is shown in Fig. 214.

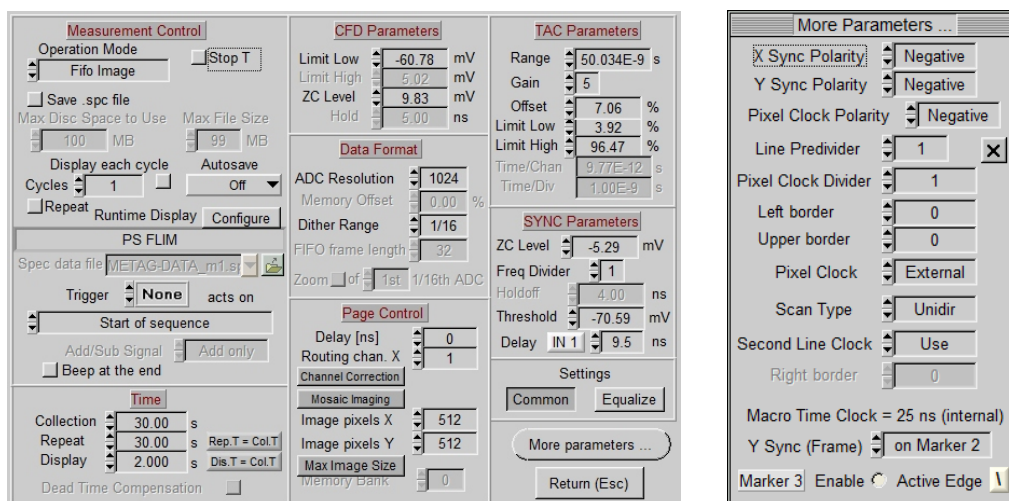


Fig. 213: Left: System parameters, FLIM image 512 x512 pixels. Dual-SPC-180NX system. Right: Scan synchronisation parameters

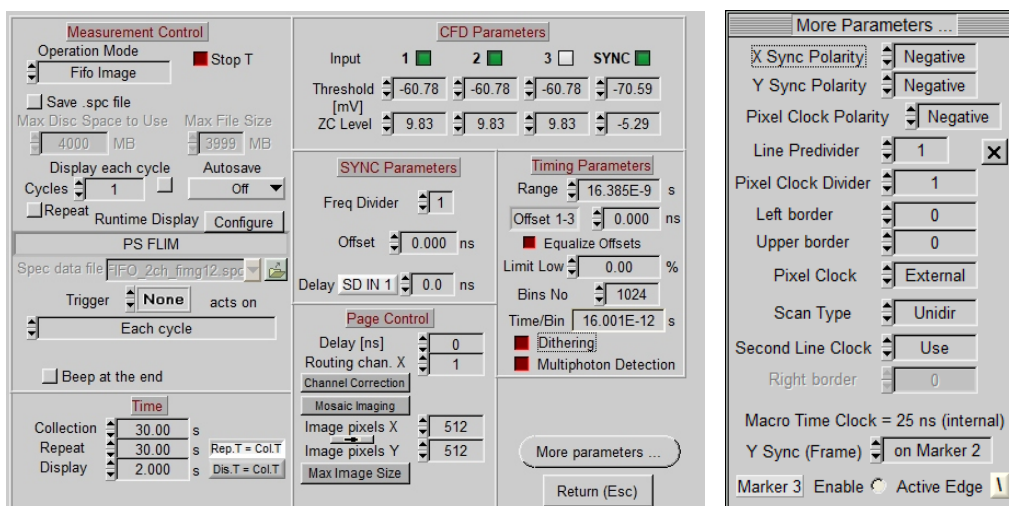


Fig. 214: Equivalent system parameters for an SPC-QC-104 system

Scan Synchronisation Parameters

The scan synchronisation parameters are shown in Fig. 213 and Fig. 214, right. The parameters allow the SPCM software to run with various scanners. There are options for line and pixel binning (line predivider and pixel clock divider), offsets of the beginning of the recording from the frame and line clock pulses, options to adapt to different scan modes (scan type) and an option to discard each second line clock in systems that deliver a line clock both at the beginning and at the end of each line. There is no need to use these options in the DCS-120 system. If you want to create special scan procedures please refer to the bh TCSPC Handbook [77], otherwise use the settings shown in Fig. 213, right.

Configure Menu

The parameters in the 'Configure' menu (Fig. 215) determine what SPCM is doing with the photon data received from the SPC modules. The system is configured to build up standard FLIM images, therefore only 'Picosecond FLIM' is enabled.

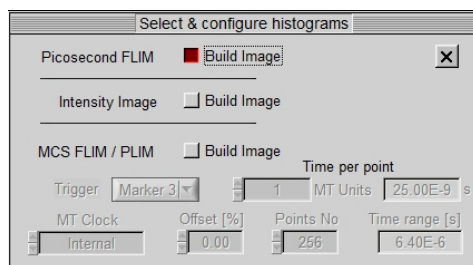


Fig. 215: Configure menu

Other Pixel Numbers

The pixel number for the FLIM acquisition are defined in the 'Page Control' section of the system parameters. The parameters in Fig. 213, left, and Fig. 214 are for a FLIM image of 512 x 512 pixels. Correct recording with the scan synchronisation parameters shown in Fig. 213, right, requires that the scanner runs a scan with the same number of pixels, i.e. 512 x 512.

Recording with other pixel numbers is either achieved by loading an appropriate setup from the predefined-setup panel, or by changing the pixel number in the system parameters and in the scanner parameters. When the pixel number is changed in the system parameters it can be transferred into scanner parameters by clicking on the  symbol. Vice versa, a pixel number defined in the scanner panel can be transferred into the system parameters by clicking on a similar symbol in the scan parameters, see Fig. 216. Automatic transfer from the scanner panel to the system parameters is performed when the little square next to the  symbol is activated, see Fig. 216, right.

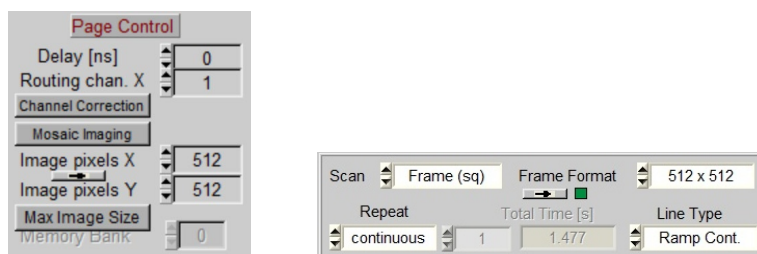


Fig. 216. Pixel format definition in the system parameters (left) and in the scanner panel (right). The format can be transferred from the system parameters to the scanner parameters and vice versa by clicking on the  symbol. Automatic transfer of the scan data is performed when the little square next to the  symbol is activated.

Numbers of Time Channels

For many years, FLIM experiments were performed with a time-axis resolution of 256 time channels. The time channel width (for a 10 ns observation interval) is then 40 ps. This may be enough for most PMT detectors, such as the Hamamatsu H7422 modules. However, it is not enough for HPM-100 detectors, especially not for the HPM-100-06 and -07 detectors. To exploit the full resolution of these detectors a time-channel width of less than 10 ps or less is necessary which means that, for a 10 ns observation-time interval, 1024 time channels should be used. A number of time channels of 1024 in combination with 512 x 512 pixels does not pose a problem for SPCM and SPCImage in a reasonable new computer. However, if the pixel number is increased to 1024 x 1024 or 2048 x 2048 it can be necessary to allocate more memory to the SPCM application. To do so, please click into 'Max Image Size' and select the maximum memory space available in your computer, see Fig. 217, right.

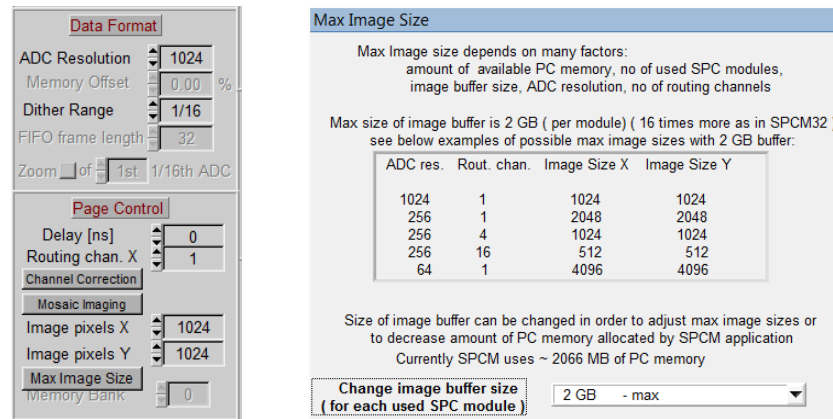


Fig. 217: Left: Data format definition for an image of 1024 x 1024 pixels and 1024 time channels. Right: Allocating memory space for large FLIM data formats.

Module-Specific System Parameters

The two TCSPC modules of a dual-channel FLIM system can - but need not - be operated with different system parameters. Different parameters are mostly used when the two channels have different detectors, requiring different CFD parameters or TAC offsets. This is not normally the case for the DCS-120. The system parameters are therefore ‘Common’ for the two TCSPC modules, see Fig. 213.

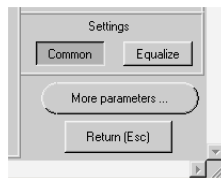


Fig. 218: Standard dual-channel DCS systems use ‘Common’ system parameters for both channels.

Window Parameters and Trace Parameters

The Trace Parameters and Window Parameters are used to display different subsets of multi-dimensional data. Images can be displayed in different time windows, or decay curves can be displayed over one of the image coordinates and in selected windows of the other coordinate. The Window Parameter panel is shown in Fig. 219. For basic FLIM operation one time window, one X and one Y window are defined. For displaying time-gated images and curves within x and y windows please see ‘3D Trace, Window and Display Parameters’, page 71 and ‘Parameter Setup for Basic FLIM Experiments’, page 103.

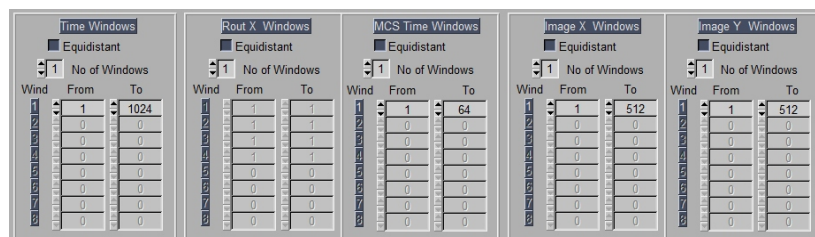


Fig. 219: Window parameters for basic FLIM system

The 3D Trace parameters are defined to display the images for both TCSPC modules. The 3D Trace definitions are shown in Fig. 220. Image type is 'Lifetime'.

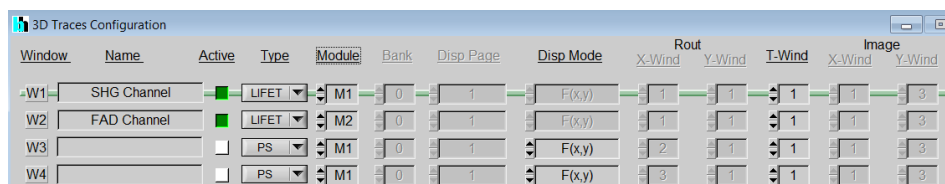


Fig. 220: 3D Trace parameters for a dual-channel FLIM system

Display Parameters

There are separate sets of display parameters for the two display windows of the main panel. Thus, different colour, different intensity scaling, or different T windows can be assigned to the individual images. The display parameters for the two display windows of Fig. 212 are as shown in Fig. 221.

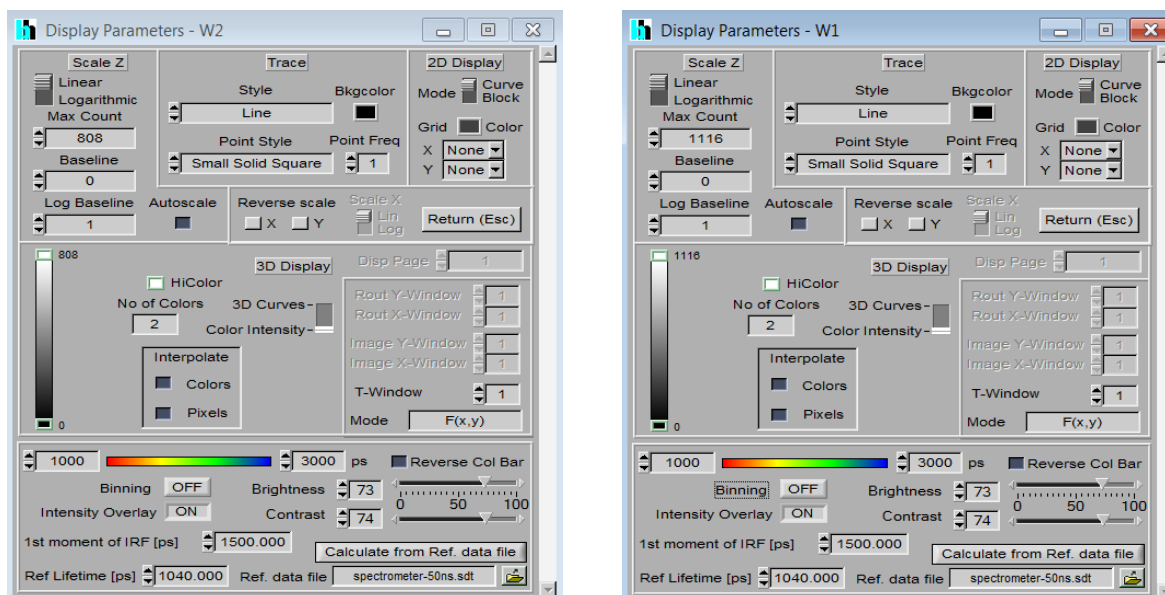


Fig. 221: Display parameters for the two display windows of the main panel shown in Fig. 212. Different colour coding, contrast, and brightness can be assigned to the individual images.

Lifetime-Intensity FLIM

Lifetime-Intensity FLIM combines intensity data from a fast counter with fluorescence decay data from the TCSPC timing electronics. The technique avoids loss in contrast when FLIM data are recorded at extremely high count rates. The technical background is described in [102]. The new 'Lifetime/Intensity' mode is available for the SPC-160 and the new SPC-180 family TCSPC modules.

Parameter Setup

The Lifetime/Intensity mode is available in the SPCM data acquisition software, Version 9.86 of June 8, 2021 or later. The implementation in the user interface and the parameter setup are shown in Fig. 222 and Fig. 223. In the 'System Parameters', click on 'Configure' and enable 'Intensity Image / Build Image'. This enables the parallel counter, puts the pixel intensities in the photon data stream, and instructs the software to build up an array of pixel intensities.

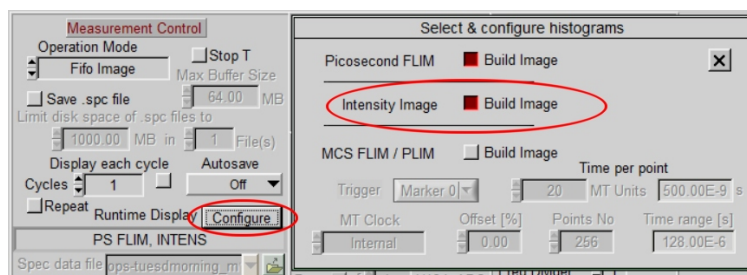


Fig. 222: System parameters for intensity imaging via the parallel counter channel. SPCM builds up an array of the pixel intensities recorded by the parallel counter.

In the 3D Trace parameters (accessible via 'Parameters' or right mouse click in an image window), select 'Lifet+I' (Lifetime + Intensity) as a data type, see Fig. 223. You can select 'Lifet+I' for one channel or for several channels simultaneously. In Fig. 223 it has been set for Display Window 1, Display Window 2 shows a 'normal' lifetime image created by the online-lifetime function of SPCM, see 'Display of Lifetime Images' page 78. The images for the second TCSPC module, M2, have been turned off. With the settings shown, SPCM displays the two images shown in Fig. 224.

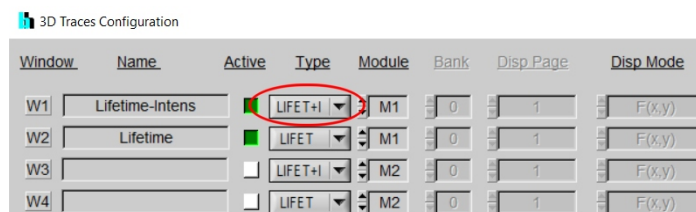


Fig. 223: Enabling the buildup of a Lifetime/Intensity image using the intensity values from the parallel counter

Example

An example of the image improvement is shown in Fig. 224. The images were recorded with a DCS-120 Confocal FLIM system using SPC-180NX TCSPC modules. The sample was a stained mouse kidney section (Invitrogen F24630). The excitation wavelength was 480 nm, the detection wavelength interval 500 to 680 nm. The images were recorded with 512 x 512 pixels, and 1024 time channels per pixel. The recorded count rate averaged over the image was 5.6 MHz. The image on the left was created by the new Lifetime/Intensity mode, the image on the right by the traditional FLIM mode. Average (recorded) count rate 5.5 MHz.

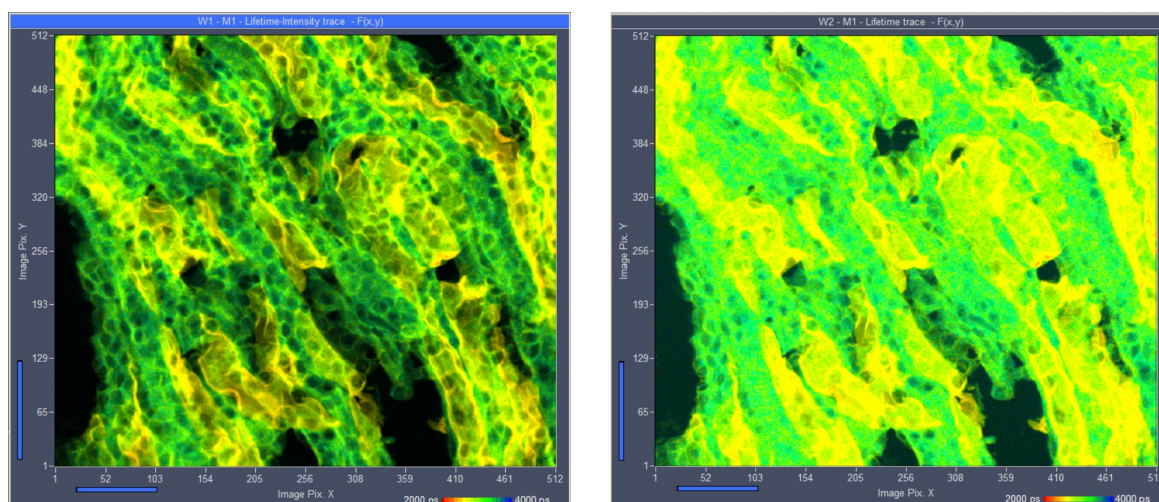


Fig. 224: FLIM images recorded from an Invitrogen F24630 Mouse kidney section. Left: Lifetime/Intensity mode. Right: Traditional FLIM mode. Average (recorded) count rate 5.5 MHz.

Analysis with SPCImage NG

Analysis of Lifetime/Intensity data is implemented in SPCImage NG, Version 8.4 or later. Data analysis is performed the usual way, see chapter 'SPCImage NG Data Analysis Software'. Right-click into the SPCM display window of interest, and select 'Send Data to SPCImage', or click into 'Main' and 'Send Data to SPCImage'. SPCImage recognises that the data are Lifetime/Intensity data, and handles them accordingly. That means, the decay curves are taken from the normal FLIM data array, but the intensities come from the array of pixel photon numbers created by the parallel counter channel. To start the data analysis, select an appropriate decay model, and start the procedure via 'Calculate', 'Decay Matrix'. The result for the data of Fig. 224, left, is shown in Fig. 225.

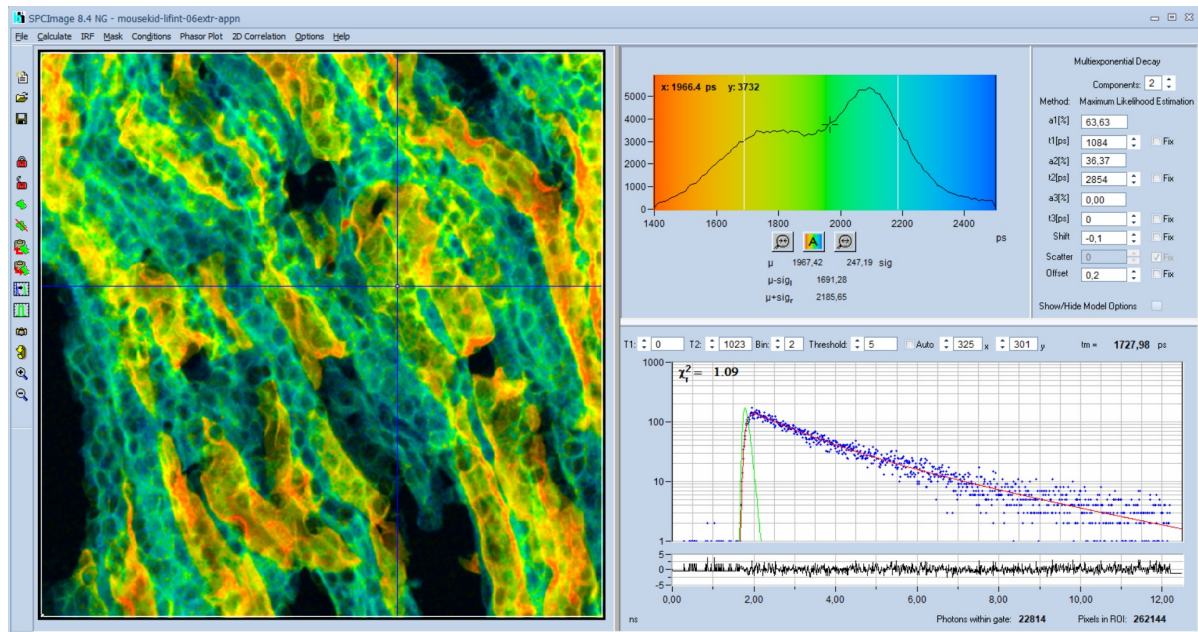


Fig. 225: Analysis of Lifetime / Intensity data by SPCImage NG. Mean lifetime of double-exponential decay, t_m , lifetime range from 1400 ps to 2500 ps.

Online-Display of Decay Curves in FLIM Images

SPCImage is able to display decay curves from points of interest or regions of interest of the recorded FLIM images online. An example is shown in Fig. 163.

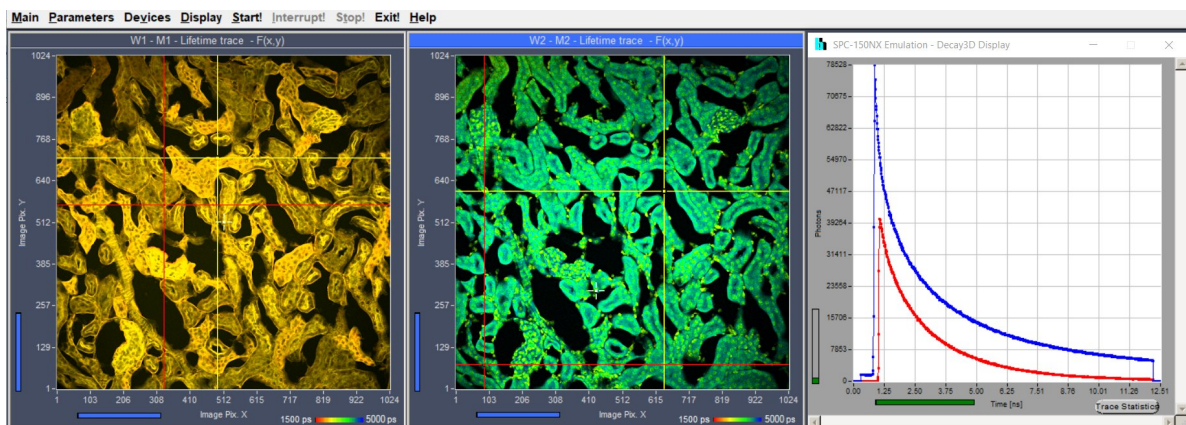


Fig. 226: A right mouse click into an image and a click on 'Show Decays' opens the decay curve panel shown on the right

The function is controlled by a special 'Decay3D Trace Attributes' panel, see Fig. 227. The panel opens by a right mouse click into one of the image windows and selecting 'Show Decays', see Fig. 227, left. The panel allows you to select the colours of the cures, assign the curves to the individual image windows, select whether the curves are from a point of interest (POI), a region of interest (ROI), or from the entire image (ALL). Size and position of the point or region of interest can be linked to the cursors in the image windows.

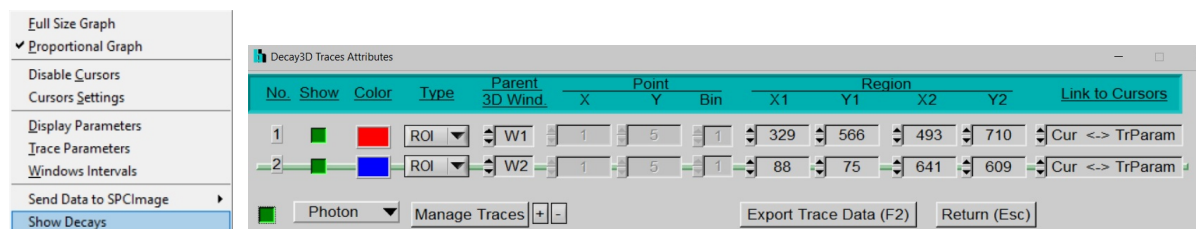


Fig. 227: Decay3D Trace Attributes panel for control of the online-decay function. The panel opens by a right mouse click into an image windows and selecting 'Show Decays'.

The display of the decay curves themselves is controlled by the 'Display Parameters' the same way as for decay curves recorded in the 'Single', 'Oscilloscope', or 'FIFO' mode. The display scale can be linear or logarithmic, an autoscale function is available, and the curves can be displayed as individual data points, lines, or data points connected by lines.

Fast Lifetime Preview

A fast lifetime preview can be obtained by using the normal FLIM mode with lifetime display in combination with short acquisition time and the repeat function of the SPCM software. A typical main panel is shown in Fig. 228. It shows images in two wavelength channels of a dual-channel FLIM system. Both images are calculated by the online-FLIM function of the SPCM software [49, 77]. The images size in Fig. 228 is 512 x 512 pixels. The acquisition time of the images was 1 second.

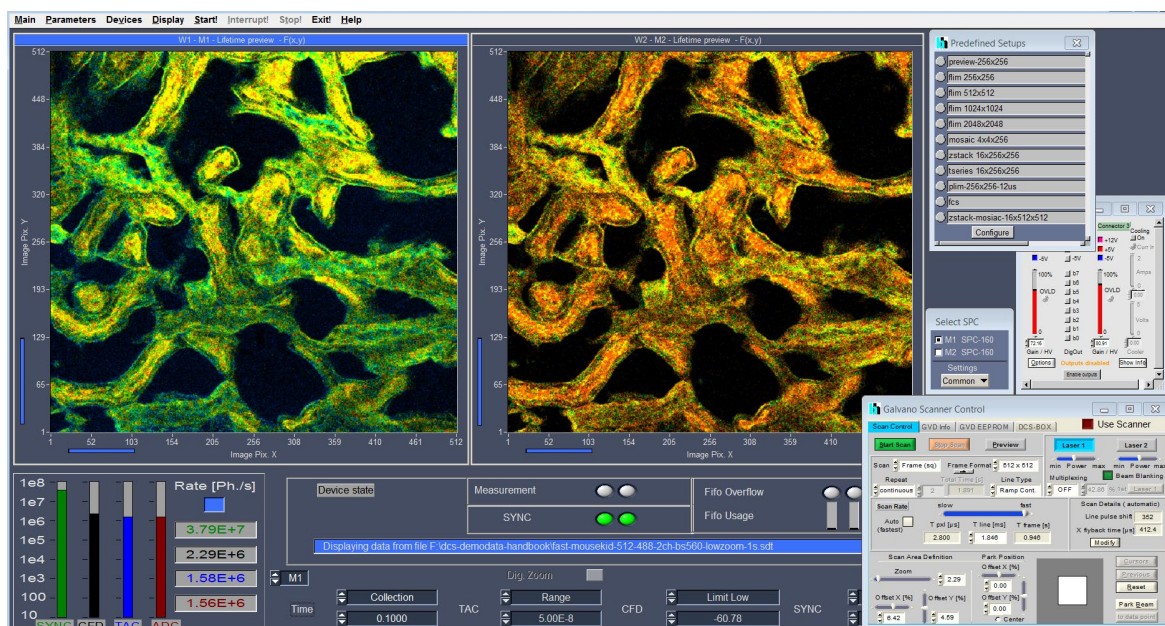


Fig. 228: SPCM main panel, fast lifetime preview. 512 x 512 pixels, two wavelength channels, lifetime range for both images 1000 ps to 3000 ps.

System parameters

The system parameters are essentially the same as for the normal FIFO imaging setup, see 'Basic Dual-Channel FLIM', page 123. The only difference is that 'Stop T', and 'Repeat' are active, and that a short 'Collection' and a short 'Repeat' time are defined. The system then records single frames of the scan, calculates the lifetimes, and displays the data.

Trace, Window, and Display Parameters

The Trace and Window Parameters can be defined as shown in for lifetime display with the standard dual-channel system, page 123. For the Display Parameters the settings shown in Fig. 221, page 127 can be used.

Recording Fluorescence Decay Curves with a DCS-120 System

FLIM experiments often have to be supported by precision measurements of the decay functions of special fluorophores. FLIM users then often resort to an additional fluorescence lifetime spectrometer for cuvette-based measurements of decay functions. In most cases, however, such fluorescence decay data can better be recorded with the FLIM system.

There are two ways to record decay curves with a DCS-120 system. You can use the 'Single' mode and collect the data directly in a single curve, or you can use the 'FIFO Imaging' mode, record an image, and later combine the data of all pixel in a single decay curve.

For collecting the data into a single curve, switch the SPCM software to the 'Single' mode, and increase the 'ADC resolution' to the number of time channels you need. Leave the other system parameters as in the FLIM (FIFO Imaging) mode. Typical system parameters and scan parameters are shown in Fig. 229.

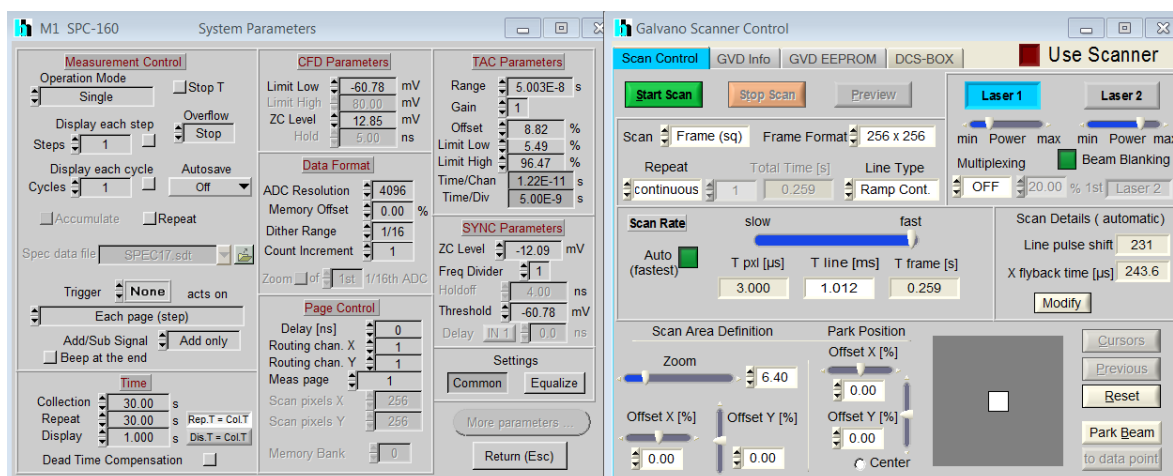


Fig. 229: System parameters and scan parameters for lifetime spectrometer mode

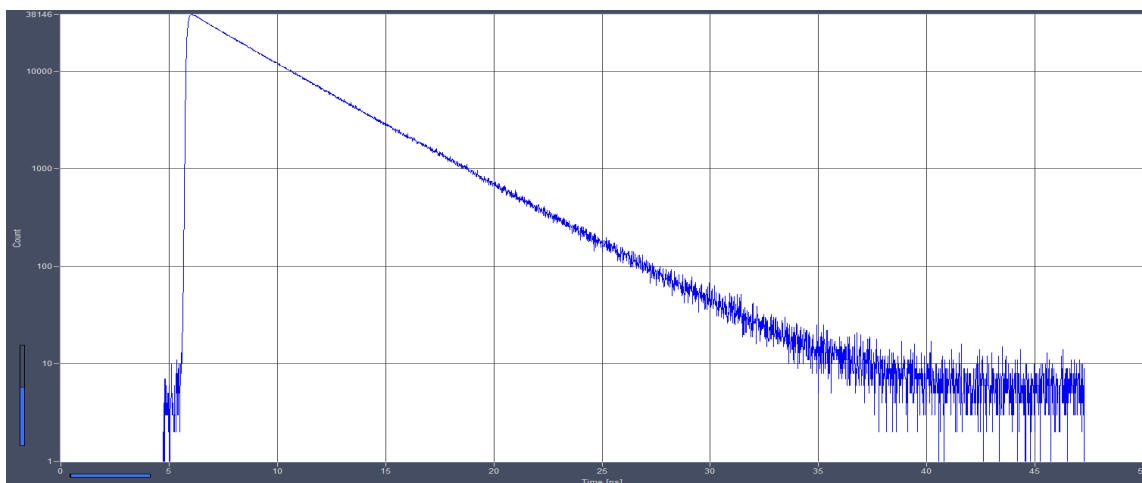


Fig. 230: Decay curve of Atto 425 recorded with DCS-120 confocal scanning FLIM system

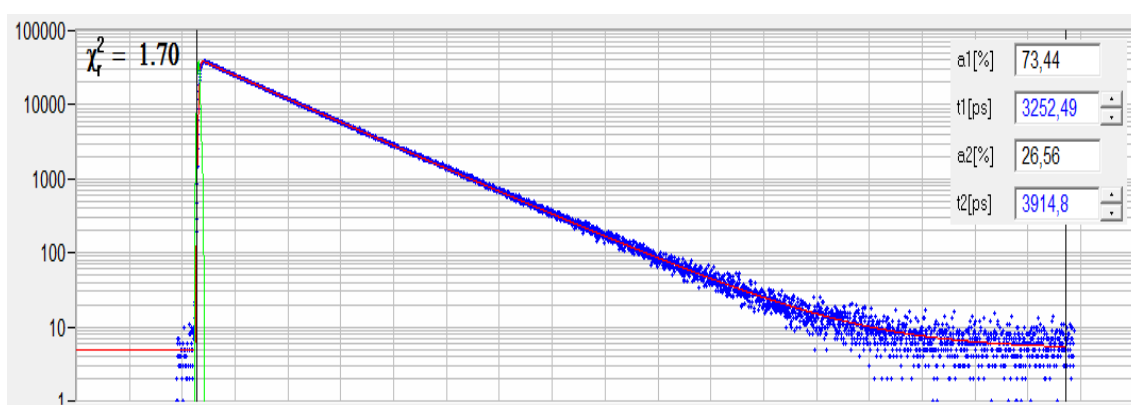


Fig. 231: Fluorescence decay curve, analysed by SPCImage

For collecting the decay data into an image, scan the dye solution with the laser of the desired wavelength and record a FLIM image. Use a FLIM data format with a large number of time channels (4096 or 1024) but a low number of pixels (128 x128). Load the data into SPCImage as described under 'Link to SPCImage Data Analysis'. Select the entire image by the two image cursors, and click on the  (Lock) button. The result is a decay curve containing the data from all pixels, see Fig. 232. Deconvolution and fitting of the combined data is performed the same way as for single pixels of a FLIM image.

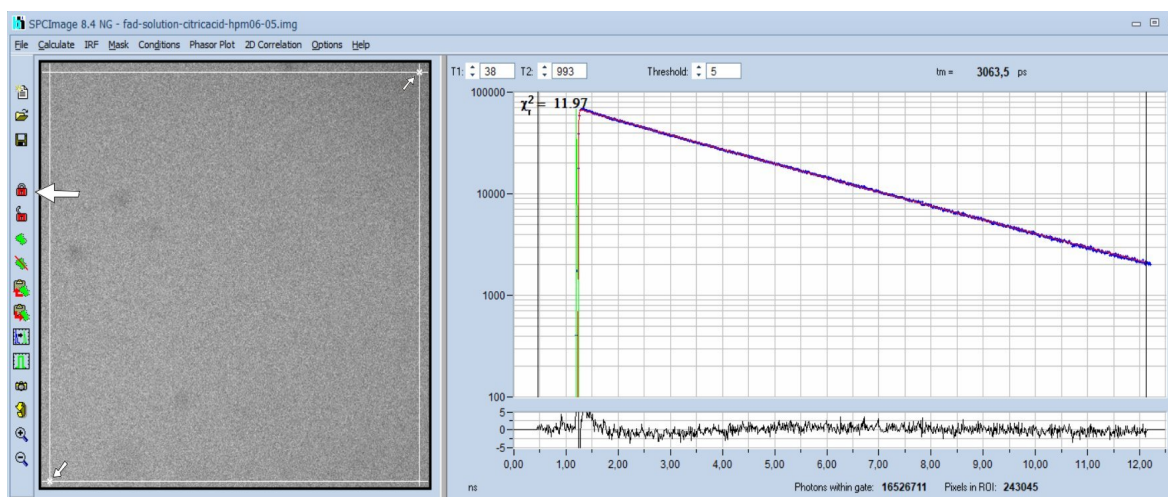


Fig. 232: Combining pixel data into a single decay curve in SPCImage NG

The procedures outlined above have the advantage that the decay curves are recorded under exactly identical conditions as in the FLIM measurement. Possible anisotropy-decay effects are cancelled by the high NA of the microscope lens, and there is virtually no re-absorption. Moreover, phosphorescence and delayed-fluorescence decay curves can be recorded. Please see bh TCSPC Handbook [77] for further details. Another advantage is the extremely high time resolution. Two-photon systems with HPM-100-06 or -07 detectors reach an IRF width of <20 ps FWHM. Compared with a standard fluorescence-lifetime spectrometer this can make the difference between accurately detecting a fast decay component or missing it. An example for an FAD solution is shown in Fig. 233. FAD has long been suspected to have a fast decay component with a lifetime around 50 ps [600], but the component has never been detected directly. The bh DCS-120 MP system with a HPM-100-06 detector shows it without problems, see the peak at the maximum of the decay curve.

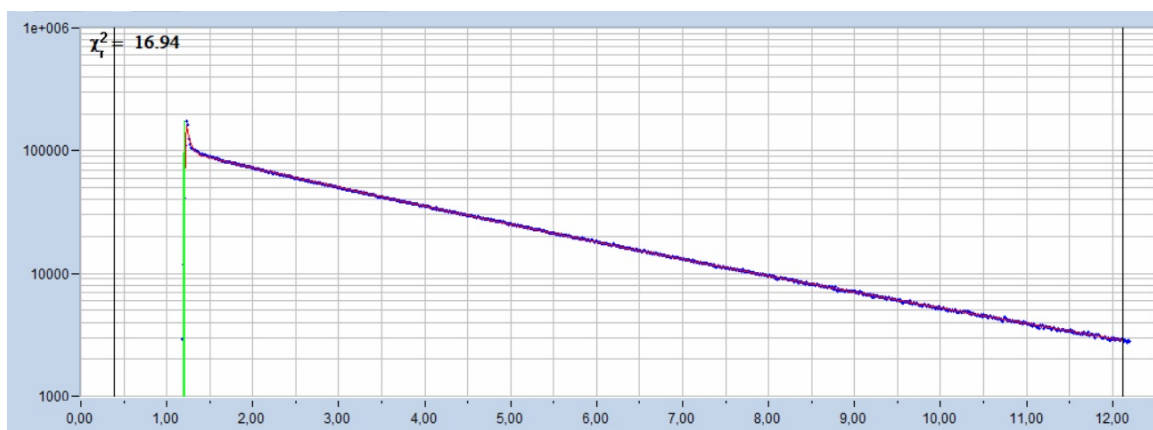


Fig. 233: Fluorescence decay curve of FAD in water, detected with bh DCS-120 MP FLIM system. The system shows that there is a 59-ps decay component with an amplitude of 29%.

Oscilloscope Mode

The Oscilloscope Mode repetitively records fluorescence decay curves and displays them in short periods of time. The Oscilloscope Mode is not directly used in FLIM experiments. It is, however, an extremely useful tool for system alignment, setup and troubleshooting.

Main Panel

The main panel of a dual-channel (SPC-180N) system in the oscilloscope mode is shown in Fig. 234. The fluorescence decay curves detected in both channels are displayed in the upper left. The Display parameters, the detector control panel, and the predefined setup panel are open on the right. The TAC, CFD, and SYNC parameters are accessible at the bottom of the main panel.

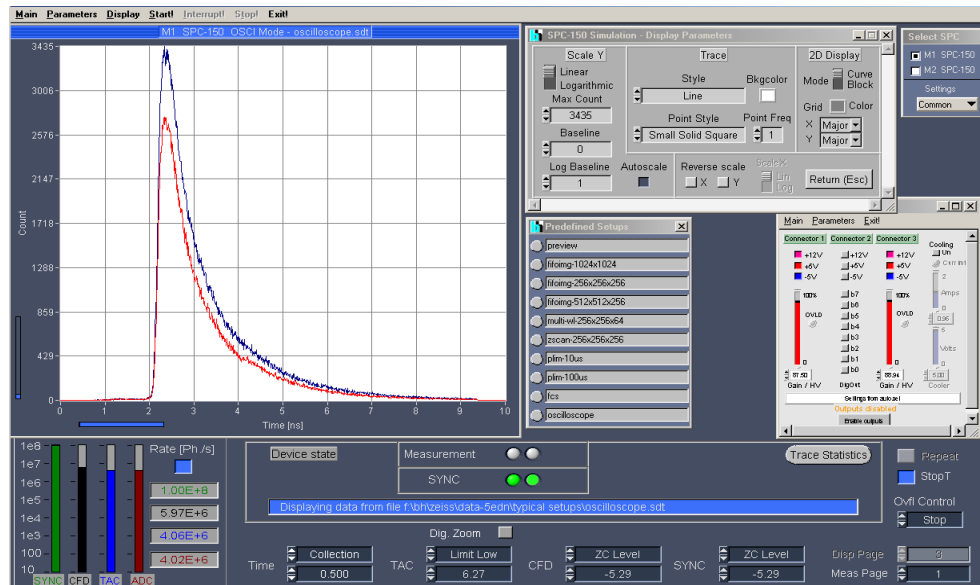


Fig. 234: Main panel for the Oscilloscope Mode

System Parameters

The system parameters are shown in Fig. 235. The setup is the standard one for a system with two SPC modules without routing. For the function of the Oscilloscope mode Stop T and a conveniently short Collection Time have been set.

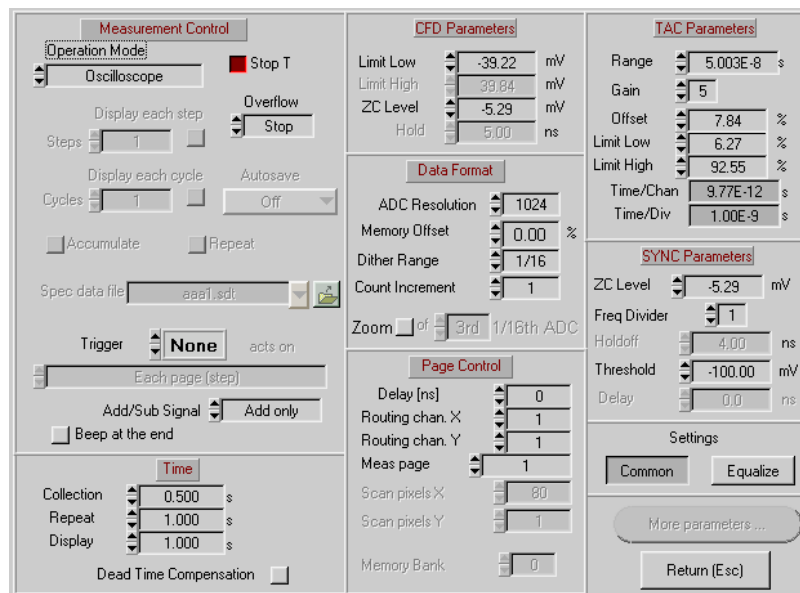


Fig. 235: System parameters for the Oscilloscope Mode

Trace parameters

The trace parameters are shown in Fig. 236. One trace for Module 1 (M1), and a second one for Module 2 (M2) has been enabled.



Fig. 236: Trace parameters for the oscilloscope mode. One trace for Module 1, and another for Module 2 are active.

Advanced Techniques and Procedures

Excitation Wavelength Multiplexing

In biological FLIM applications it is often required to record the signals of several biosensors or of several endogenous fluorophores. The fluorophores usually have overlapping emission spectra and thus cannot be separated by emission filters alone. It is then necessary to record the signals not only in different detection wavelength intervals but also at different excitation wavelengths.

In principle, the signals of different fluorophores could be recorded separately, by running subsequent measurements with different lasers and different emission filters. However, in applications where dynamic lifetime changes are involved a procedure like this would be not desirable. The two measurements then have to be performed simultaneously. To solve such problems the DCS-120 system is able to multiplex two lasers or, in the DCS-120 CMI system, even four lasers in time. The TCSPC system records the signals excited by different lasers in separate memory blocks. The multiplexing of the laser is controlled by the GVD-120 or GVD-140 scan controller of the DCS system. To avoid aliasing effects between the scan and the multiplexing the multiplexing is synchronised with the frames, lines, or pixels of the scan.

Principle

The excitation principle is illustrated in Fig. 237 and Fig. 238. There are two lasers, called 'blue laser' and 'green laser', and two detection wavelength intervals, called 'blue detection interval' and 'green detection interval'. The blue laser excites fluorescence preferentially in the blue detection interval, the green laser excites fluorescence in the green detection interval.

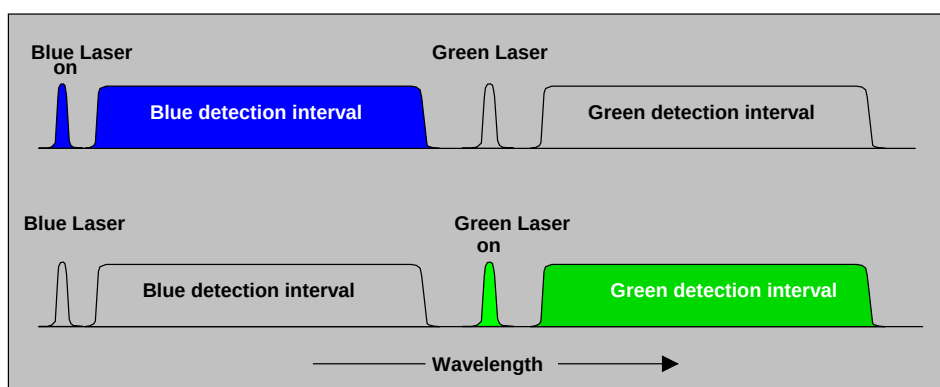


Fig. 237: Laser wavelength multiplexing. There are two lasers, 'blue' and 'green', and two detection wavelength intervals, 'blue' and 'green'. The 'blue' laser excites fluorescence preferentially in the 'blue' detection interval, the 'green' laser excites fluorescence in the 'green' detection interval.

The multiplexing scheme is shown in Fig. 238. The blue laser is turned on for the first half of the multiplexing period. It excites fluorescence in the blue detection interval. In the second half of the multiplexing period the green laser is turned on. It excites fluorescence in the green detection interval. The signals in different detection intervals are recorded by different detectors and TCSPC modules.

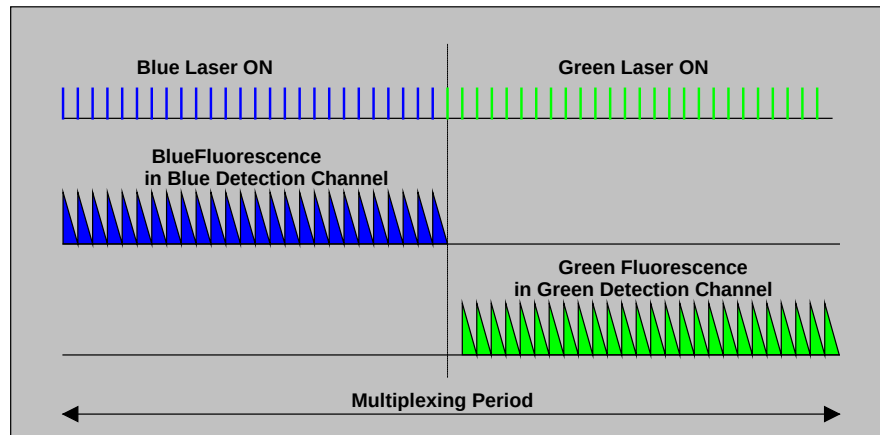


Fig. 238: Laser wavelength multiplexing. Laser ON/OFF switching and detected signals.

The detection of the signals by the TCSPC Modules is illustrated in Fig. 239. To separate the signals from the two lasers the TCSPC modules receive a multiplexing signal that indicates which laser is running at a given time. Based on the state of this signal, the TCSPC module routes the photons into separate photon distributions, see Fig. 239. The two modules of the DCS system thus record images for four combinations of laser wavelengths and detection wavelength intervals. In most cases, only two combinations contain useful data: Blue laser and blue detection interval, and green laser and green detection interval. In practice, however, it happens that the blue laser excites fluorescence also in the green detection interval. In this case, the result is that three images for three excitation-emission combinations are obtained.

Normally, the multiplexing period is synchronised with the frames, lines, or pixels of the scan. Thus, the multiplexing period can be very fast. With pixel multiplexing the multiplexing period can be as short as a few microseconds. Importantly, the signal-to-noise ratio of the result is independent of the multiplexing frequency, it only depends on the total acquisition time.

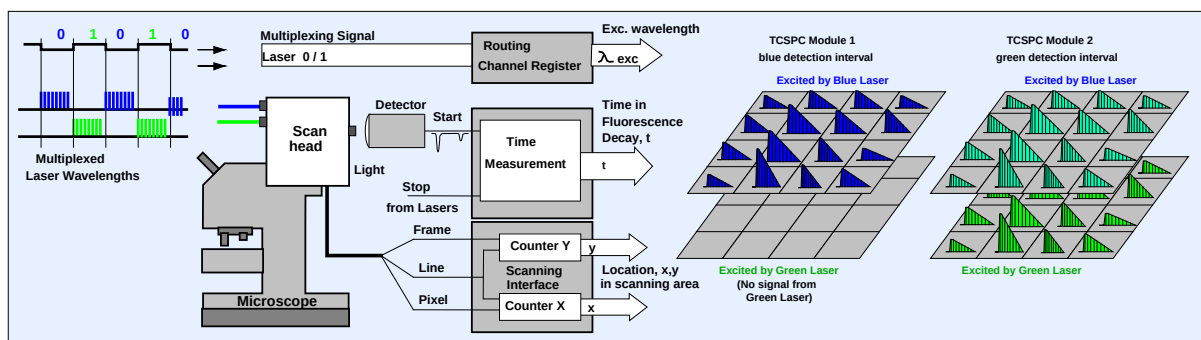


Fig. 239: TCSPC process for laser wavelength multiplexing. Two such channels are operated in different detection wavelength intervals

It can happen that both excitation wavelengths are on the short-wavelength side of the detection-wavelength intervals, see Fig. 240. The multiplexing scheme is shown in Fig. 241. Superficially, it looks similar as the one in Fig. 238. However, if the fluorophores have large stokes shift or wide emission spectra it happens that the blue laser excites some fluorescence in the green detection channel and the green laser in the blue detection channel. Nevertheless, the TCSPC process deals with the situation: The multiplexing signal routes the photons of all these combination into different data blocks so that the signals get cleanly separated.

An example of this kind of operation is NADH / FAD FLIM, see section 'Metabolic FLIM', page 189. In this case both the excitation spectra and emission spectra of the two fluorophores

overlap, see Fig. 329, page 190. Clean separation of the two signals is obtained by multiplexed excitation at 375 nm and 405 nm and simultaneous detection in intervals of 425 nm to 475 nm and 490 nm to 600 nm.

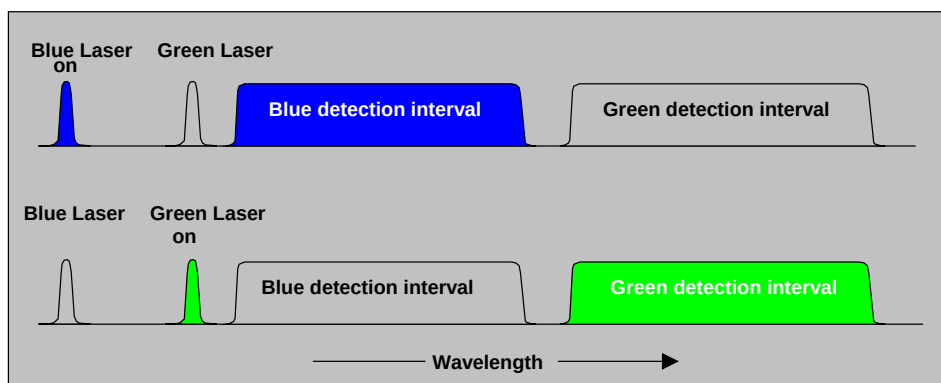


Fig. 240: Laser wavelengths and detection wavelength intervals, both excitation wavelengths on short-wavelength side of detection intervals.

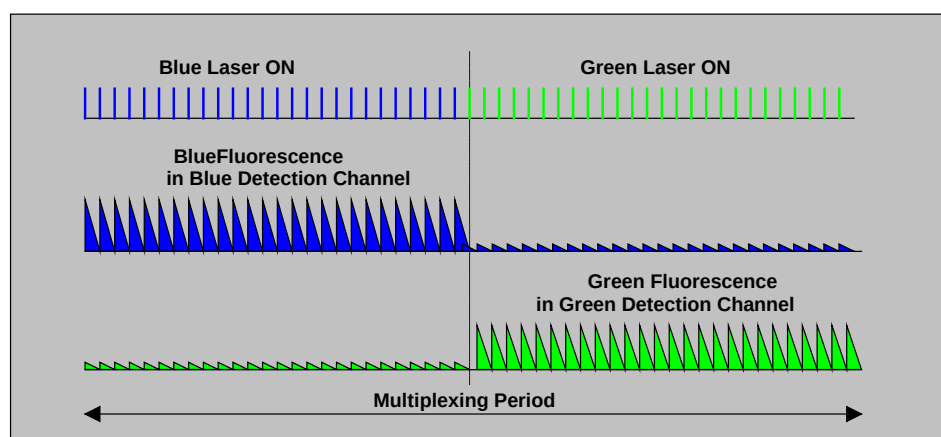


Fig. 241: Laser wavelength multiplexing. Laser ON/OFF switching and detected signals.

Multiplexed excitation is mostly performed with ps diode lasers. However, multiplexed excitation can also be achieved with other lasers. Examples are the bh 'Laser Hub', the Toptica 'FemtoFibre Dichro' dual-wavelength fibre laser, or the combination of two Toptica Femto-Fibre Smart lasers. Please see section 'Two-Photon Metabolic FLIM'. With super-continuum lasers and an AOTF it is even possible to multiplex more than two wavelengths. An example for 8 multiplexed wavelengths can be found in [77] and [611].

It should be mentioned that wavelength multiplexing can be achieved also by pulse-interleaved excitation (PIE). Examples are described in [31, 77] and [34]. In the case of PIE, the lasers are multiplexed pulse-by-pulse, not in longer time intervals as shown above. The disadvantage of PIE is that the fluorescence signals do not get entirely separated - the tail of the fluorescence excited by one laser extends into the fluorescence decay of the other one. PIE has also intensity-crosstalk by counting-loss and pile-up effects. These problems are entirely avoided by the principle described above. Please see [77] for a comparison.

User Interface and Parameter Setup

The SPCM main panel for excitation-wavelength multiplexed FLIM is shown in Fig. 242. The two diode lasers of the DCS system, with wavelengths of 405 nm and 473 nm, were multiplexed frame by frame. Display windows were defined for (left to right) 473 nm excitation

and 510 to 650 nm detection, 405 nm excitation and 435 to 460 nm detection, and for 405 nm excitation and 510 to 650 nm detection.

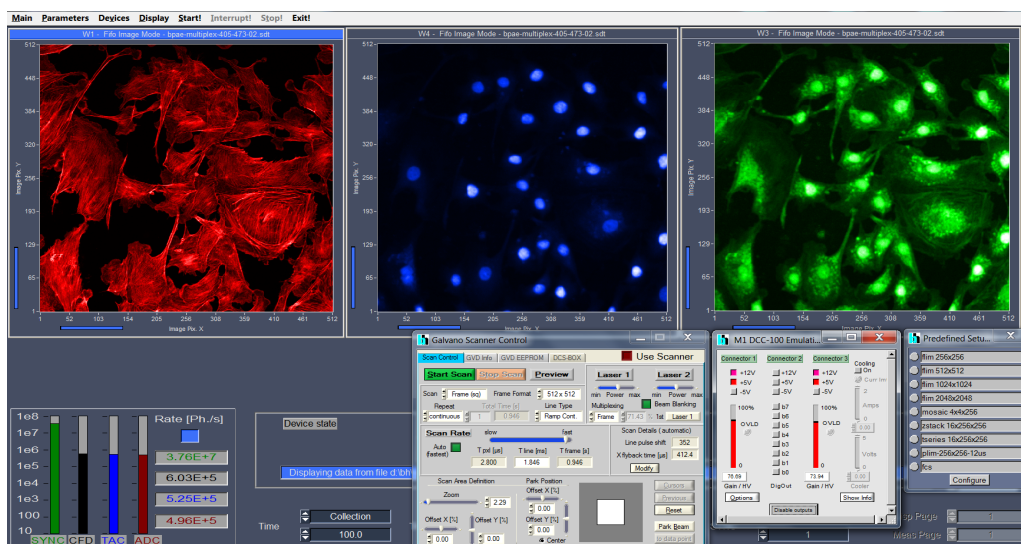


Fig. 242: SPCM main panel for excitation-multiplexed FLIM. The two diode lasers of the DCS system, with wavelengths of 405 nm and 473 nm, were multiplexed. Images were detected from 435 to 460 nm and 510 nm to 650 nm. The excitation emission combinations are 473nm - 510 to 650nm (left, red), 405nm - 435 to 460 nm (middle, blue) and 405nm - 510 to 650nm (right, green)

Scanner and Laser Control Parameters

The scanner and laser control parameters, page control parameters, and scan synchronisation parameters for different multiplexing modes are shown in Fig. 245 through Fig. 244. All setups are shown for images sizes of 512 x 512 pixels.

A setup for frame-by-frame multiplexing is shown in Fig. 243. 'Multiplexing' is 'Frame'. The page control and 'more' parameter settings are shown middle and right.

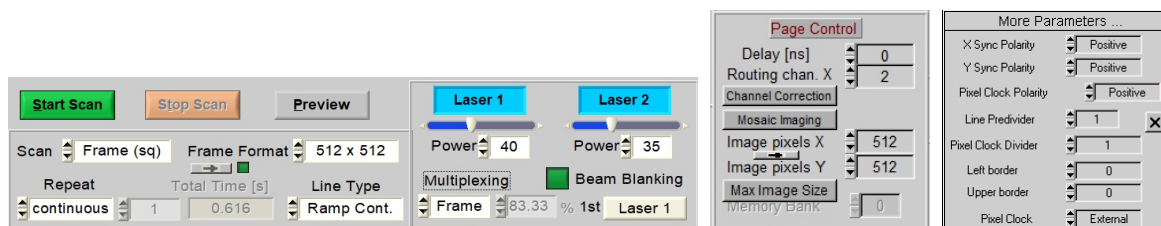


Fig. 243: Parameters setup for laser multiplexing, frame by frame

For the line multiplexing mode the setup has to be different. With line multiplexing, the lasers are running alternately in every second line. Laser one is running in the uneven lines, laser 2 in the even lines. To build up a correct image the SPC modules have to combine two lines of the scan into one line of the photon distribution. The corresponding parameters are shown in Fig. 244.

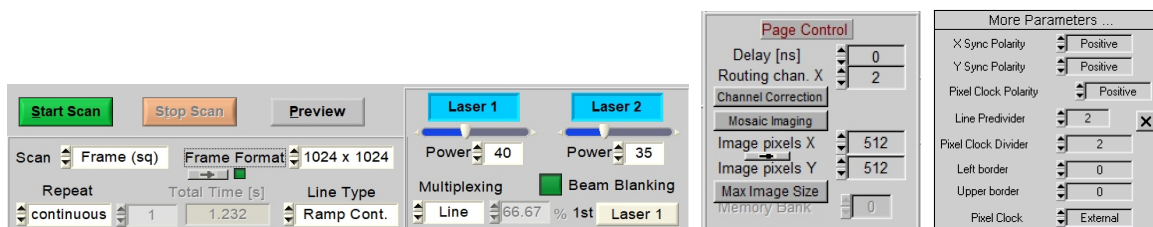


Fig. 244: Parameters setup for laser multiplexing, line by line

The scanner runs frames of 1024 x 1024 pixels. 'Line Predivider' = 2 and 'Pixel Predivider' = 2 bin every two pixels and every two lines into one pixel and one line of the image. The final images are thus 512 x 512, as specified in the page control parameters. Two such images exist for each TCSPC module, each containing photons excited by different lasers.

Fig. 245 shows the setup for pixel multiplexing. Different than frame and line multiplexing, Pixel multiplexing does not switch the lasers pixel by pixel. Instead, it switches from one to the other laser *within one pixel*. Left to right, Fig. 245 shows the upper part of the scan control panel, the page control section, and the more parameter panel of the SPC system parameters.

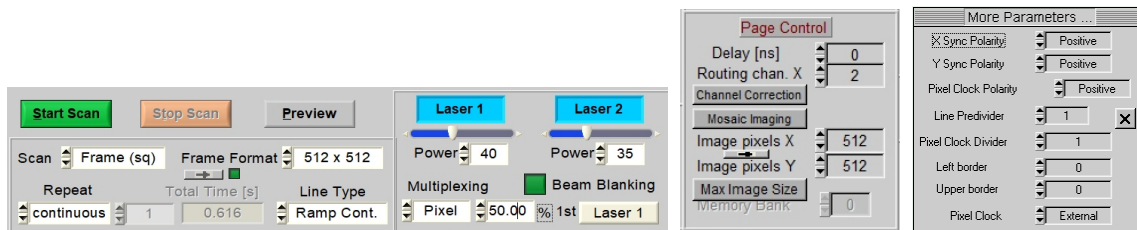


Fig. 245: Parameters setup for laser multiplexing, pixel by pixel

The scanner scans frames of 512 x 512 pixels. Both lasers are turned on, and are multiplexed within the same pixel. Laser 1 is running for 50% of the pixel time, laser 2 for the rest of the pixel. The corresponding TCSPC system parameters are shown in Fig. 245, middle and right. The 'Page control' parameters (middle) are set to 2 routing channels and an image size of 512 x 512 pixels. In the 'More Parameters' panel (right) line and pixel clock dividers are set to 1, i.e. no pixel and line binning are used.

Configuration of DCS Switch Box

Laser multiplexing requires that the bits of the laser multiplexing signal from the GVD-120 scan controller arrive at the correct routing inputs of the SPC modules. The connection of the bits is different for laser multiplexing and for multi-wavelength FLIM. Therefore, the DCS box can be electronically configured from the scan control panel. Click on the tab 'DCS-Box' to open the configuration panel, and activate 'Laser Routing Enabled'. Please see Fig. 246. The configuration data are stored in the .sdt and .set data and setup files. When a system configuration is loaded via the predefined setup panel, or a setup or date file is loaded the routing configuration is loaded with the data and sent to the DCS box.

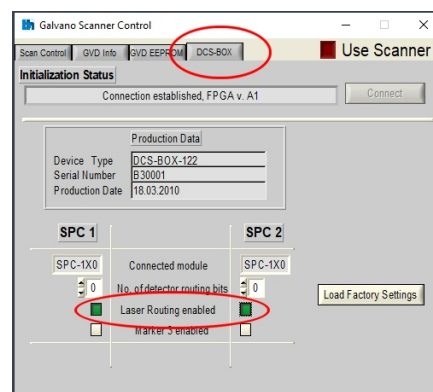


Fig. 246: Definition of the routing configuration of the DCS box

Display of the Images

To display these images simultaneously the 'Window Intervals' and '3D Trace Parameters' shown in Fig. 247 are used. The window parameters (left) define two routing windows both of

which contain just one routing channel. The 3D Trace Parameters define four display windows, W1 through W4. W1 and W2 are for 'Routing Window 1', i.e. for images excited by Laser 1, W3 and W4 for 'Routing Window 2', i.e. laser 2. W1 and W3 are for Module M1 (detecting in the long wavelength range), W2 and W4 for Module 2 (detecting in the short wavelength range). The main panel obtained with these settings is shown in Fig. 242.

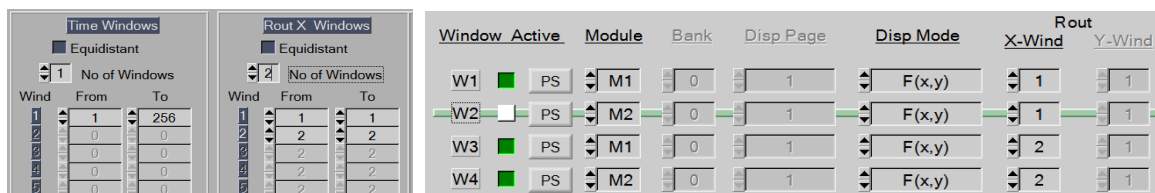


Fig. 247: Window Intervals and 3D Trace parameter definitions for laser multiplexing

Online-Lifetime Display

Laser multiplexing can be combined with online-lifetime display. The function is enabled in the 3D Trace parameters, see Fig. 248. The main panel then becomes as shown in Fig. 249.

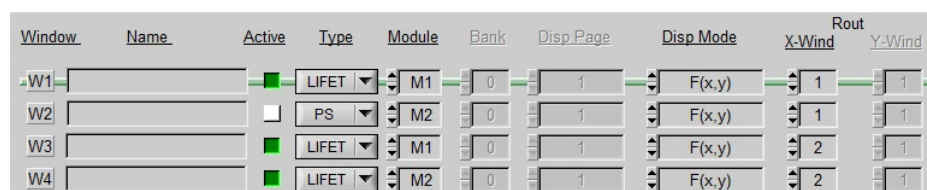


Fig. 248: Trace parameters for online-lifetime display

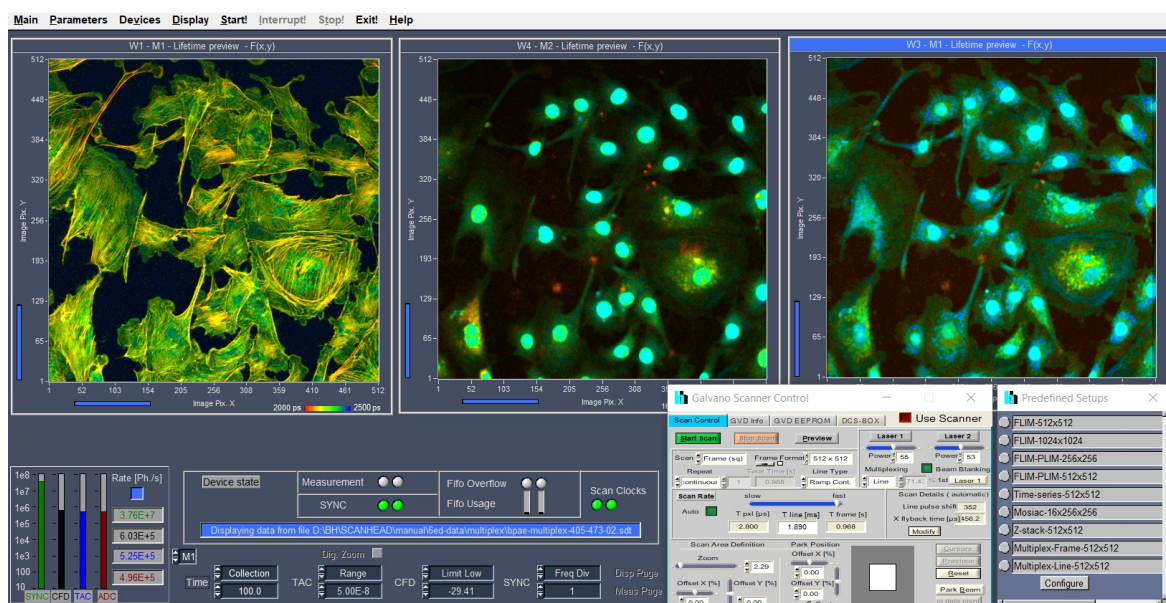


Fig. 249: Main panel with online-lifetime display

Data Analysis

Laser-multiplexed FLIM data are analysed by the same procedures as normal FLIM images. The images in the SPCM panel can either be sent to SPCImage all at once, or separately. Data analysis in SPCImage is performed the normal way. The analysed images of the data displayed in the main panel of SPCM (Fig. 242 and Fig. 249) are shown in Fig. 250.

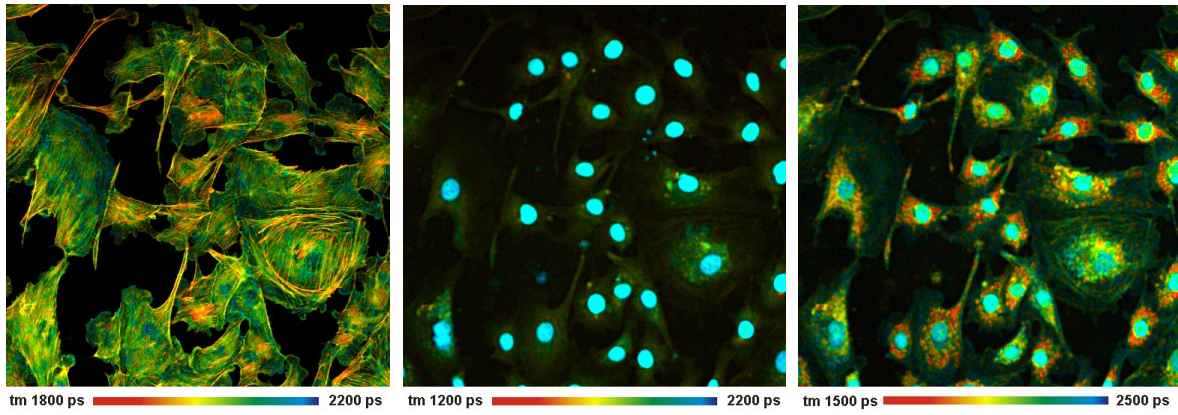


Fig. 250: Analysed images of the data shown in Fig. 242. SPCImage, amplitude-weighted lifetime of double-exponential decay model.

Multi-Wavelength FLIM

Principle

Multi-wavelength FLIM simultaneously delivers 16 lifetime images for 16 wavelength intervals within the spectrum of the fluorescence. A spectrum of the fluorescence light is spread over the input channels of an MW-FLIM multi-wavelength detector assembly. The assembly consists of a spectrograph, a 16-channel GaAsP PMT, and the electronics that determines in which detector channel a photon was detected, see Fig. 103 page 59.

For every photon, the detector thus delivers a timing pulse and the number of the channel that detected the photon. Together with the spatial position of the scanner in the moment of the photon detection, x and y , the TCSPC module determines the time of the photon in the laser pulse period and reads the detector channel number. It uses these pieces of information to build up a photon distribution over the time of the photons in the fluorescence decay, the wavelength, and the coordinates of the image [67, 69, 89, 80]. The principle of the recording process is illustrated in Fig. 251. The result is an image that contains several decay curves for different wavelengths in each pixel.

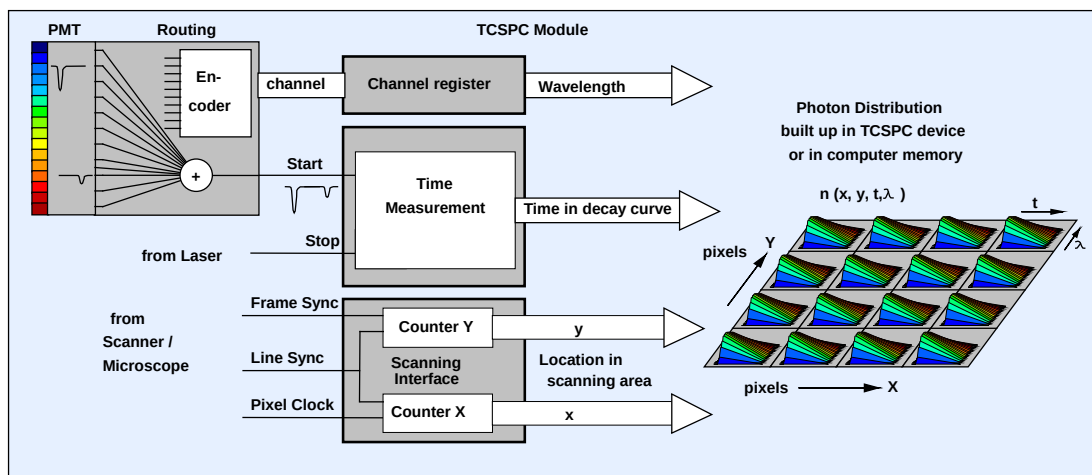


Fig. 251: Multi-wavelength FLIM. The recording process builds up a photon distribution over x, y, t , and λ in the device memory. The result is an array of pixels, each containing a number of fluorescence decay curves for different wavelength.

The result can also be interpreted as a number of FLIM images for different wavelength [67, 77]. The entire assembly is connected to one of the optical ports of the DCS-120 scanner via a

fibre bundle. For multi-wavelength FLIM one HPM-100 detector is replaced with the MW-FLIM GaAsP detector. No other hardware changes are required. The detector connected to the DCS-120 scan head is shown in Fig. 252.

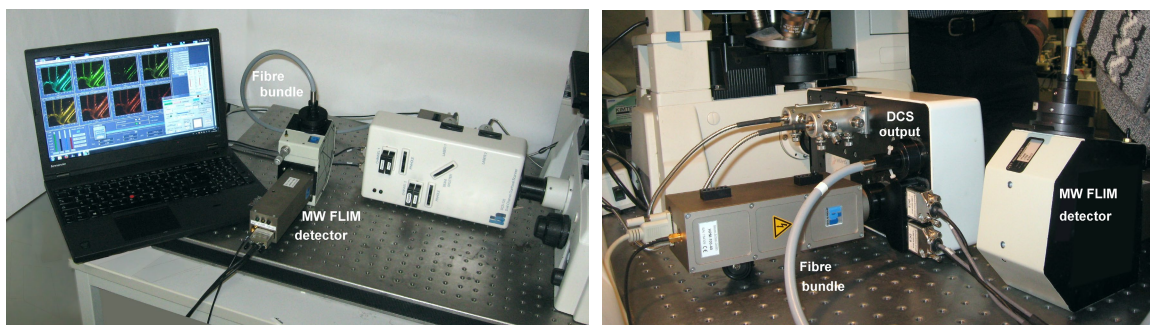


Fig. 252: MW FLIM GaAsP multi-wavelength detector connected to DCS-120 scanner

System Parameters

The TCSPC system parameters of a FLIM measurement with the MW-FLIM detector are shown in Fig. 253 and Fig. 254.

Fig. 253: System parameters for a multi-wavelength FLIM measurement, SPC-180N series. FIFO Imaging Mode, 16 detector channels, 512 x 512 pixels, 256 time channels

Fig. 254: System parameters for a multi-wavelength FLIM measurement, SPC-QC series. FIFO Imaging Mode, 16 detector channels, 512 x 512 pixels, 256 time channels

Measurement Control

The measurement runs in the FIFO Imaging mode. The measurement control parameters are essentially the same as for FIFO Imaging with single-channel detectors. With the settings shown in Fig. 253 and Fig. 254, intermediate results are displayed every 2 seconds.

Data Format and Page Control

The image size and the number of wavelength channels are specified under ‘Page Control’. Until 2016, the image format was limited by the amount of memory provided by Windows 32 bit. Typical data formats were 256x256 pixels and 64 time channels, or 128x128 pixels and 256 time channels. With the introduction of SPCM 64 bit this memory limitation does no longer exist. With the system parameters shown in Fig. 253 and Fig. 254 the DCS-120 system delivers images of 512x512 pixels and 256 time channels for all 16 wavelength channels.

Main Panel Configuration

The traditional way to display multi-Wavelength FLIM data is by using the normal display functions of SPCM. Eight images are displayed, each for two subsequent channels of the MW FLIM detector. The SPCM Main Panel for multi-wavelength FLIM is shown in Fig. 255. The predefined setup, the scanner control, and the detector control panels are kept open on the right. The display colours have been adjusted to the spectral colours of the wavelength intervals. The data can also be displayed as lifetime images, please see Fig. 150, page 83.

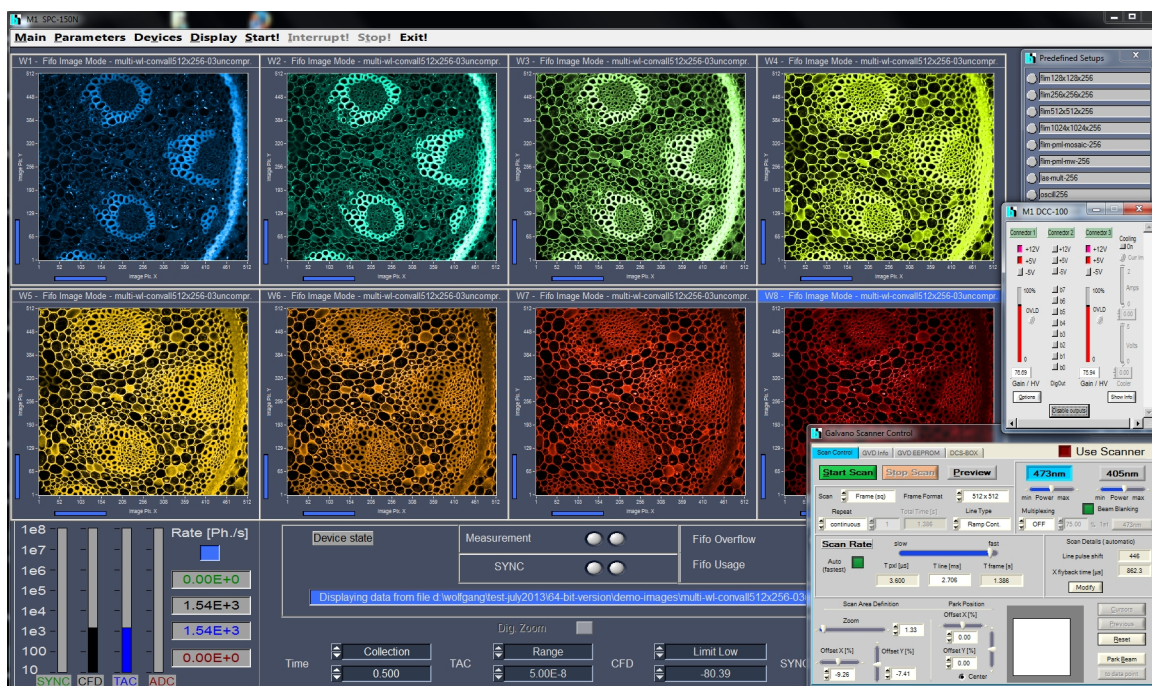


Fig. 255: SPCM main panel for multi-wavelength FLIM

Window Parameters and 3D Trace Parameters

The Window Parameters and 3D Trace Parameters define how many images are displayed in the SPCM main panel, and which data they contain. The Window parameters for Multi-wavelength FLIM differ from those for normal FIFO imaging in that several routing windows are defined. The routing windows define wavelength intervals in which images are to be displayed. The window parameters shown in Fig. 256 define eight routing windows, each containing two subsequent wavelength channels.

Fig. 256: Window parameters for multi-wavelength FLIM

The 3D Trace parameters shown in Fig. 257 define independent display windows for the eight routing windows. Thus, eight images for subsequent wavelength intervals are displayed. Each image contains the data of two wavelength channels of the MW FLIM detector.

Fig. 257: 3D Trace parameters for multi-wavelength FLIM, display as coloured intensity images

Fig. 258: 3D Trace parameters for multi-wavelength FLIM, display as lifetime images

The parameters shown in Fig. 256 and Fig. 257 assume that there is only one MW detector, and that it is connected to SPC module 1. In a dual-SPC-180NX system, the MW detector can also be connected to module 2. 'Module' in the 3D Trace parameters would then be 'M2'.

Please note that the combination of the detector channels by the window intervals act *only on the way the data are displayed*. The .sdt file generated by the SPCM software contain the data of all individual wavelength channels, and for detectors and all SPC modules used.

DCS Box Settings

The DCS switch box (see Fig. 106, page 61) must be configured to transfer the routing bits of the MW detector to the SPC modules. The DCS box is configurable via the configuration panel shown in see 147, right. The settings in the configuration panel are stored in the SPCM data and setup files. When a data or setup file is loaded via the predefined setup panel or via the load panel the DCS box is automatically re-programmed according to the information stored in this file.

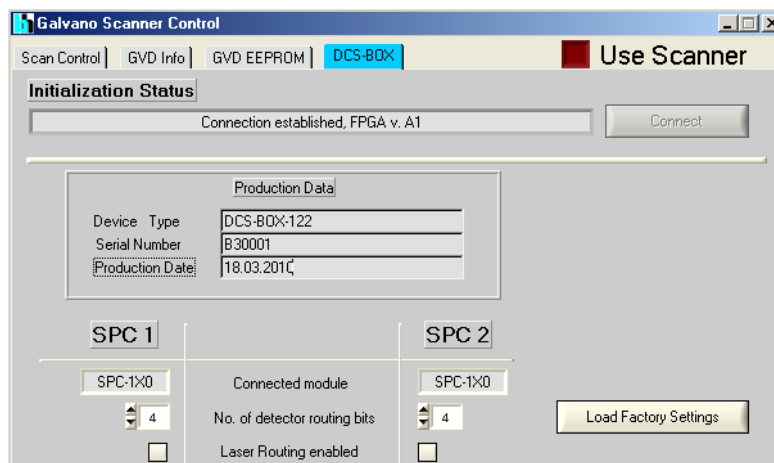


Fig. 259: Configuration of the DCS box for multi-wavelength FLIM

Multi-Wavelength FLIM by Mosaic Function

Mosaic FLIM records several (usually a large number of) FLIM measurements into the elements of a single, large data array. The individual measurements can represent different lateral positions on the sample, different longitudinal positions, different times after the start of the experiment, or data in different wavelength channels of a multi-wavelength detector.

System Parameters for Multi-Wavelength Mosaic FLIM

The system parameters for multi-wavelength Mosaic FLIM are shown in Fig. 260. The basic FLIM parameters are the same as for classic multi-wavelength FLIM, see 'Multi-Wavelength FLIM', page 143. The difference is that the Mosaic Imaging is enabled in the Mosaic Imaging panel. Mosaic Type 'Routing Channels' has been selected, and a mosaic layout of 4 x 4 elements has been defined.

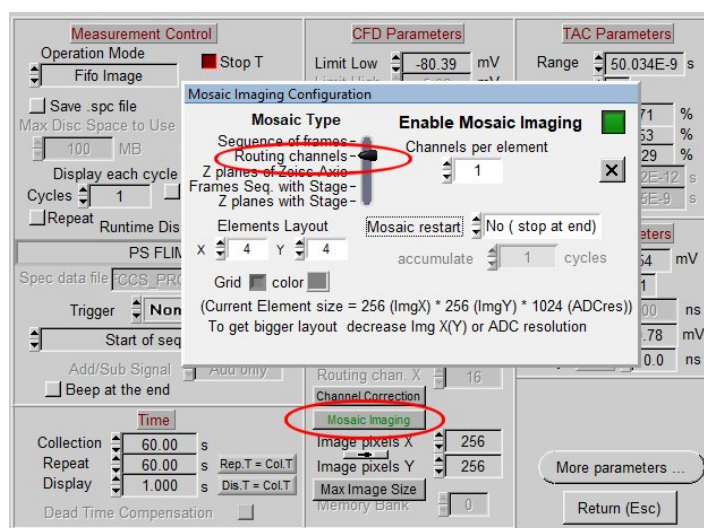


Fig. 260: System parameters for multi-wavelength Mosaic FLIM with Mosaic configuration panel

Main Panel Configuration

A typical main panel configuration for Multi-Wavelength Mosaic FLIM is shown in Fig. 261. The detector control panel is open in the upper right, the scanner panel and the predefined setup panel are open in the lower right. The online decay-curve display (see page 129) is used to display a decay curve from a point of interest in the 7th wavelength element (second row, third column).

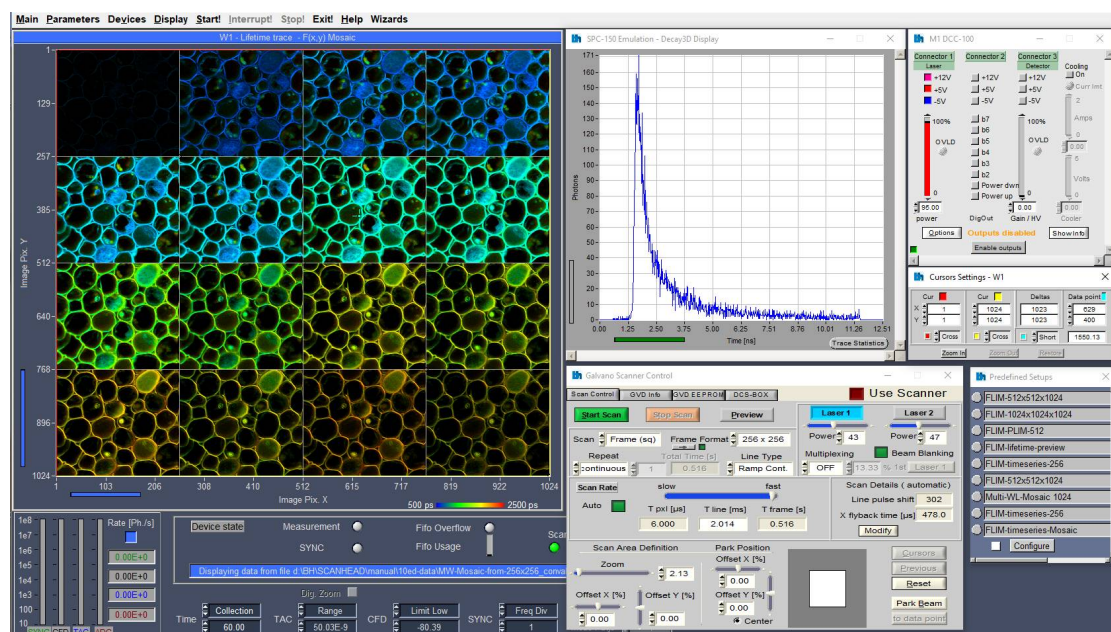


Fig. 261: Typical Main Panel configuration for Multi-Wavelength Mosaic FLIM

3D Trace Parameters and Window Parameters

The 3D Trace Parameters define the Display Mode 'Mosaic', the Window Parameters are irrelevant, see Fig. 262.

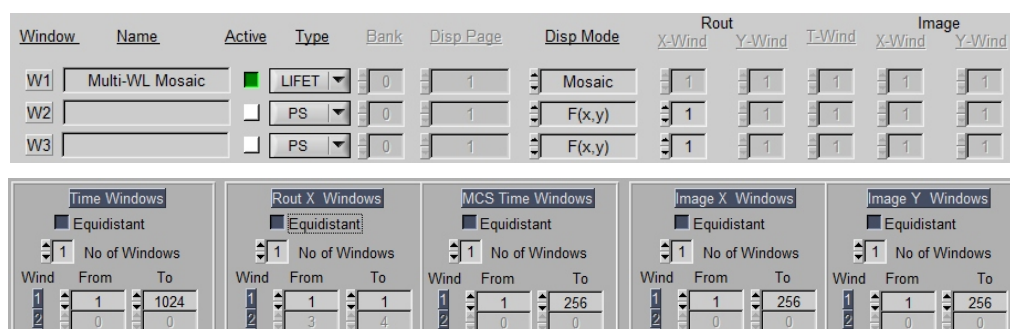


Fig. 262: 3D Trace and Window Parameters for multi-wavelength Mosaic FLIM

Time-Series FLIM

The bh FLIM systems are able to automatically record a series of consecutive measurements and save the data into subsequent data files. The individual measurements may be steps of a time series of measurements, subsequent planes of a FLIM Z-stack, or any other series of

measurements over which an external parameter of the experiment is changed. The procedure is controlled by the Cycle and Autosave functions of the SPCM software.

For time-series FLIM, SPCM records an image, saves the data into a file, records a new image, saves it in a new file, and so on. The advantage is that a virtually infinite number of time steps can be recorded, and that the individual images can be obtained at pixel numbers up to the maximum resolution of the FLIM system. The disadvantage is that some time is required to save the data between the recordings. The speed is therefore limited to about one image per second. For faster recording and accumulation of the data (e.g. after a periodic stimulation of the sample) please see ‘Time-Series FLIM by Mosaic’, page 151.

Measurement Control Parameters

The record-and-save procedure is organized by the measurement control functions of SPCM. The operation mode is FIFO Imaging. A number of measurement ‘Cycles’ is defined, corresponding to the number of images to be recorded. The results of the cycles are saved into subsequent files by the ‘Autosave’ function. The names of these data files are specified under ‘Spec data file’. The file name specified is ‘time-series’, the software extends the file name by ‘_c01’, ‘_c02’, and so on. The acquisition time for each cycle is defined by ‘Collection Time’ the time from one cycle to the next by ‘Repeat Time’. The measurement control section of the SPCM system parameters for time-series FLIM is shown in Fig. 263.

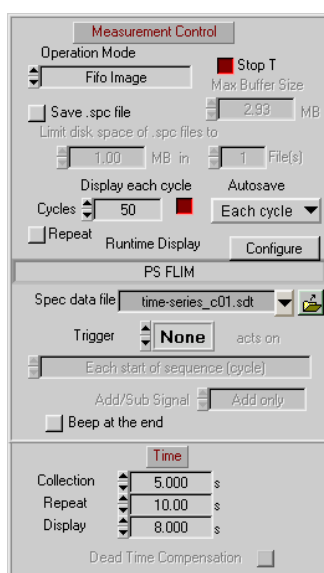


Fig. 263: Recording a FLIM sequence

With the parameters shown, the FLIM system records 50 cycles, resulting in 50 images. The collection time for each measurement cycle is 5 seconds, the measurements are repeated in intervals of 10 seconds. The data of the subsequent recording cycles are saved into data files named time-series_c01.sdt, time-series_c01.sdt, and so on.

There is a number of possible modifications of this procedure. Fig. 265, left, shows a setup where the individual measurement cycles are triggered by an external trigger signal. The acquisition time per step is 20 seconds, the recording is triggered by a positive pulse edge on the Experiment trigger line. An input connector for the trigger pulse is available at the front of the DCS switch box, see Fig. 264. When the cycles are triggered the ‘Repeat Time’ does not control the start of the next recording, it only enables the system to react to the next trigger edge.

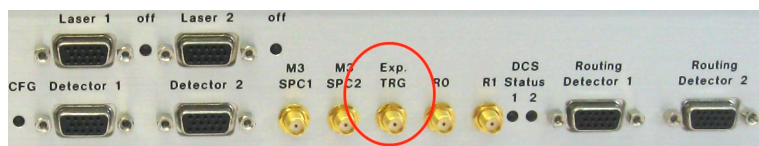


Fig. 264: Trigger input at the DCS switch box

Single frames are recorded by the setup shown in Fig. 265, middle. To obtain single-frame recording the collection time has been set shorter than the frame time. The recording then starts with the start of the next frame, and continues until the end of this frame (see ‘Start-Stop Synchronisation with the Scanning’, page 111). Please note that some time is necessary to save the data, which means that at least one frame is lost during the save operation. (For recording extremely fast time series please see ‘Time-Series FLIM by Mosaic Imaging Function’, page 151).

For recording fast sequences of large images ‘display each cycle can’ be turned off to have more computation power for the data recording and data saving processes available, see Fig. 265, right.

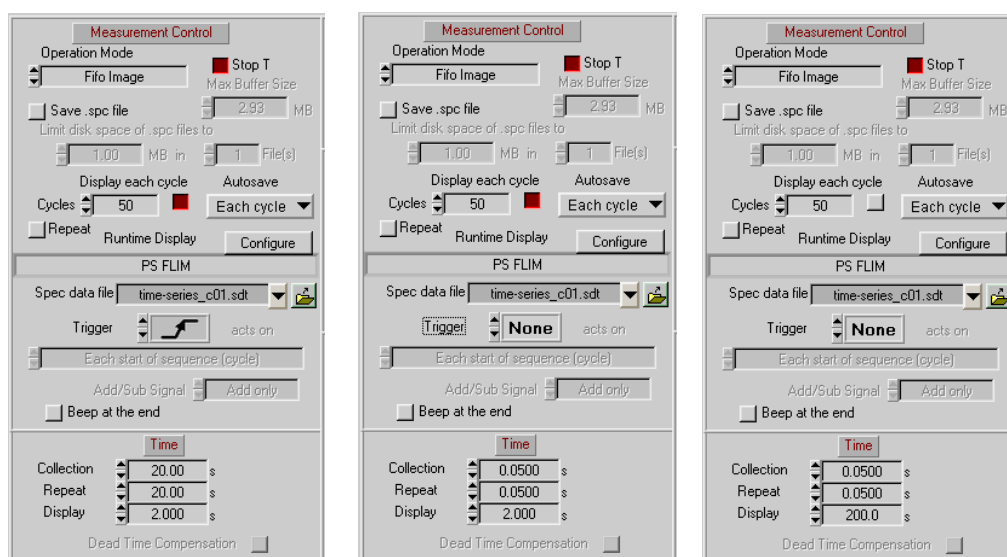


Fig. 265: Left: Triggered time series, each cycle is triggered by a positive pulse edge at the experiment trigger input of the SPC modules. Middle: Single-frame recording. The collection time is selected shorter than the frame time. Right: Fast sequence, single frame recording, display of individual cycles turned off to save computation power.

Display of Time Series Data

Time-series data can be displayed by the Multi-File View functions of SPCM. The function is accessed via the ‘Main’ pull-down menu of the SPCM software, see Fig. 166.

Analysis of Time Series Data

Time-series data produced by a record-and-save procedure consist of (usually large) number of FLIM data files. Data analysis of such data can be conveniently performed by the batch processing function of SPCImage. Batch processing loads the .sdt FLIM files one after another, analyses the data by a selected model and with selected fit parameters, and writes them into subsequent .img files. Please see chapter ‘SPCImage NG Data Analysis Software’, ‘Batch Processing’.

Time-Series FLIM by Mosaic Imaging Function

Principle

The principle of time-series Mosaic FLIM is shown in Fig. 266. The sample is scanned repetitively in the same spatial position, but subsequent images are recorded in consecutive elements of the FLIM mosaic. The result is a time series, the time step of which is a multiple of the frame time.

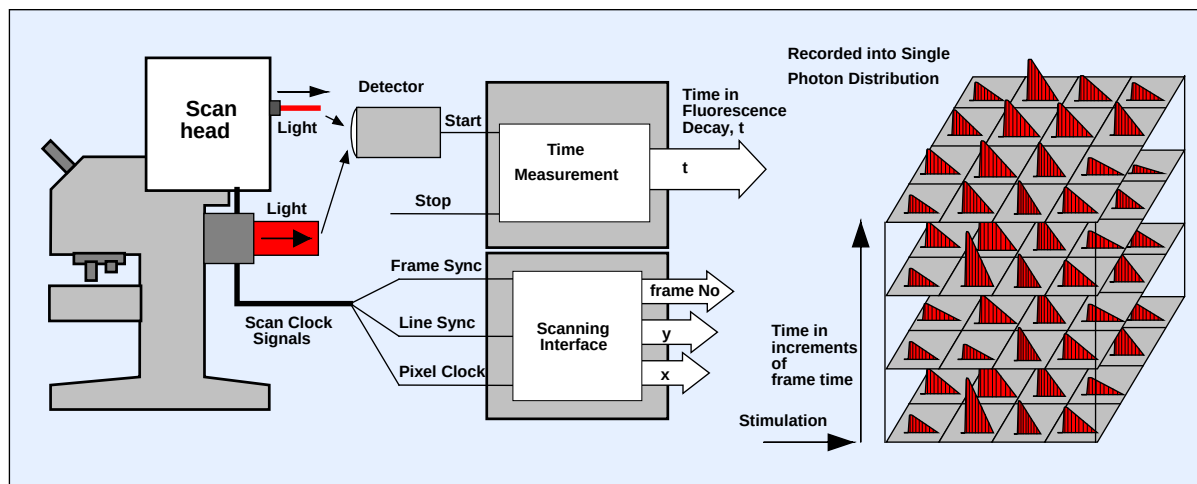


Fig. 266: Temporal Mosaic FLIM

Mosaic Definition

The mosaic FLIM definition panel is shown in Fig. 267, right. The setup shown records a time series of 64 images with 256 x 256 pixels and 256 time channels into a mosaic of 8x8 elements. Each element of the mosaic represents one frame of the microscope scan. The size of the result is equivalent to a 2048x2048 pixel image with 256 time channels. It may therefore be required to allocate more memory to the SPCM application, see Fig. 267, left.

The speed of the time series can be changed by selecting different frame times in the DCS-120 scan control panel or by defining several 'Frames per Element' in the mosaic definition panel.

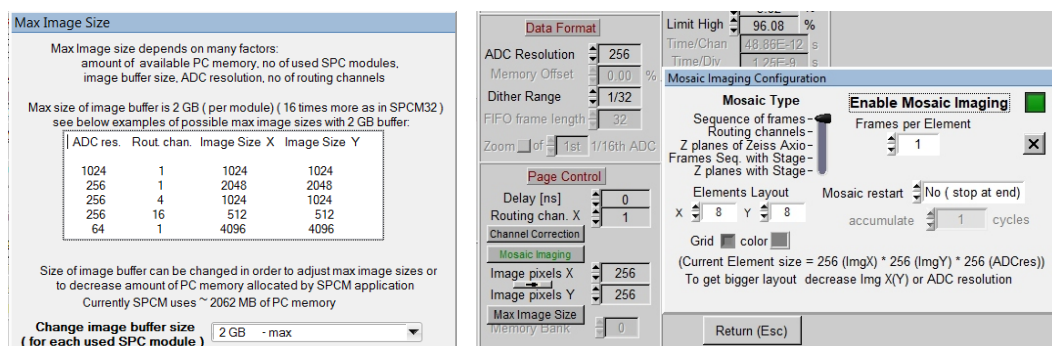


Fig. 267: SPCM system parameter setup for temporal mosaic FLIM. The setup records a time series of 64 256x256 pixel images into a mosaic of 8x8 elements. Each element of the mosaic represents one frame of the microscope scan.

Example

An example of a mosaic time series is shown in Fig. 268. The sample was a fresh moss leaf. The time runs from lower left to upper right. The excitation light causes a non-photochemical transient in the chlorophyll fluorescence [258, 259, 311], resulting in a decrease in the fluorescence lifetime with the time of exposure, compare elements lower left an upper right.

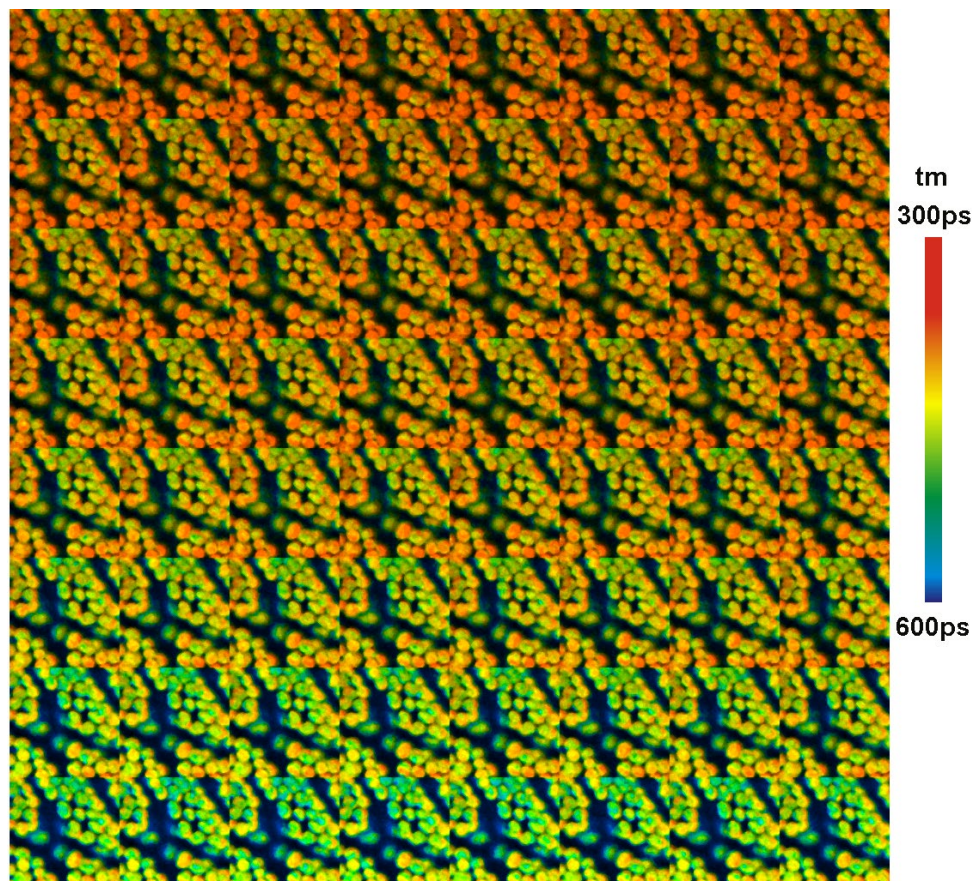


Fig. 268: Time series recorded by mosaic imaging. Non-photochemical transients in chloroplasts of a moss leaf. 64 mosaic elements for consecutive times after turn-on of excitation light. Acquisition time per element 1 second, total time of sequence 64 seconds, image size of each element 128 x 128 pixels, 256 time channels. Time runs from lower left to upper right.

Triggered Accumulation

Mosaic time-series recording has several advantages over the conventional record-and-save procedure. First, the transition from one mosaic element to the next occurs instantaneously. No time has to be reserved for saving the data of the individual steps of the series. Moreover, the result is not a sequence of files but a single, large photon distribution. The data can thus be analysed in a single SPCImage data analysis run. This makes it easier to use fit procedures with global parameters.

The most significant advantage is, however, that a mosaic time series data can be accumulated: A sample would be periodically stimulated by an external event, and the start of the mosaic recording be triggered by the stimulation. With every new stimulation the recording procedure runs through all elements of the mosaic, and accumulates the photons. Accumulation allows data to be recorded without the need of trading photon number and lifetime accuracy against the speed of the time series [80]. Consequently, the time per step (or mosaic element)

is only limited by the minimum frame time of the scanner. For a 128x128-pixel scan and large zoom factor the frame time is on the order of 100 milliseconds, for 64x64 pixels it can be less than 50 ms.

An SPCM setup for triggered accumulation of a time series is shown in Fig. 269. With the setup shown, a series of 64 images of 64 x 64 pixels is recorded into a mosaic of 8x8 elements. The recording of the sequence is repeated for 100 accumulation cycles, the start of each cycle is indicated by a trigger pulse at the 'Marker 3' input of the SPC module(s).

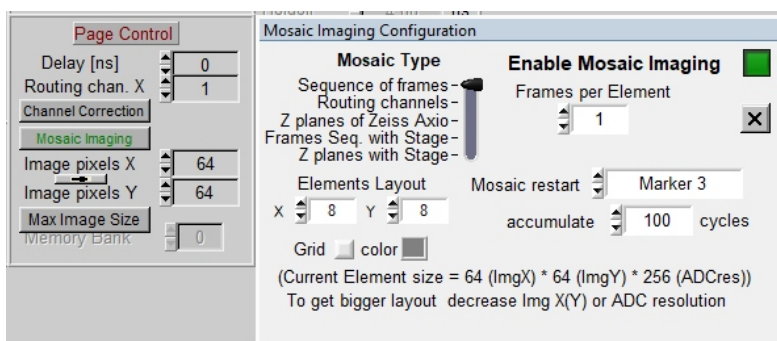


Fig. 269: SPCM setup for triggered accumulation of a time series. A series of 64 images of 64x64 pixels is accumulated over 100 cycles, the start of each cycle is triggered by an input at 'Marker 3'.

The Mosaic FLIM function of the bh FLIM systems is able to record Ca^{2+} transients in cultured neurons after stimulation with an electrical signal. Fig. 270 shows an example. The mosaic format is 8 x 8 elements. Each element was scanned with 64 x 64 pixels, and a frame time of 38 ms. The stimulation period was 3 seconds. 150 milliseconds before every stimulation a recording through the entire 64-element mosaic was started. 100 such acquisition runs were accumulated. The result shows clearly the increase of the fluorescence lifetime of the Ca^{2+} sensor in the mosaic elements 4 to 6, and a return to the resting state over the next 10 to 15 mosaic elements (380 to 570 ms).

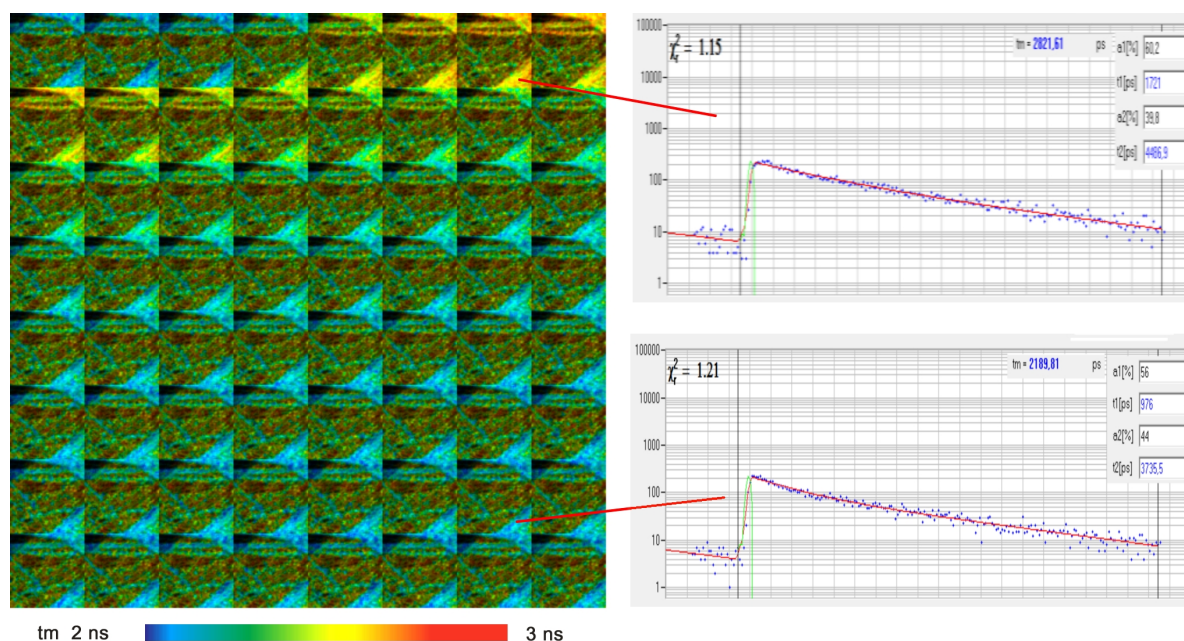


Fig. 270: Temporal mosaic FLIM of the Ca^{2+} transient in cultured neurons after stimulation with an electrical signal. The time per mosaic element is 38 milliseconds, the entire mosaic covers 2.43 seconds. Experiment time runs from upper left to lower right. Photons were accumulated over 100 stimulation periods.

Z Stack FLIM

Z stack FLIM records a series of FLIM images in subsequent focal planes in the sample. That means the microscope must have a Z drive that can be controlled by the FLIM system. This is the case for the Zeiss Axio-Observer Z1 and Axio Examiner Z1 microscopes. For recording the data, two procedures are available. The images from different focal planes can either be saved in subsequent data files by a 'record-and-save' procedure, or stored in a single, large photon distribution, see 'Z Stack Recording by Mosaic FLIM', page 158. Mosaic FLIM data are easier to handle. The record and save procedure has the advantage that the number of Z planes is virtually unlimited. Moreover, it does not require more main memory than a normal FLIM measurement with the same pixel number. Therefore the individual images of the Z stack can, in principle, be recorded with pixels numbers up to the maximum resolution of the DCS system.

Connection to Microscope Z Drive

A panel for controlling the Z drive of the Zeiss microscopes is opened as shown in Fig. 271. Click on 'Connect to Axio Observer Z1' to build up the USB connection to the microscope.

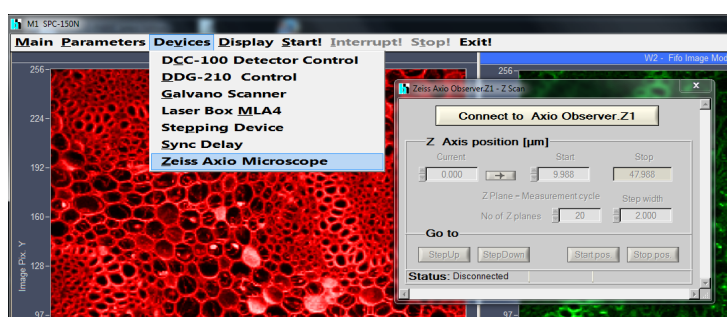


Fig. 271: Activating the panel for the Z control of the microscope

SPCM Main Panel

The SPCM main panel for Z stack FLIM is shown in Fig. 272. In addition to the usual panels for the predefined setups, the detector control, the scanner control the panel for controlling the Z drive of the microscope are open.

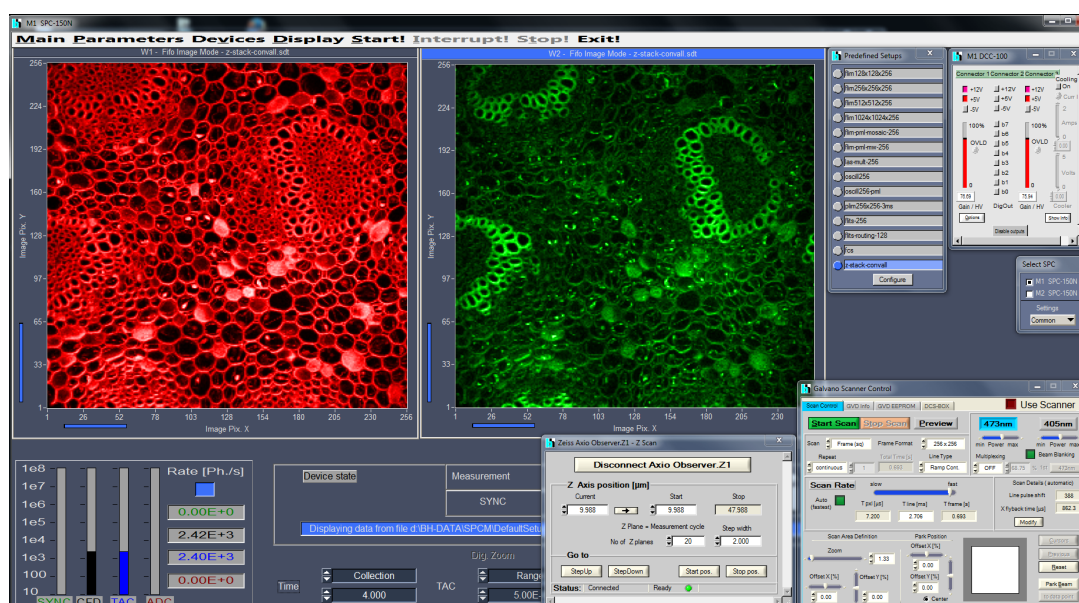


Fig. 272: Main panel for Z stack FLIM

SPCM System Parameters

The system parameters are shown in Fig. 273, left. The procedure is defined by the parameters under 'Measurement Control'. 'Collection Time' defines the acquisition time for each plane of the Z stack. In the setup shown the collection time is 4 seconds, 'Stop T' is set to stop the acquisition after this time. 20 measurement cycles with the defined acquisition time are performed. The images for each measurement cycle (i.e. each Z plane) are displayed (Display each cycle is on), and saved into subsequent data files by the 'Autosave' function. The file names are defined under 'Spec. data file'. The SPCM software extends the file names with '_C01 ... _C10' for the subsequent measurement cycles.

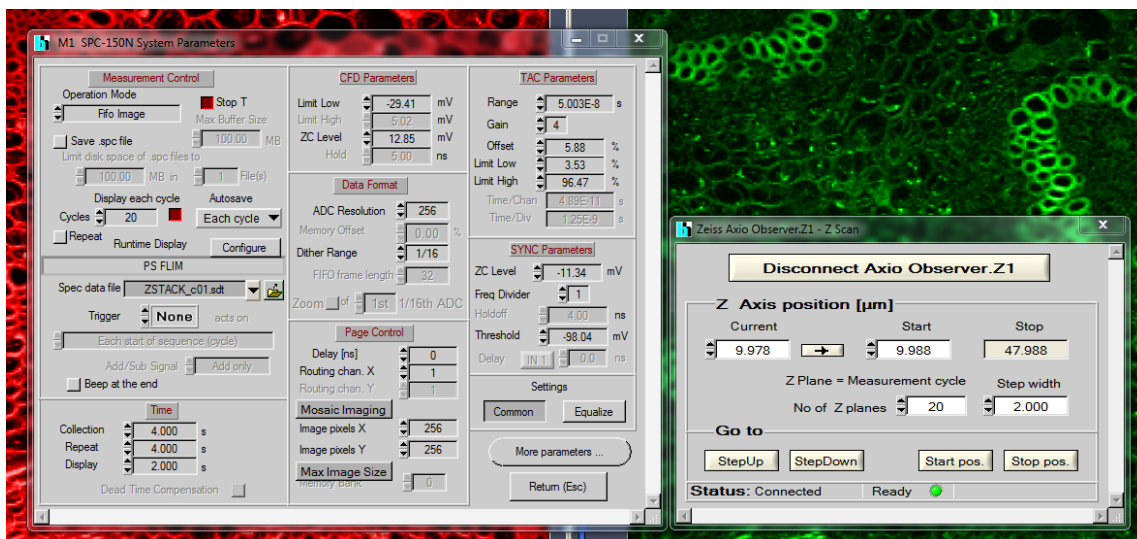


Fig. 273: System parameters for Z stack FLIM

Fig. 273, right, shows the control panel for the Z drive of the microscope. It shows the current Z position, the start position of the Z scan, and the stop position. The current position can be changed by typing in a new position, by clicking on the symbols, by clicking on the Step Up or Step Down buttons, or by turning the focusing knob of the microscope. The stop position is automatically calculated from the number and the width of the Z steps. The number of Z steps is identical with the number of measurement cycles in the system parameters, see Fig. 273, left. Clicking on defines the current position as a start position of the scan. By clicking on the buttons in the lower part of the Z drive panel you can perform single steps, and go to the start position and to the stop position.

Scanner Control

Z scanning is performed with the ordinary x-y scan parameters, see Fig. 274. The scan format used in Fig. 274 is 256 x 256 pixels. In principle, you can use any frame format up to the maximum frame size of 2048 x 2048. Since the images are saved into data files after being recorded Z stack FLIM does not require more main memory than a normal FLIM acquisition with the same number of pixels. However, the amount of data produced by recording a large number of large-size images (and the time needed to analyse them!) can be enormous. We therefore recommend to restrict the frame size to pixel numbers no larger than absolutely needed. Typical frame sizes for Z scanning are 256 x 256 and 512 x 512 pixels, both with 256 time channels.

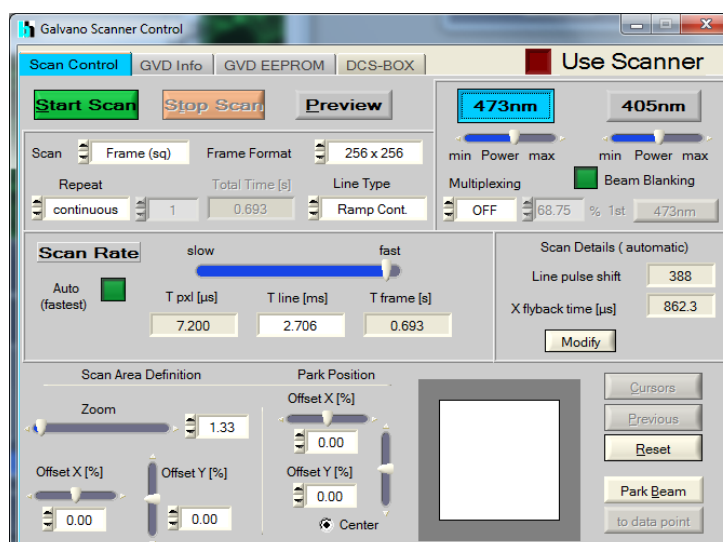


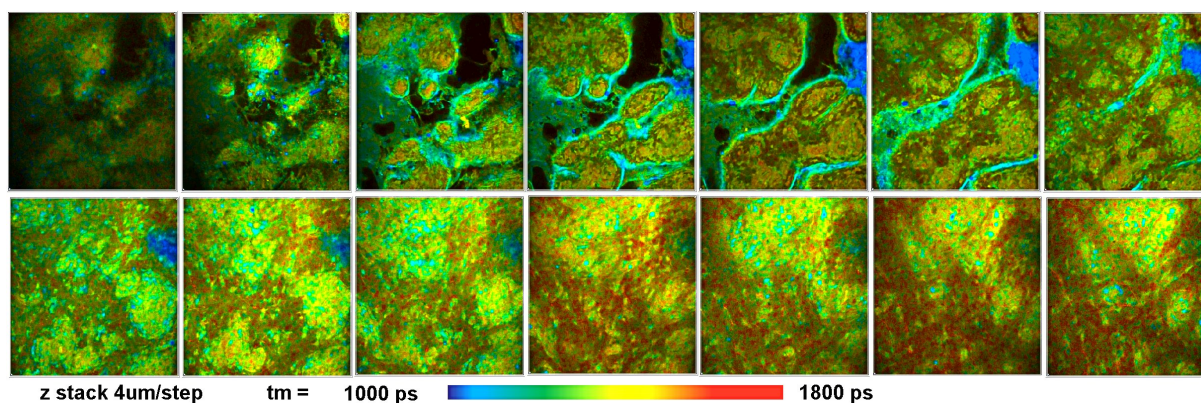
Fig. 274: DCS-120 scanner setup used for the scans shown in Fig. 275

Procedure

To run a Z scan sequence, first run a fast repetitive scan in the 'Preview' mode. With the Preview running, select the Z start position for the Z scan. You can do this by turning the focusing knob at the microscope, or by changing the position via the Z control panel. When you have found the right Z position, click on  to define it as a start of the Z scan. Then do a few steps up and down in Z direction to find out whether the step width you defined is appropriate. Go to the Stop position to see whether the total Z interval of the scan is appropriate. Then go back to the start position. Switch off the Preview, and start the measurement. The SPCM software will record the defined number of images for the subsequent Z positions and save them into consecutive files.

Examples

An example of a Z scan is shown in Fig. 275. A pig skin sample was scanned by the procedure described above, with 256x256 pixels and 256 time channels, and a Z step width of 4 μm . The acquisition time per Z plane was 30 seconds, the average count rate about $4 \cdot 10^5$ photons/second. The data were analysed by the batch processing function of SPCImage.

Fig. 275: Z stack recording of a pig skin sample. Images 256x256 pixels, 256 time channels. 14 steps in Z, step width 4 μm . DCS-120 MP system, FIFO Imaging mode, 256x256 pixels, 256 time channels.

A high-resolution scan of a fly (*Musca domestica*) is shown in Fig. 276 and Fig. 277. The entire Z scan contains 289 planes, each with a resolution of 1024 x 1024 pixels and 1024 time

channels. Three arbitrarily selected planes are shown in Fig. 276. A vertical projection of all 286 planes is shown in Fig. 277.

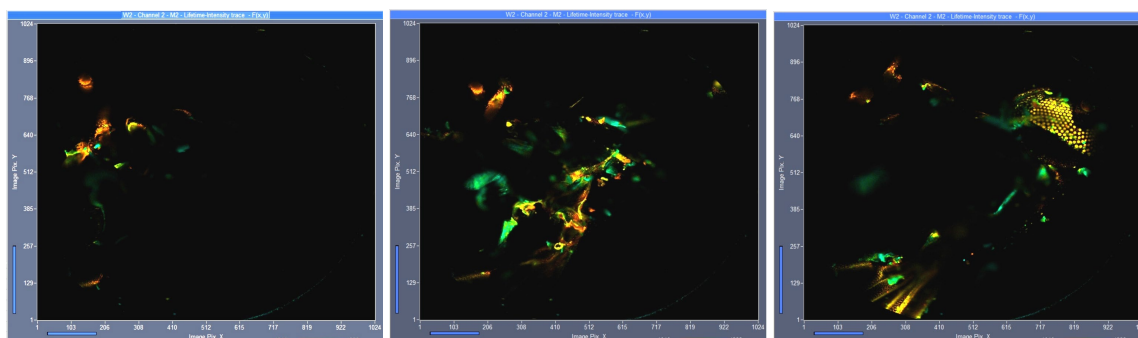


Fig. 276: Three images from different Z planes. Left to right: Plane 120, plane 150, plane 180. Online-lifetime display of SPCM.

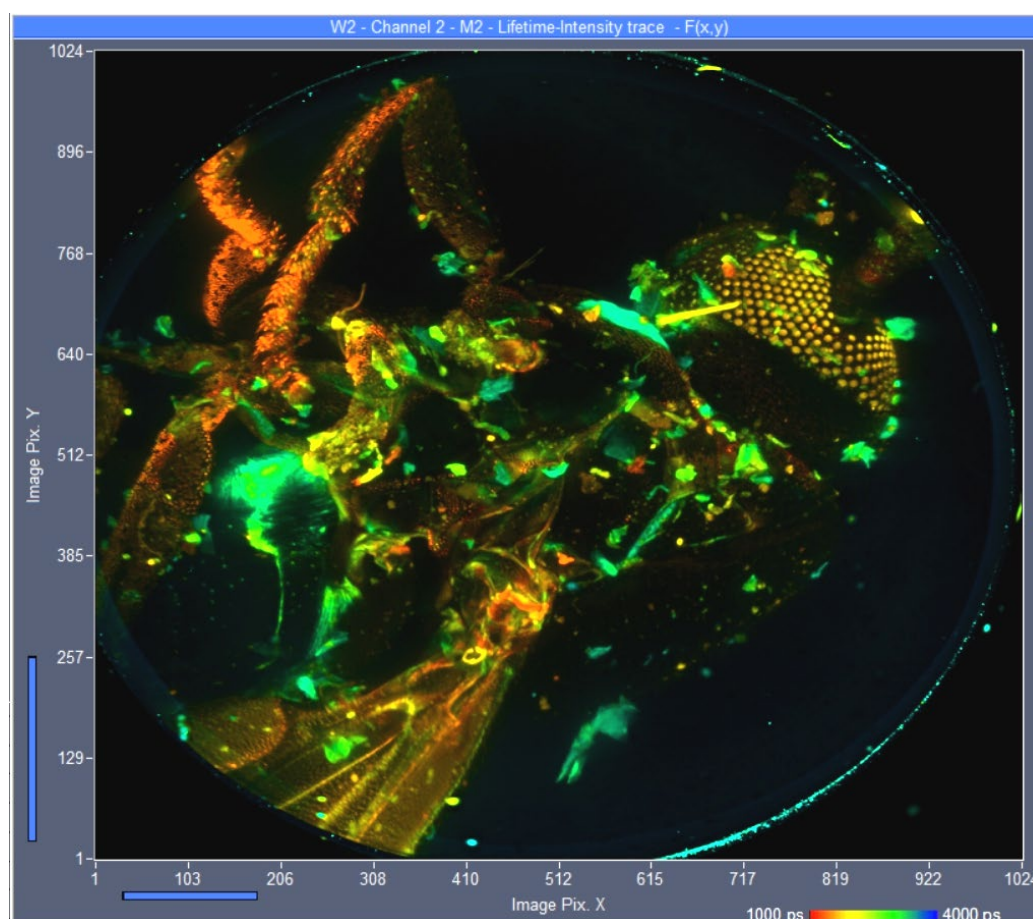


Fig. 277: Vertical projection of all 289 planes into a single FLIM image. Planes added by Multi-File View of SPCM, image displayed by Online-Lifetime function of SPCM.

A 3D reconstruction of the data by Image J is shown in Fig. 278. The individual planes were analysed by the batch-processing function of SPCImage and exported as bitmap files by SPCImage's batch-export function. The bitmap files were loaded into ImageJ, and combined by ImageJ's Image Stacks 3D Project routine.

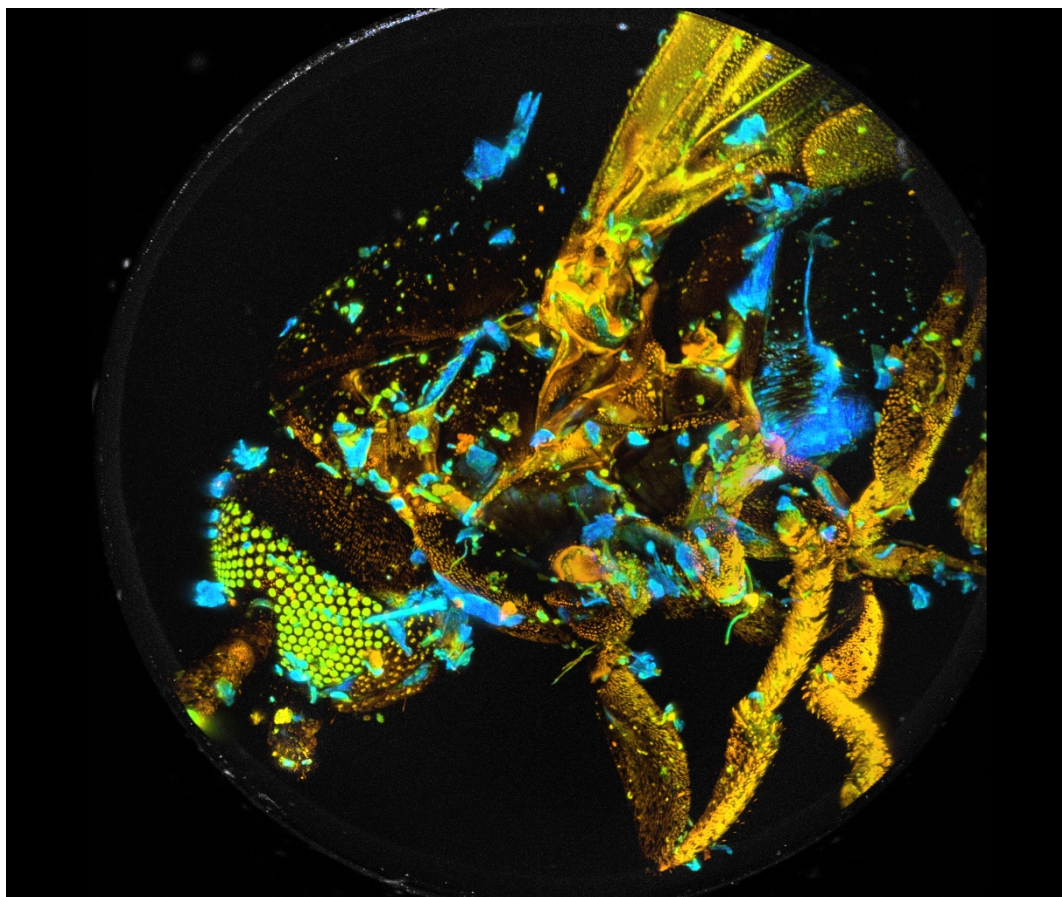


Fig. 278: 3D reconstruction by ImageJ. The data of the individual planes were analysed by SPCImage with a double-exponential model, converted into bmp files, and combined by ImageJ. The colour represents the amplitude-weighted lifetime of a double exponential decay.

Z Stack Recording by Mosaic FLIM

Principle

The mosaic FLIM function of SPCM can be used to record Z stacks of FLIM images. The FLIM procedure for Z-stack mosaic FLIM is illustrated in Fig. 279.

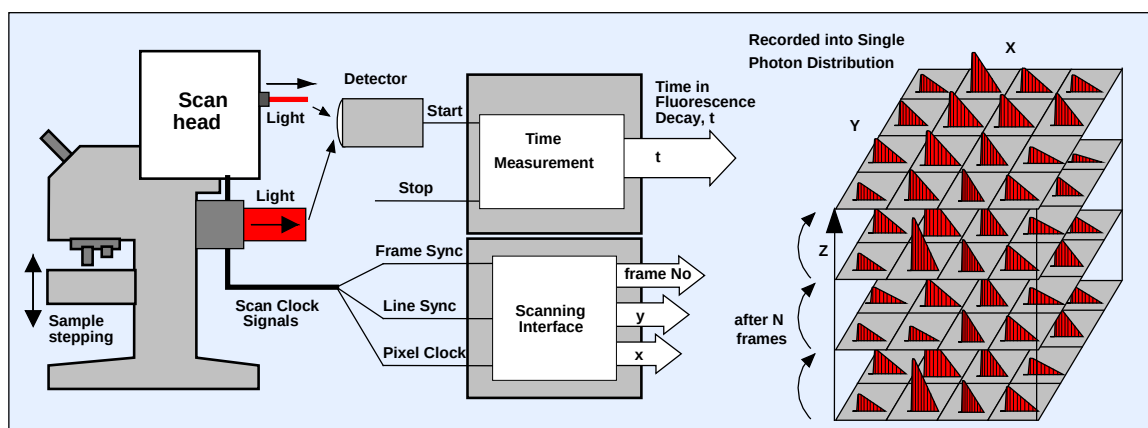


Fig. 279: Z Stack recording by the Mosaic FLIM

The number of mosaic elements is made identical with the number of Z planes the microscope scans. The number of frames per mosaic element is selected according to the number of

frames per Z plane. Compared with a Z-stack FLIM procedure by a record-and-save procedure (see page 154) Mosaic FLIM has the advantage that no time has to be reserved for the saving actions. Moreover, the data of all z planes are recorded in one and the same photon distribution. The data analysis can therefore be run for the data of all z planes together. This makes it easier to maintain exactly the same fit conditions and model parameters for all Z planes, and to extract global parameters from the entire array.

SPCM Main Panel

The SPCM main panel for Z stack recording by Mosaic FLIM is shown in Fig. 280. Images in 16 Z planes were recorded and displayed as a 4x4 mosaic. The control panels for the microscope Z drive, the scanner, and the detectors are open. The predefined setup panel has been placed in the upper right.

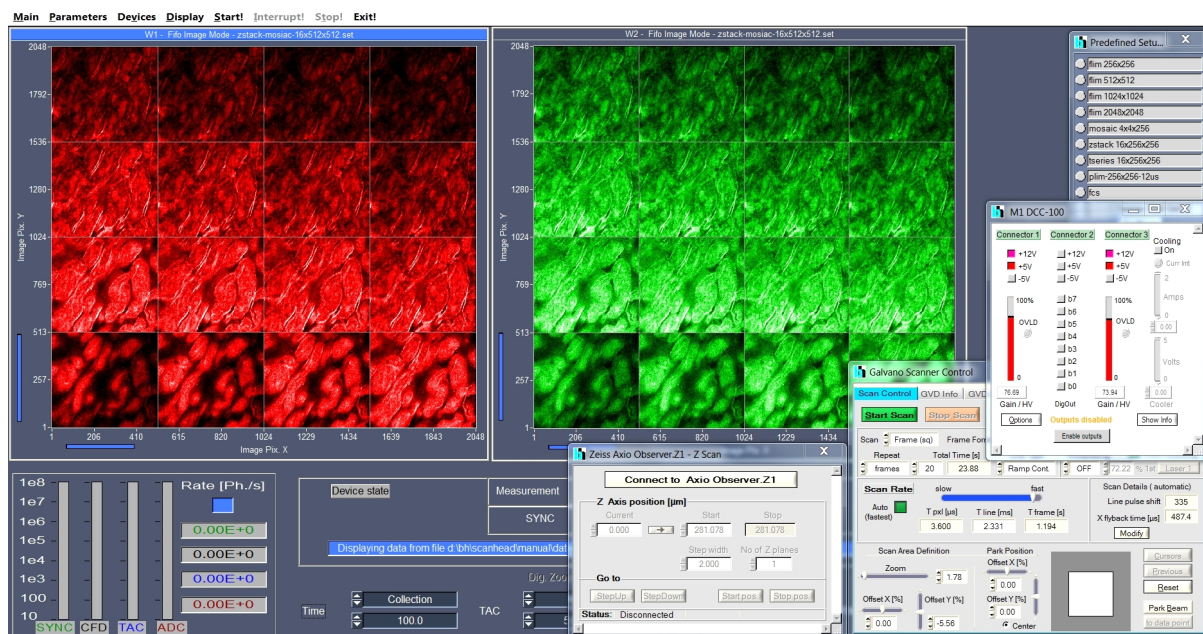


Fig. 280: SPCM main panel for Z-Stack Mosaic FLIM. Two TCSPC channels, each channel 16 Z planes displayed as a 4x4 element mosaic. Control panels for microscope Z drive, scanner, and detectors open.

Mosaic Definition

The mosaic definition parameters are shown in Fig. 281. To create a Mosaic FLIM setup, load a setup of the normal (FIFO Imaging) FLIM mode with the desired number of pixels per image. Then open the System Parameters, click the Mosaic FLIM button, and define the number of elements of the mosaic, and the number of frames per element, see Fig. 282, left. Select Mosaic Type ‘Z planes of Axio Observer’, and define the number and arrangement of mosaic elements. If the desired number of elements is not available go back to the system parameters, click into ‘Max Image Size’ and allocate more memory to the SPCM software (see Fig. 183, page 105 or Fig. 267, page 151). Alternatively, you can reduce the number of pixels or the number of time channels.

When this is done, open the panel for the Z drive of the Axio Observer and define the start position and Z step width, see Fig. 282, right. The number of Z planes is automatically adjusted to number of mosaic elements in the ‘Elements Layout’ (X·Y) in the Mosaic definition panel.

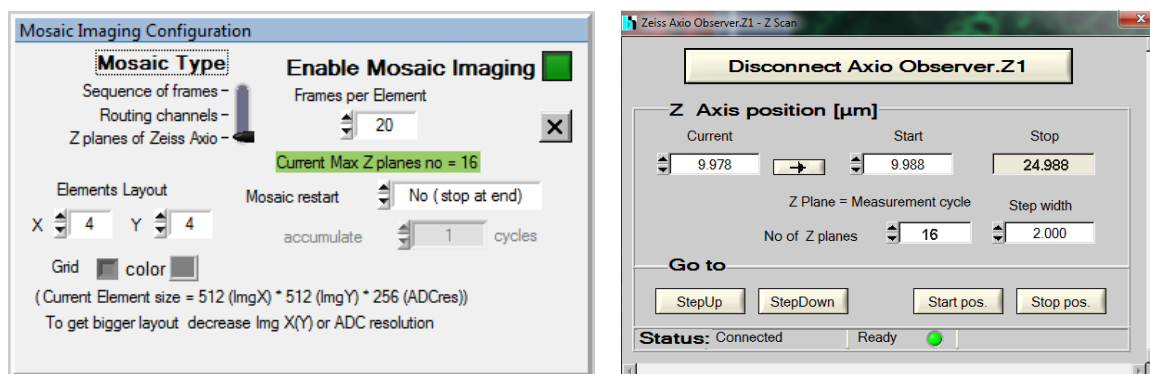


Fig. 281: SPCM setup for Mosaic FLIM Z stack. Mosaic Imaging Configuration (left) and Z-drive panel (right).

Procedure

The procedure for recording a mosaic Z stack is the same as for a conventional Z stack. First run a fast repetitive scan in the 'Preview' mode. With the Preview running, select the Z start position for the Z scan. You can do this by turning the focusing knob at the microscope, or by changing the position via the Z control panel. When you have found the right position, click on  to define it as a start of the Z scan. Then do a few steps up and down in Z direction to find out whether the step width you defined is appropriate. Go to the Stop position to see whether the total Z interval of the scan is appropriate. Then go back to the start position. Switch off the Preview, and start the measurement. The SPCM software will record the defined number of images for the subsequent elements of the mosaic.

Data Analysis

Mosaic FLIM data can be considered a single, large photon distribution containing the images of all Z planes. The data can thus be analysed like normal FLIM images: Send the data to SPCImage, select an appropriate model, set appropriate fit parameters, and start the analysis. There is only one difference to the analysis of 'normal' FLIM images: Z scan mosaic data can be extremely large. For example, the data shown in Fig. 280 are equivalent to two 2048 x 2048 images with 256 time channels in each pixel. In other words, this is the same amount of data as in 512 2048 x 2058 pixel intensity images. Data analysis of such large images can take a long time. We therefore suggest to analyse a small part of the data first, and check whether the model and the fit parameters were appropriately selected. A fast analysis can be run with the '1st Moment' calculation. It delivers the fluorescence lifetime of a single-exponential approximation of the data. The lifetimes are obtained with the ideal photon-number limited standard deviation ($1/N^{1/2}$) but can contain a systematic bias if the fluorescence did not decay completely in the recording time interval. Please see chapter 'SPCImage NG Data Analysis Software'.

Analysed images of the data of Fig. 280 are shown in Fig. 282. Images of single planes are shown in Fig. 283.

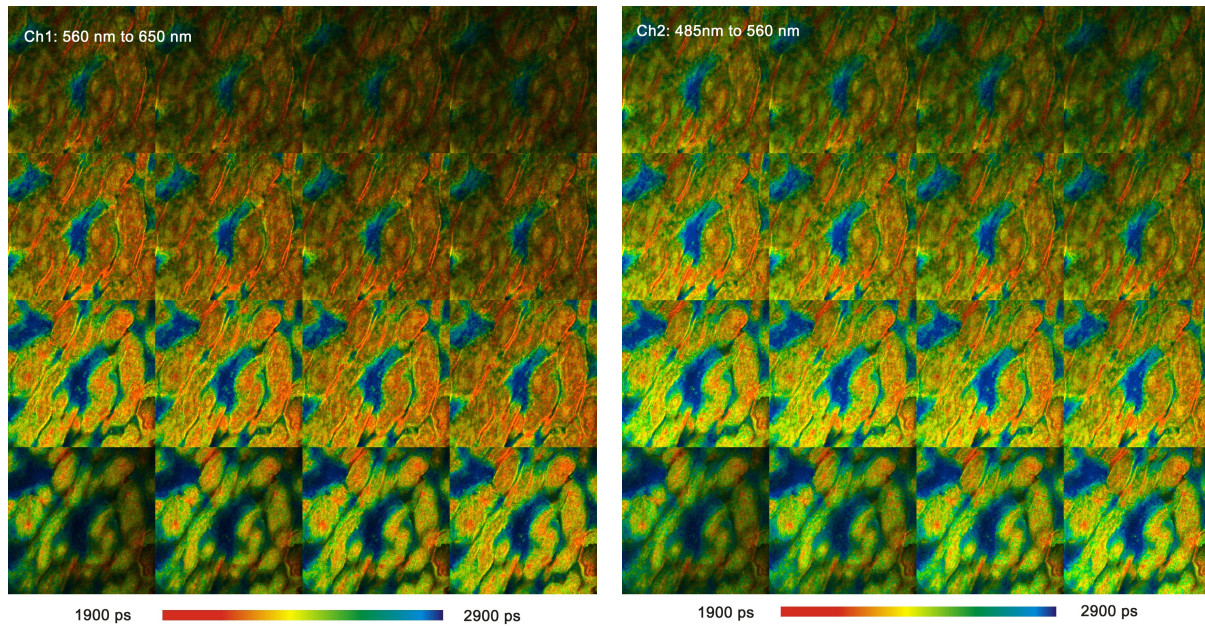


Fig. 282: Mosaic Z Stack, data from Fig. 280, analysed by SPCImage. Pig skin sample, excited at 473 nm. 16 Z planes recorded into 4x4 element mosaic. Each element 512 x 512 pixels, 256 time channels. Red channel (left) and green channel (right).

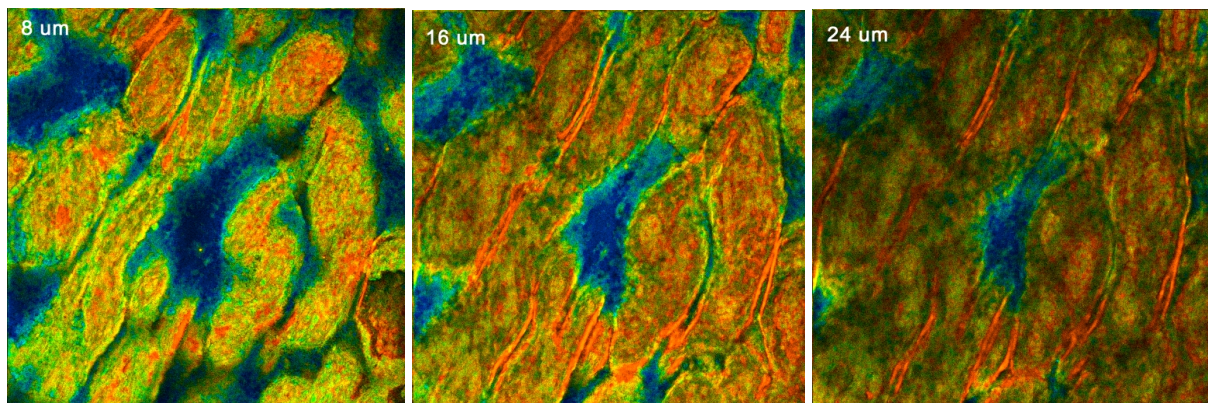


Fig. 283: Images of single Z planes from the array shown in Fig. 282, left. Left to right: Depth in the sample 8 μm , 16 μm , 24 μm

X-Y Mosaic FLIM

With software version 9.76, the control of a motorised sample stage has been integrated in the bh SPCM TCSPC/FLIM data acquisition software. The stage can be used to record X-Y mosaic FLIM. The microscope scans an image at one position of the sample, then offsets the sample by the size of the scan area, and scans a new image. The process is repeated, and the images of the individual frames are fit together into one large FLIM data set. The result is a FLIM image that covers a sample area larger than the field of view of the microscope lens.

The recording process is illustrated in Fig. 284. The recording principle for a single mosaic element is similar to the normal TCSPC FLIM procedure [77, 80]. However, for Mosaic FLIM memory space is provided not only for the photon distribution of a single mosaic element but for the entire mosaic. Recording starts in the data space of the first mosaic element. After a defined number of frames, the sample is shifted by the size of the scan area, and the recording continues in the next data element. The procedure continues until all mosaic elements are filled with data. The result is a FLIM data array that contains all elements of the mosaic. The data structure is the same as for a single FLIM image with a pixel number equal to the total pixel number of the mosaic. For details of the parameter setup please see sections ‘Motorised Sample Stage’, page 114, and ‘Mosaic Imaging Function’, page 107.

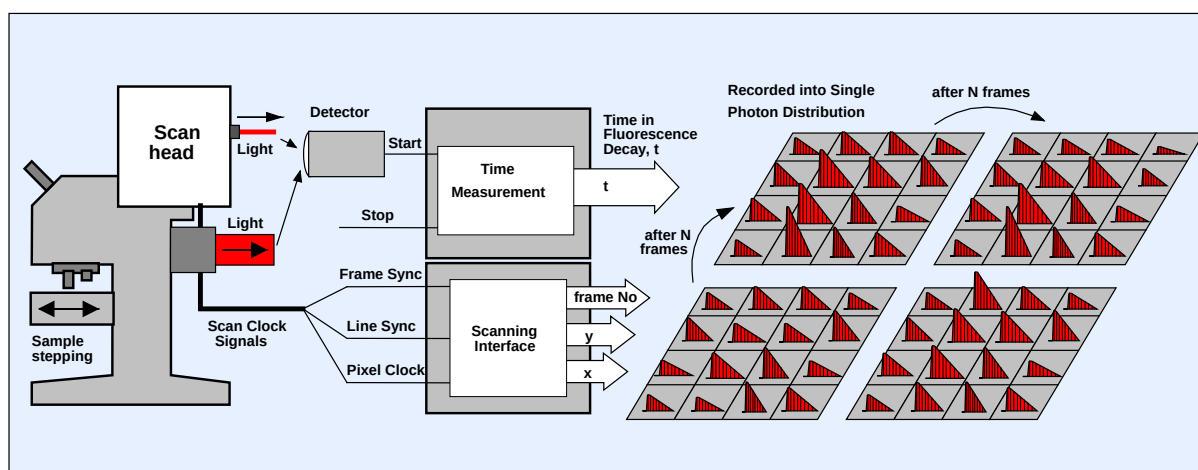


Fig. 284: Mosaic FLIM, recording of a X-Y mosaic

Example

An example of a Mosaic-FLIM image is shown in Fig. 285. The image was recorded by a DCS-120 MP (multiphoton) system in combination with a bh SPC-160 TCSPC system [77]. The mosaic has 4 x 4 elements, each element has 512 x 512 pixels with 256 time channels. The complete mosaic has thus 2048 x 2048 pixels, each pixel containing 256 time channels. The sample area covered by the mosaic is 0.8 mm x 0.8 mm.

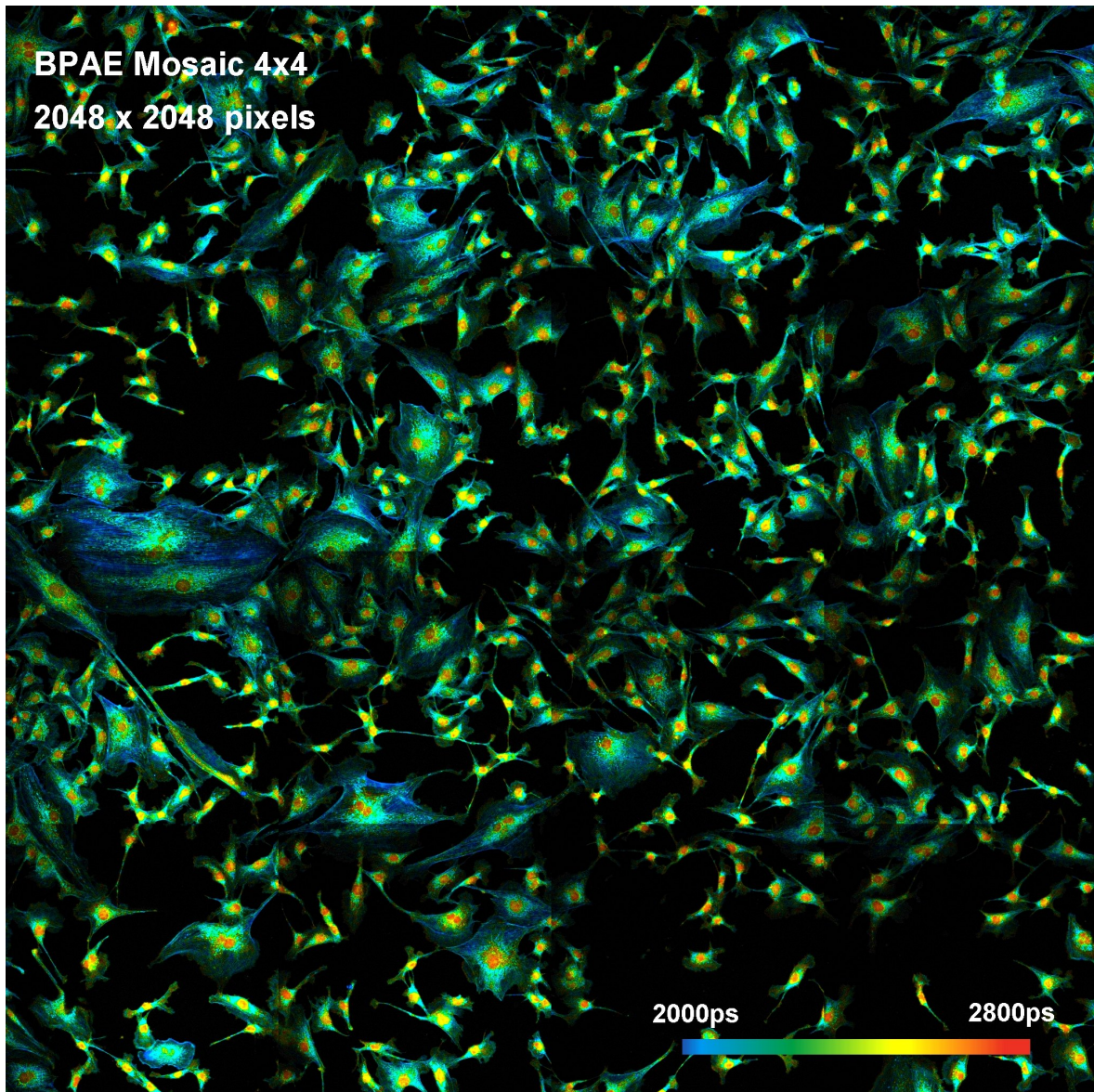


Fig. 285: Mosaic FLIM of a BPAE cell sample. The mosaic has 4x4 elements, each element has 512x512 pixels, each pixel has 256 time channels. DCS-120 MP (multiphoton) system. Data analysis by bh SPCImage.

Advantages

Large-area FLIM images can also be recorded by simply using low-magnification microscope lenses. However, such lenses have low NA (numerical aperture). Low NA results in low light collection efficiency, low excitation efficiency for multiphoton excitation, and poor optical resolution. With mosaic FLIM, large image areas can be covered with high-NA objective lenses. The result is high efficiency, both for excitation and collection, and high spatial resolution. Since the entire mosaic is recorded into a single, large FLIM photon distribution, standard SPCImage FLIM data analysis can be applied to the data.

FLITS: Fluorescence Lifetime-Transient Scanning

Principle

FLITS (Fluorescence Lifetime-Transient Scanning) records transient effects in the fluorescence lifetime of a sample with spatial resolution along a one-dimensional scan [46, 76, 77, 229]. The technique is based on building up a photon distribution over the distance along the scan, the experiment time after a stimulation of the sample, and the arrival times of the photons after the excitation pulses. The maximum resolution at which lifetime changes can be recorded is given by the line scan time. With repetitive stimulation and triggered accumulation transient lifetime effects can be resolved at a resolution of about one millisecond.

Fig. 286, left, shows the photon distribution built up by normal TCSPC FLIM. It is a distribution of photon numbers over x , y , and t . In Fig. 286, right, one spatial coordinate (y) has been replaced with an ‘experiment time’, T . The experiment time, T , is the time after a stimulation of the sample, or after any other event that is temporally correlated to a lifetime change in the sample. X is the distance along a spatially one-dimensional scan.

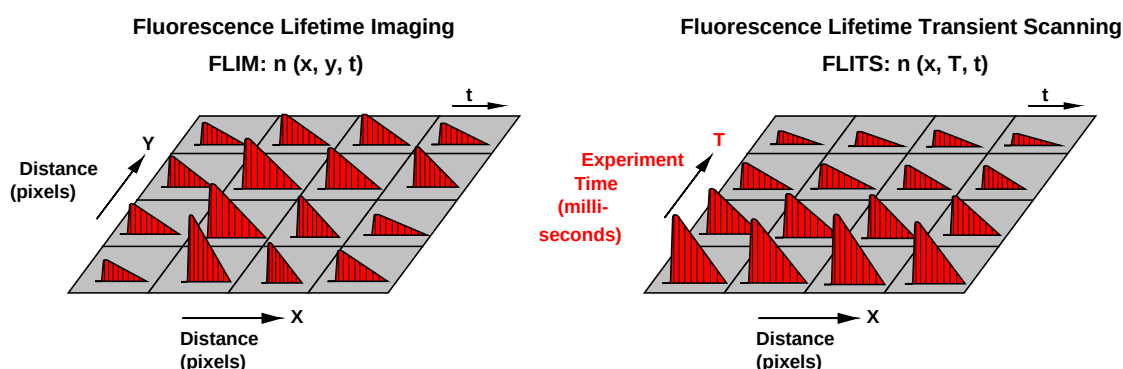


Fig. 286: Left: Photon distribution built up by standard FLIM. Right: Photon distribution built up by fluorescence lifetime-transient scanning.

As long as the stimulation occurs only once the recording process of FLITS is simple: The sequencer of the TCSPC module [77] starts to run with the stimulation, and puts the photons in consecutive time channels along the T axis. The result is equivalent to a time-series of line scans.

However, there is an important difference to a simple time series: The data of all lines are stored in a single data array. The data are still in the memory (either in the on-board memory of the TCSPC module or in the computer) when the sequence has been completed. Thus, the recording process can be made repetitive: The sample would be stimulated periodically, and the start of T triggered by the stimulation. The recording then runs along the T axis periodically, and the photons are accumulated into one and the same photon distribution.

With this ‘triggered accumulation’ process, it is no longer necessary that each T step acquires enough photons to obtain a complete decay curve in each pixel and T channel. No matter when and from where a photon arrives, it is assigned to the right location in x , the right time T , and to the right arrival time, t , after the laser pulse. The signal-to noise ratio depends only on the total acquisition time, not on the line time. This behaviour is an equivalent of the capability of FLIM to deliver a signal-to-noise ratio independent of the pixel time if only the total acquisition time remains the same. Thus, the resolution at which FLITS resolves lifetime changes is limited only by the period of the line scan. This is about one millisecond for the galvanometer mirrors used in the DCS-120 scanner.

Technical Solution

Fluorescence lifetime-transient scanning (FLITS) can be performed in the commonly used FLIM modes of the bh TCSPC modules [77]. That means, the SPC module records the fluorescence photons in the Scan Sync In or in the FIFO Imaging mode. The synchronisation with the scan is accomplished the usual way, via the pixel clock, line clock, and frame clock pulses. However, different than for x-y-t imaging, the frame clock for FLITS does not come from the scanner but from the stimulation of the sample. All that is needed to perform FLITS is a way to switch the frame clock source between the frame clock from the scan controller and a trigger pulse from the source of stimulation. In the simplest case, this can be done by swapping the cable connector between the two sources.

A more comfortable way is to connect the frame clock for FLIM to the Marker 2 input, the frame clock for FLITS to the Marker 3 input, and define two different setups to select the clock source, see Fig. 287. The change between FLIM and FLITS can then be performed by selecting the corresponding setup from the predefined setup panel.

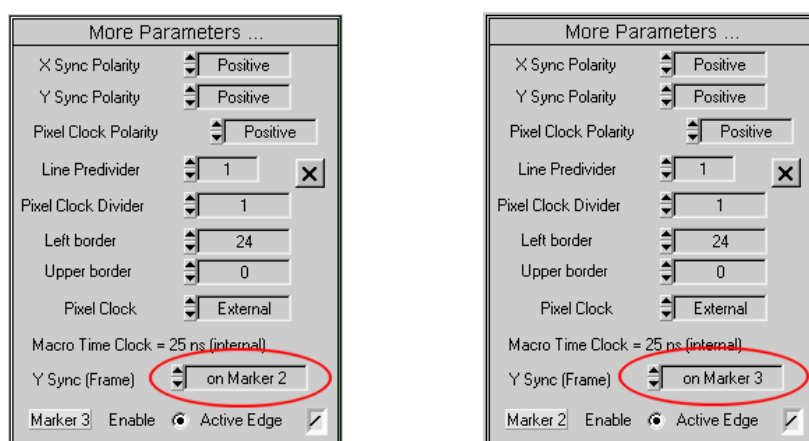


Fig. 287: Selection of the frame clock source via the More Parameters panel in the FIFO imaging mode. FLIM shown left, FLITS shown right.

In the DCS-120 system the frame clock from the scanner is internally connected to ‘Marker 2’. ‘Marker 3’ inputs to the SPC modules of the DCS-120 system are provided at the DCS-120 switch box, see Fig. 288. Thus, all that is needed to get FLITS running is a TTL pulse from the stimulation of the sample, and a connection to these inputs.

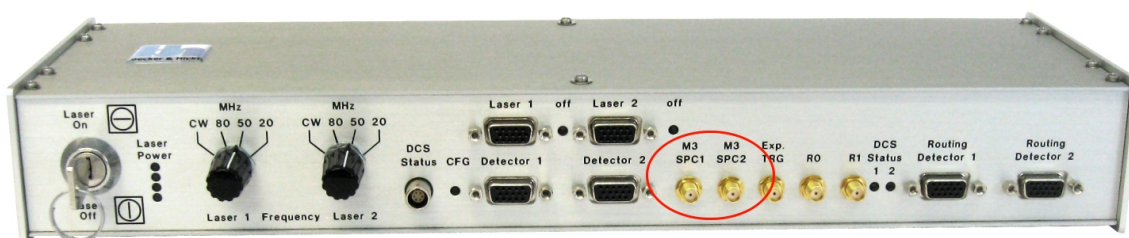


Fig. 288: Marker 3 inputs at the DCS switch box

SPCM Main Panel and System Parameters

An SPCM main panel for FLITS is shown in Fig. 289, System parameters for FLITS in the FIFO Imaging Mode are shown in Fig. 290.

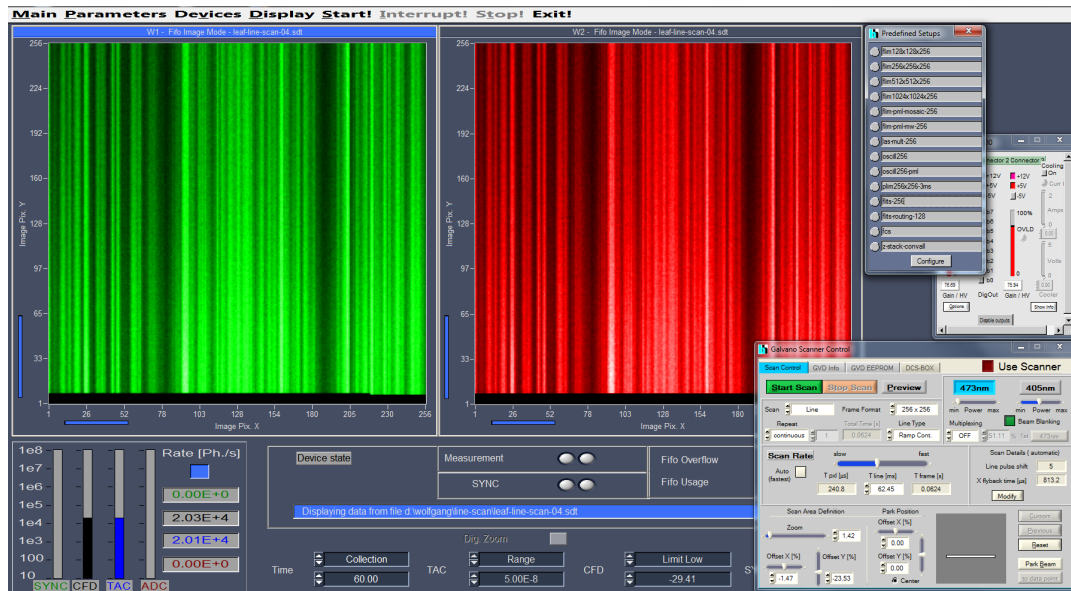


Fig. 289: Main panel, FLITS in the FIFO imaging mode

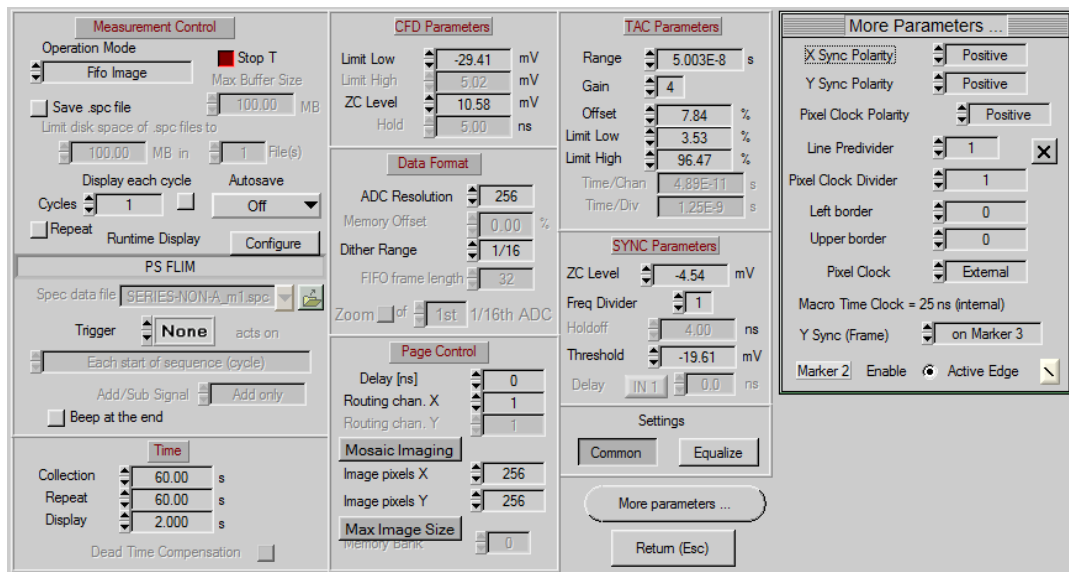


Fig. 290: System parameters, FLITS in the FIFO imaging mode

FLITS procedure

Before the FLITS recording is started it is recommended to run a normal FLIM acquisition to obtain an image of the sample. The result of the FLIM measurement is an image as shown in Fig. 292, left. Then the FLITS setup is loaded. This switches the scanner in the line mode, and switches the source of the frame clock to the stimulation. The location of the line scan is selected within the image recorded before.

The scanner control panel for the line mode is shown in Fig. 291, left. Note that the scan rate selection is not in the 'Auto' mode. Instead, a line time is specified that yields the desired time scale for the FLITS time axis. Please note that even at this stage you can start a fast preview scan, see Fig. 291, right. When you stop the preview the scanner will switch back into the line mode and to the selected scan speed.

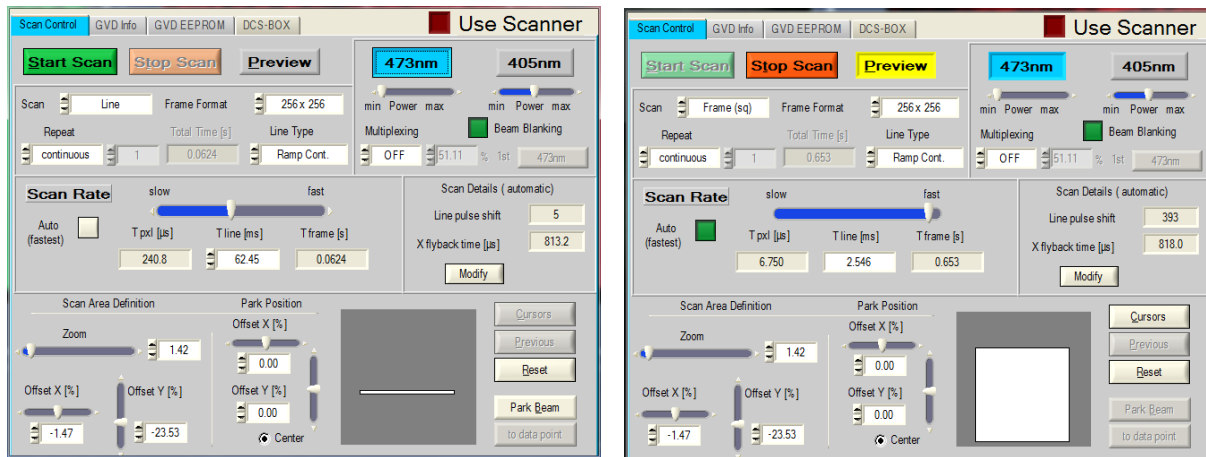


Fig. 291: Left: FLITS setup, scanner in line mode. Right: Preview scan, scanner in frame mode. The scanner switches back to line mode when the preview is stopped.

When the sample is in the correct position and correct focal plane and the scan parameters are set, the FLITS recording can be started. Start the measurement first, either by 'Start Scan' in the Scanner panel or 'Start Measurement' in the main panel. Then start the stimulation sequence.

The SPC module now records a photon distribution $n(x, T, t)$ the coordinates of which are the distance, x , along the scanned line, the transient time, T , after the event that stimulates the sample, and the time, t , of the photon after the laser pulses. The principle is illustrated in Fig. 292, right. The transient-time, T , is given in multiples of the line time.

There is no need to synchronise the start of the recording with the start of the stimulation sequence: The recording in the TCSPC modules does not start until the first trigger pulse from the stimulation arrives. It is only required that the stimulation sequence be started *after* the start of the FLITS recording.

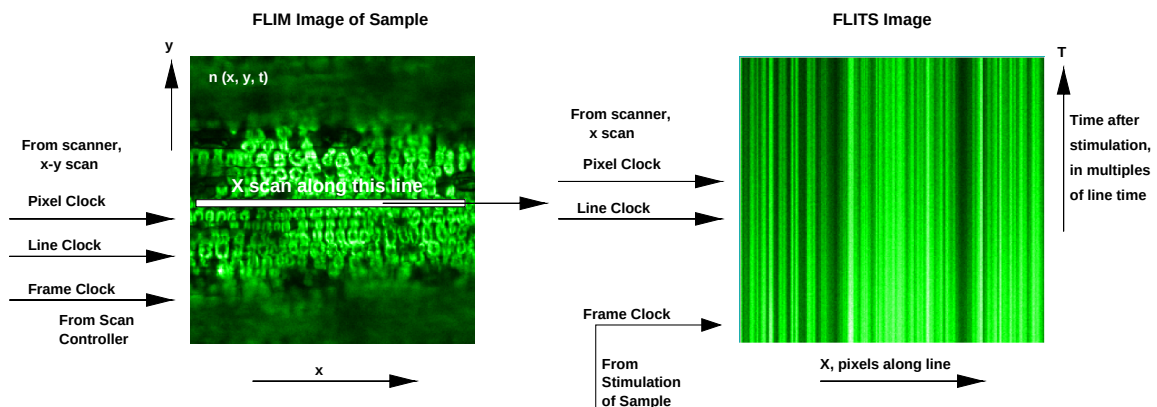


Fig. 292: Principle of recording transient lifetime effects by line scanning

The time scale of T can be varied either by varying the scan rate, or by defining 'line clock divider' values larger than one in the scan parameter section of the TCSPC system parameters, see [77]. When FLITS data have been recorded they can be analysed by standard SPCImage FLIM analysis procedures.

Example: Chlorophyll transients

FLITS can be demonstrated most easily by recording the 'chlorophyll transients' that occur when a live plant is exposed to light [258, 391]. Upon illumination, the fluorescence lifetime (and intensity) first increases. The increase happens within a few milliseconds or tens of mil-

liseconds. It is attributed to the progressive saturation of photosynthesis channels, and a corresponding decrease in fluorescence quenching. The increase in the fluorescence quantum efficiency is therefore called 'photochemical transient'. After a few seconds of exposure, the fluorescence lifetime starts to decrease again. At intensities comparable to sunlight, it reaches a steady-state level after a few tens of seconds. It is assumed that the decrease of the lifetime is a protection mechanism of the plant. The slow decrease is called 'non-photochemical transient'.

Non-Photochemical Transient

Recording the non-photochemical transient is relatively easy: The transient is slow and can even be recorded by conventional time-series FLIM [77, 310], see 'Time-Series FLIM', page 148. It is therefore easy to record it by FLITS.

An example is shown in Fig. 293. The sample was a fresh grass blade. The excitation intensity during the initial FLIM recording was kept low so that no noticeable lifetime change was induced. The FLITS recording was started by manually generating a single frame clock pulse. The excitation laser was switched on 180 ms later. The horizontal coordinate is the distance along the scanned line, the vertical coordinate is the time, T , after the line clock. The line time was 60 ms, the number of lines along the T axis is 256. Thus, the total time along the vertical axis is 15.4 seconds. It can clearly be seen that both the fluorescence intensity and the fluorescence lifetime decrease with the time of illumination.

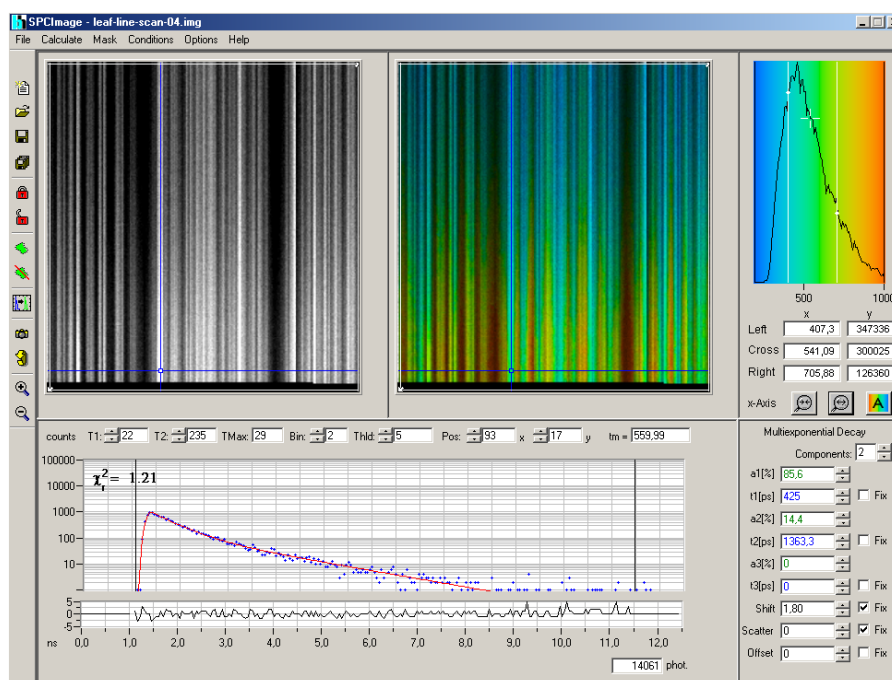


Fig. 293: FLITS image of non-photochemical chlorophyll transient. Horizontal: Distance along the line scanned. Vertical, bottom to top: Time, T , after the start of illumination. 256 pixels along the line, line time 60 ms, 256 lines, total time interval recorded 15.36 seconds. Left: Intensity image. Right Lifetime image. Colour represents lifetime, blue to red corresponds to 200 ps to 1 ns. Amplitude-weighted lifetime of double-exponential decay. Bottom: Decay curve at cursor position.

The lifetime change is shown in more detail in Fig. 294. It shows decay curves for a selected spot in the line scan (2 pixels wide) for times of 0.5 s, 7.5 s, and 13.4 s after the turn-on of the laser. The mean lifetimes, t_m , obtained from a double-exponential fit are 560 ps, 385 ps, and 311 ps, respectively. The changes in the decay profiles are clearly visible.

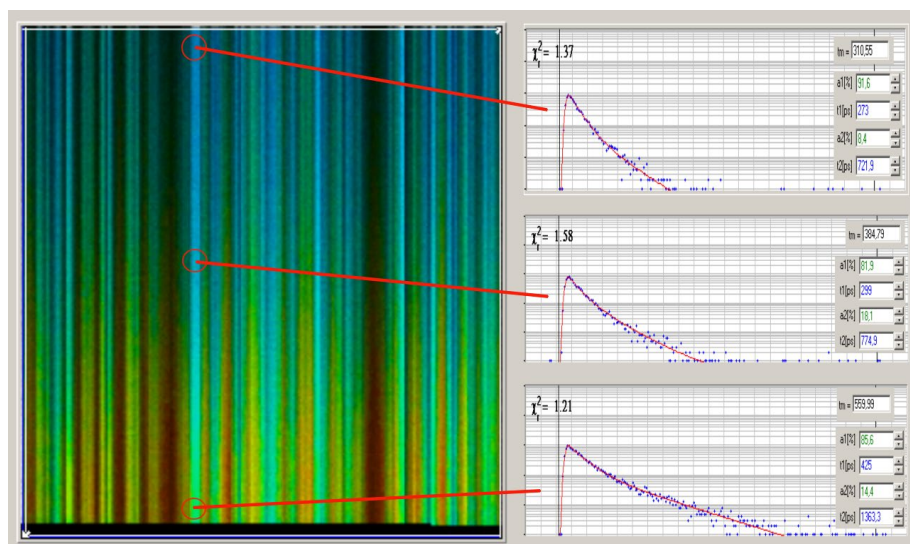


Fig. 294: Decay curves for a selected spot in the line scan for times of 0.5 s, 7.5 s, and 13.4 s after the turn-on of the laser

The changes in the fluorescence decay over the experiment time are nicely displayed also in the Phasor plot of SPCIMage. Fig. 295, left, shows the phasor plot of the entire FLITS data array. Fig. 295, right, shows the phasors for the same lateral spot in the line as selected for the decay curves in Fig. 294.

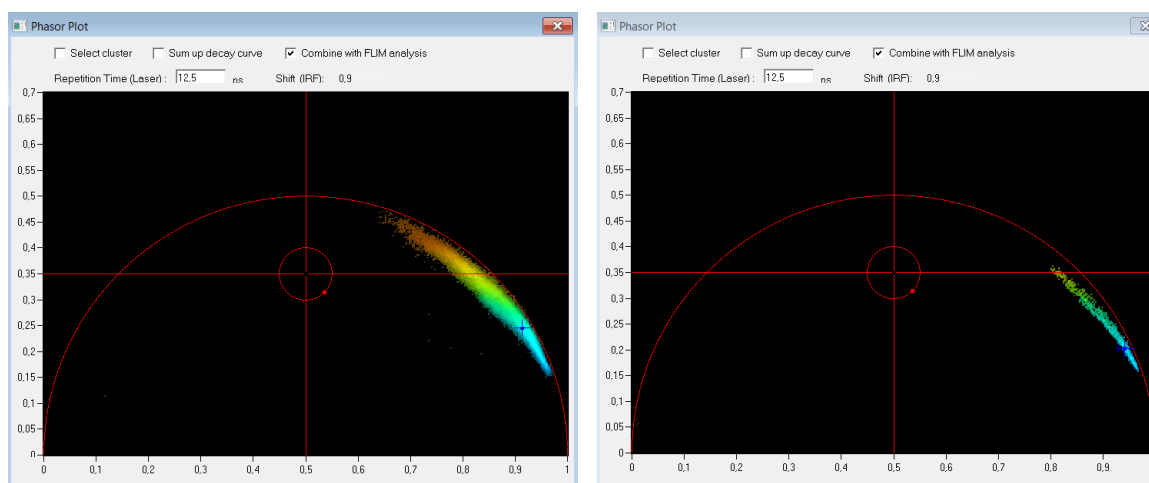


Fig. 295: Phasor plot of the FLITS data shown above. Left: Entire image. Right: Phasor plot of selected spot in the line scan.

Photochemical Transient

Recording the photochemical transient requires periodical stimulation and acquisition of the data over a longer period of time. Both the laser on-off signal [30] and the FLITS frame clock were therefore generated by a bh DDG-210 pulse generator card. (Any standard pulse generator works as well.) The on-off period was 1 second, the ‘on’ time within the period was 200 ms. Each turn-on of the laser initiates a photochemical transient, i.e. a temporary decrease in the fluorescence lifetime. In the laser-off period the leaf partially recovers, so that the next laser-on initiates a new transient. The total acquisition time was 40 seconds, i.e. 40 on-off periods were accumulated.

The result is shown in Fig. 296. The intensity image is shown on the left, the lifetime image on the right, and a decay curve at the cursor position at the bottom. The horizontal axis of the images is the distance along the line scanned. The vertical axis (bottom to top) is the time after the start of illumination. The line time was 1 ms, 256 lines, i.e. 256 ms in total, were

recorded in T direction. The Laser was turned on at $T = 0$, and turned off at $T = 200$ ms. The FLITS image shows the amplitude-weighted lifetime obtained from a double-exponential fit. The lifetime range is from 450 ps (blue) to 650 ps (red).

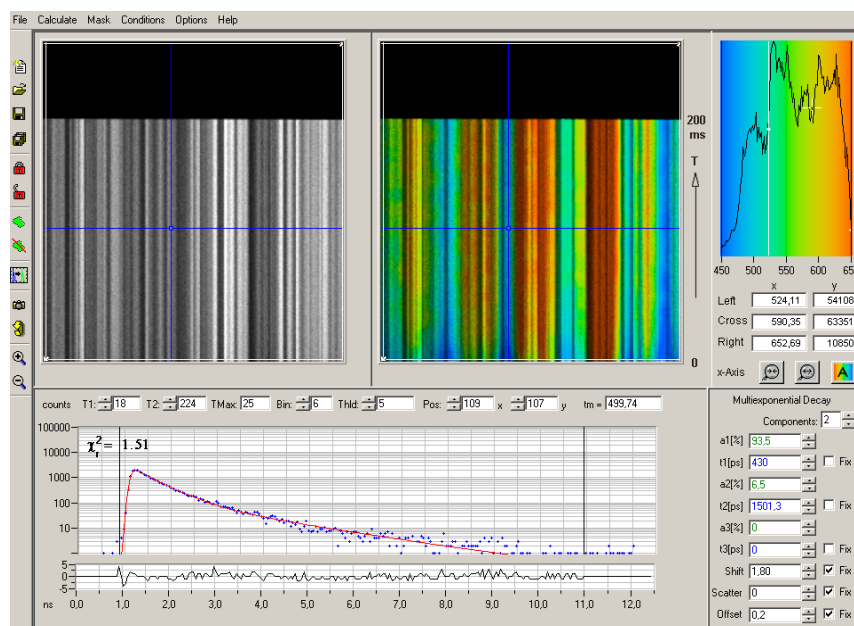


Fig. 296: FLITS image of photochemical chlorophyll transient. Horizontal: Distance along the line scanned. Vertical, bottom to top: Time, T , after the start of illumination. Line time 1 ms, 256 lines, total time interval recorded 256 ms. Laser turned on at $T=0$, laser-on time 200 ms. Left: Intensity image. Right Lifetime image. Colour represents lifetime, blue to red corresponds to 450 ps to 650 ps. Amplitude-weighted lifetime of double-exponential fit. Bottom: Decay curve at cursor position.

Decay curves and lifetimes for a selected pixel in the scan and for two points on the T axis are shown in Fig. 297. From $T = 3$ ms to $T = 191$ ms the amplitude-weighted lifetime of the double-exponential fit increases from 480 ps to 530 ps.

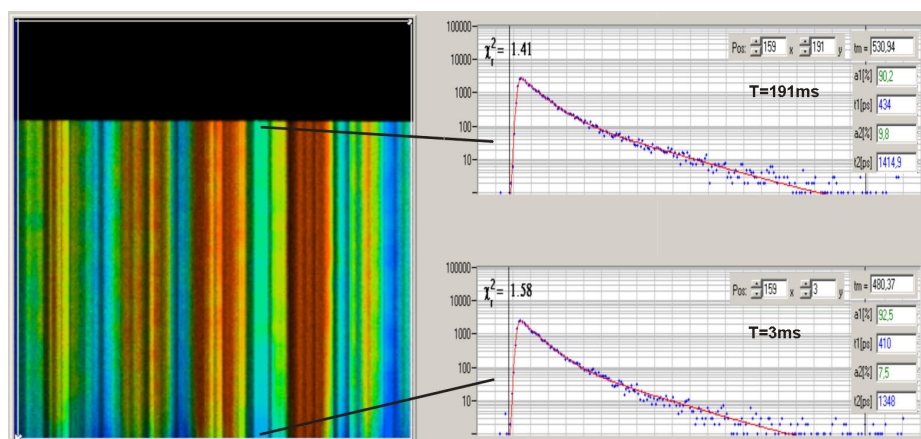


Fig. 297: Decay curves for a selected pixel ($x=159$) within the line for $T=3$ ms and $T=191$ ms into the laser-on phases. Double-exponential fit. The amplitude-weighted lifetime increases from 480 ps to 530 ps

Although the lifetime change is less pronounced than for the non-photochemical transient an increase can clearly be seen. Changes occur especially in the first 10 to 20 ms after the turn-on of the laser, see bottom of the lifetime image.

Despite the small duty cycle of the laser the illumination necessarily also causes a non-photochemical transient. When the photochemical transient is complete, activity essentially shuts down, and no non-photochemical transient is observed any more. Perfect recording of

the non-photochemical transient therefore requires optimisation of the laser intensity, the laser duty cycle, and the total accumulation time.

Calcium Transients

Other applications are in electro-physiology, especially the measurement of neuronal activity. When neurons are stimulated electrically the Ca^{++} concentration in the cells changes within milliseconds. These changes can efficiently be recorded by FLITS, please see [34, 76, 77, 229]. Data recorded with a bh SPC-152 FLIM system are shown in Fig. 298.

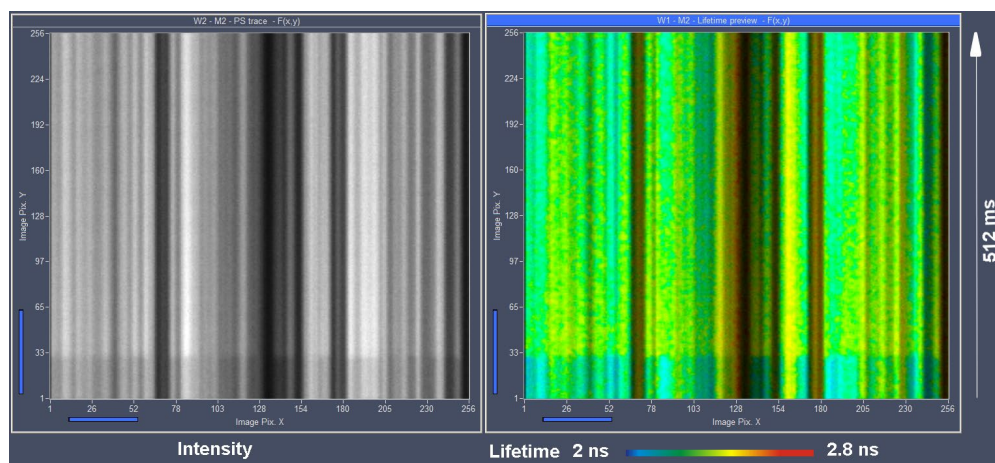


Fig. 298: FLITS of Ca^{++} transient in live neurons. Left: Intensity. Right: Lifetime, calculated by online lifetime function of SPCM software. Vertical scale 2 ms per line, time from 0 to 512 ms. Data courtesy of Inna Slutsky and Samuel Frere, Tel Aviv University.

Simultaneous FLIM / PLIM

There is a number of radiative relaxation mechanisms which occur on a much longer time scale than fluorescence. The commonly known one is phosphorescence, i.e. emission from the triplet state of organic dyes. Phosphorescence of organic dyes is usually weak at room temperature but can be strong at low temperatures, or if the dyes are embedded in a solid matrix. Strong emission in the microsecond and millisecond range is obtained also for lanthanide complexes [148] and organic complexes of ruthenium [360], platinum [221, 360, 364, 486], terbium, and palladium [364]. The time scales of phosphorescence and fluorescence are vastly different. Consequently, phosphorescence and fluorescence can easily be distinguished in PLIM data. Phosphorescent compounds can therefore be used as labels for background-free (autofluorescence-free) imaging [19, 20].

Most PLIM applications are, however, based on the fact that the luminescence of most of these complexes is strongly quenched by oxygen. This makes them excellent oxygen sensors [188, 245, 294, 360, 364, 435, 486, 539, 590]. Slow emission is also obtained from a large number of inorganic nanoparticles, quantum dots, and semiconductors. The long decay times are used to identify the particles in human skin and observe possible migration into deeper tissue layers [373, 455, 491]. There are also reports on applications as FRET donors [148], although it is not clear how the triplet state of the excited phosphor should interact with an acceptor in the ground state.

Principle

The measurement of long-lifetime luminescence by laser scanning systems faces a number of problems. The first one is related to the excitation of the phosphor. Obviously, the laser pulse period must be longer than the luminescence lifetime. The lifetime for ruthenium is in the lower microsecond range; for europium and terbium dyes it can be in the millisecond range. FLIM with these dyes requires a laser repetition rate no faster than 100 kHz or 100 Hz, respectively. Low repetition rate pulses can, in principle, be obtained from the picosecond diode lasers of the DCS system. However, this leads also to a proportional decrease in average excitation power. Moreover, any sample that emits phosphorescence necessarily also emits fluorescence. Because fluorescence is fast the relative peak power of fluorescence becomes very high. This causes transient overload effects in the detectors, preventing the detection of phosphorescence in the first microseconds after the laser pulse.

Another problem is related to scanning. The time the scanner stays within the excited sample volume must be longer than the luminescence lifetime. If the scanner runs off the excited volume within the luminescence decay time photons in the tail of the decay function would be lost, and the recorded decay profile be distorted. Reasonable recording, even of pure intensity images, can thus only be obtained by very slow scanning. However, if both the pixel time and the pulse repetition period is long there are only a few excitation pulses within the pixel time. The number of excitation pulses in the pixels varies systematically. This induces Moiré effects in the images.

The bh PLIM technique avoids all these problems by using the multi-pulse excitation principle shown in Fig. 299. A high-frequency pulsed laser is on-off modulated at a time scale in the microsecond or millisecond range. The modulation is synchronised with the pixel frequency of the scanner. The laser is turned on for a short period of time, t_{on} , at the beginning of each pixel [50, 77, 73, 486]. For the rest of the pixel time the laser is turned off. Within the on-time, t_{on} , the laser excites fluorescence, and builds up phosphorescence. Within the rest of the pixel dwell time, t_{off} , pure phosphorescence is obtained.

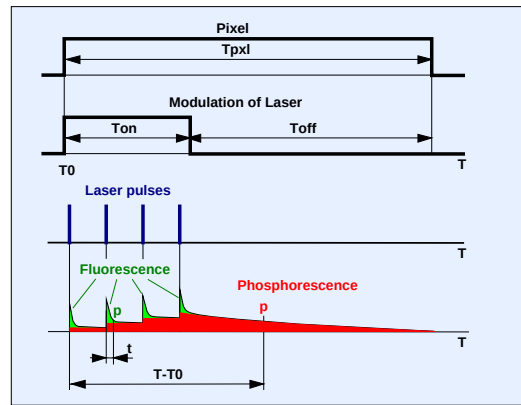


Fig. 299: Simultaneous FLIM/PLIM by multi-pulse excitation

Compared with single-pulse excitation the principle shown in Fig. 299 achieves a much higher level of phosphorescence excitation. Consider a phosphorescent dye with a lifetime of $100\ \mu\text{s}$, excited with a laser-on time, T_0 , of $20\ \mu\text{s}$. With a laser pulse repetition rate of $50\ \text{MHz}$, there are 1000 laser pulses in the laser-on time. That means the excitation level (and thus the emission intensity) is almost 1000 times higher than for excitation with a single pulse.

The principle not only delivers excellent sensitivity, it leads also to another important feature. By measuring both the times, $T-T_0$, in the modulation period and the times, t , in the excitation pulse period, *phosphorescence decay data can be recorded simultaneously with fluorescence decay data.*

TCSPC Process

The build-up of the photon distributions in the TCSPC process is shown in Fig. 300. For each photon, the SPC module determines the time, t , within the laser pulse period, and the time, $T-T_0$, after the modulation pulse. A fluorescence lifetime image is obtained by building up a photon distribution over the 'micro times', t , of the photons, and the scanner position, x, y , during the T_{on} periods. The phosphorescence lifetime image is obtained by building up a distribution over the time differences, $T-T_0$, between the photon times, T , and the 'laser on' pulse edges, T_0 . The spatial coordinates come from the scanner position in the moment of the photon detection. Thus, fluorescence and phosphorescence lifetime images are obtained simultaneously, in the same scan, and from photons excited by the same laser pulses.

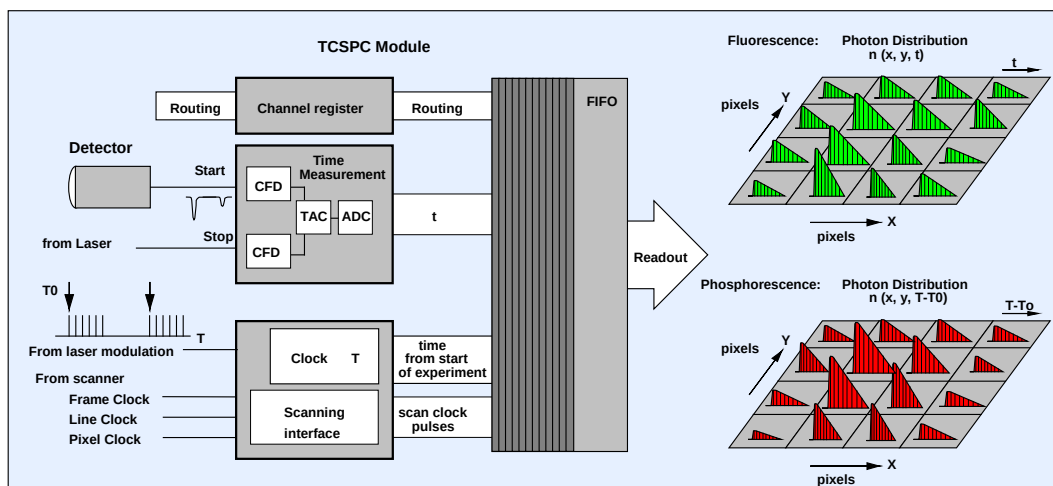


Fig. 300: Simultaneous fluorescence and phosphorescence lifetime imaging. Photon times are determined both with respect to the laser pulse period and with respect to the laser modulation period.

SPCM System Parameters

FLIM / PLIM is performed in the FIFO Imaging mode of the SPC-180 or SPC-QC-104 TCSPC modules. The System Parameters are shown in Fig. 301 and Fig. 302.

The FIFO imaging mode records individual photons with their times in the laser pulse sequence, their time from an external event (in this case the laser modulation), detector channel number, and information of whether the laser was active when the photon was detected. These data are transferred to the computer together with the pixel, line, and frame transitions. What the SPC software does with this information is defined in the 'Select & Configure Histograms' panel see panel on the right of Fig. 301. (Click on the 'Configure' button to open it.) Enable both 'Picosecond FLIM' and 'MCS FLIM/PLIM'. MCS FLIM/PLIM builds up the phosphorescence lifetime image from the photon times after the laser modulation. Select a reasonable time per point for the phosphorescence lifetimes expected, and a reasonable number of time channels. Time per point is given in units of the macro time clock, in this case 25 ns [77]. You can define an 'Offset' for the photon times to shift the phosphorescence decay curves in time. The offset can be negative to record also photons shortly before the laser modulation pulse. 'Trigger' must be 'Marker 0', which is the pixel clock. MT clock must be 'internal'.

The page control section of the System parameters defines the number of pixels recorded. For the DCS-120 these should correspond to the number of pixels in the scanner control panel. This can be achieved by clicking the  symbol. 'Routing channels X' should be '2'. The two routing channels are used to send photons during the laser-on and laser-off periods into different photon distributions.

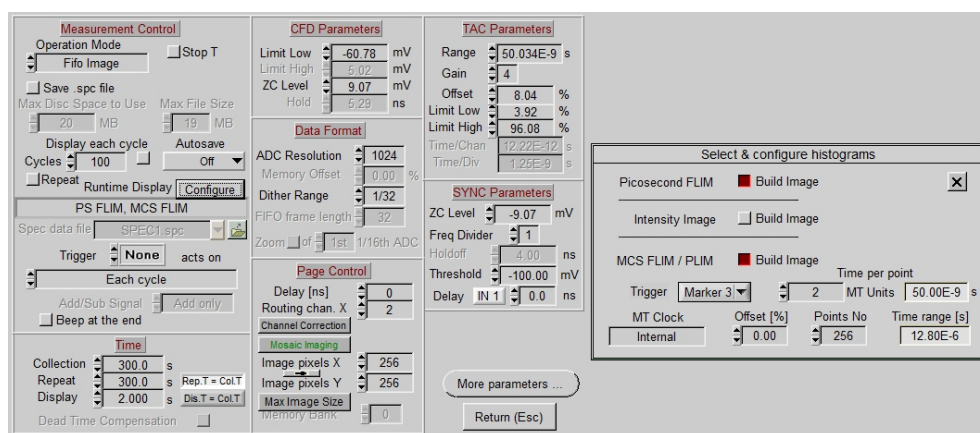


Fig. 301: System parameters for PLIM / FLIM operation, SPC-180N family

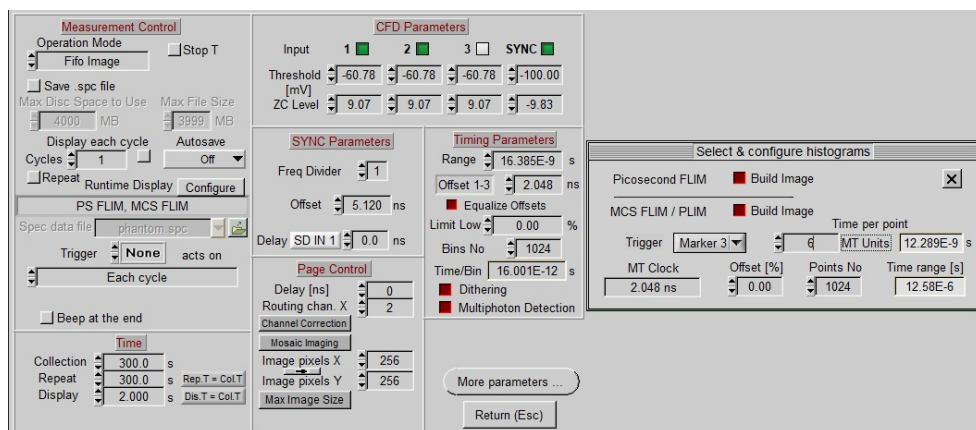


Fig. 302: System parameters for PLIM / FLIM operation, SPC-QC-104

Scan and Laser Control Parameters

The scan control panel with the parameters for FLIM/PLIM is shown in Fig. 303. The differences to standard FLIM recording are indicated. Laser multiplexing is turned on, the scanner runs in steps along the lines, and a relatively slow scan rate is used.

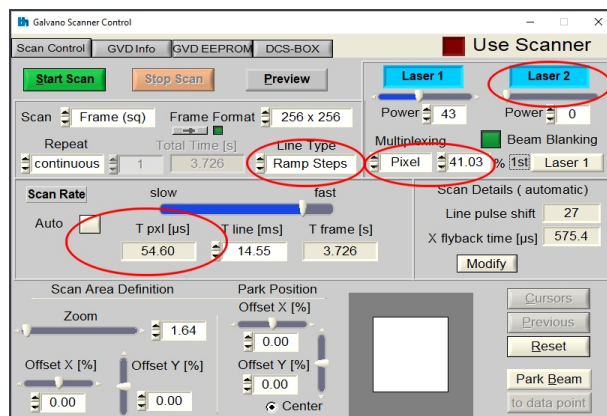


Fig. 303: Scan and laser control parameters for PLIM

Laser Modulation

The modulation of the laser is achieved via the laser multiplexing function. 'Multiplexing' is set to 'Pixel'. The fraction of the pixel time in which 'Laser 1' is on is defined in the field left of '% for 1st laser'. This is the time when the laser is running, and fluorescence is recorded. For the rest of the pixel time the laser is off, and phosphorescence is recorded.

The TCSPC module needs Sync pulses from the laser to complete the time-measurement cycle for the individual photons. However, the diode lasers do not deliver Sync pulses when they are in the 'Off' state. Therefore, the second laser is used to deliver Sync pulses to the SPC modules in the 'OFF' periods of the first laser. The multiplexing function switches it on when the first laser is off. To avoid unintentional excitation by Laser 2 the laser power is fully pulled down. The optical attenuator for the unused laser should be turned down to the 'closed' position. The lasers can be swapped by changing '1st Laser' in the scan control panel. The parameter defines which laser is the first one in the modulation period. The principle of the laser swapping is illustrated in Fig. 304.

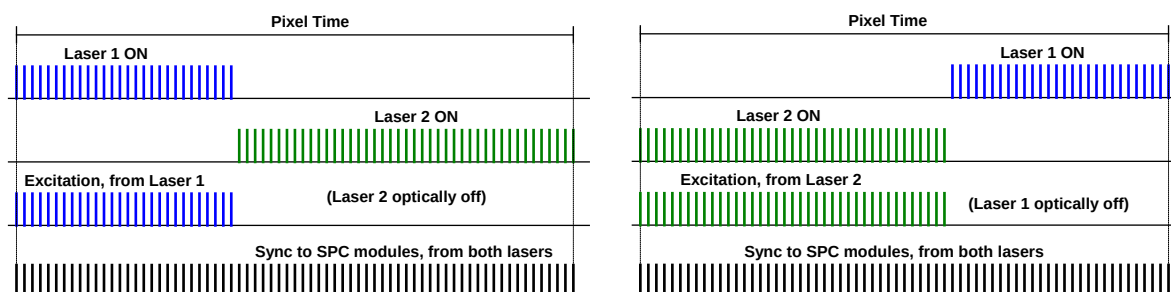


Fig. 304: PLIM Laser modulation sequence. Left: Laser 1 is first in the modulation period. Right: Laser 2 is first in modulation period. Bottom: Sync signal to SPC modules

Scanning

In contrast to fluorescence lifetimes, phosphorescence lifetimes can not be considered to be short compared to the pixel time of the scanner. It must therefore be avoided that the scanner

moves off the excited spot during the time the phosphorescence in the current pixel is recorded. In principle, this can be obtained by defining a pixel time several times longer than the phosphorescence lifetime. However, to obtain maximum speed in fast scans galvanometer scanners normally move along the lines continuously. That means the scanner can run off the excited spot even if the pixel time is longer than the phosphorescence decay time. To avoid that the phosphorescence decay functions are unpredictably distorted by this effect the DCS-120 scanner has the option to run along the lines in steps of the pixels size.

The scan parameter settings for PLIM are indicated in Fig. 303. 'Line Type' is set to 'Steps'. The 'Auto' button for the scan rate is disabled. The pixel time, T_{pxl} , is set a few times longer than the expected phosphorescence decay time. For efficient recording, T_{pxl} should be a bit longer (but not too much) than the 'Time Range' selected in the Configure panel, see above in Fig. 301, middle.

DCS Box Configuration

When the GVD-120 scan controller of the DCS-120 system multiplexes the lasers it also generates a routing bit that tells the SPC-150 modules which laser is currently running. This 'Laser Routing' bit is sent to the SPC modules via the DCS switch box (see page 61). The DCS box is configured in the 'DCS Box' section of the scan control panel, see Fig. 305. The 'Laser Routing' buttons for both SPC modules must be enabled. 'No. of detector routing bits' is normally zero. It is used for multi-wavelength detection systems only.

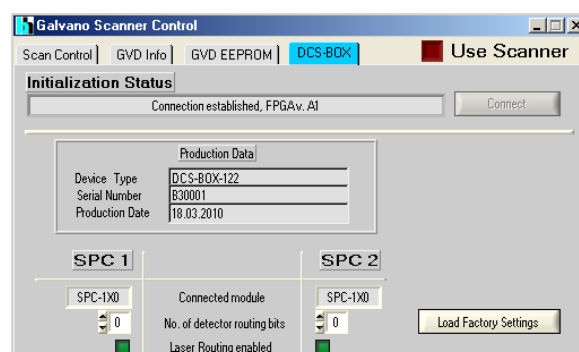


Fig. 305: Configuration of the DCS box. Laser routing must be enabled

User Interface

With the settings described above the system records the FLIM and PLIM data but does not necessarily display them conveniently. To display separate fluorescence and phosphorescence images the SPCM software uses 'Window Intervals' in combination with '3D Trace Configurations', see Fig. 306. The Window Intervals define within which intervals of time channels and routing channels images are to be displayed. The recommended definition for FLIM PLIM is shown in Fig. 306, left. There is only one time windows for the FLIM images, and one time window for the MCS (Phosphorescence) Images. Both contain all time channels, in this case 256. There are three routing windows. Window 1 contains only photons from routing channel 1. These are the photons that are recorded during the laser-on periods. Window 2 contains photons recorded during the laser-off periods. These are phosphorescence photons. Window 3 contains photons from all routing channels. An image built up in this window contains all photons, i.e. the total luminescence intensity.

The Trace Configuration parameters (Fig. 306, right) define which images are displayed. The configuration shown displays a ps FLIM image, an MCS (phosphorescence) image, an image of the total luminescence, and an image showing the decay curves of the total luminescence over the x coordinate of the scan area. All images are for the data recorded in TCSPC module 1. Similar images were defined for module two but were not turned on for simplicity.

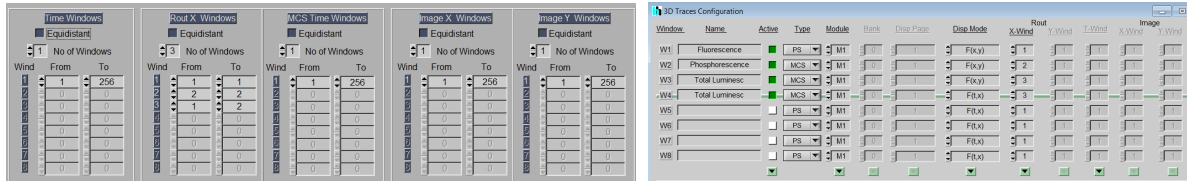


Fig. 306: Window intervals (left) and 3D Trace Configuration (right)

The SPCM Main panel with these configuration parameters is shown in Fig. 307. Left to right, it shows a fluorescence image, a phosphorescence image, and an image of the total luminescence intensity. The window middle left shows phosphorescence decay curves over the x coordinate of the image. The scan control panel is placed on the lower right.

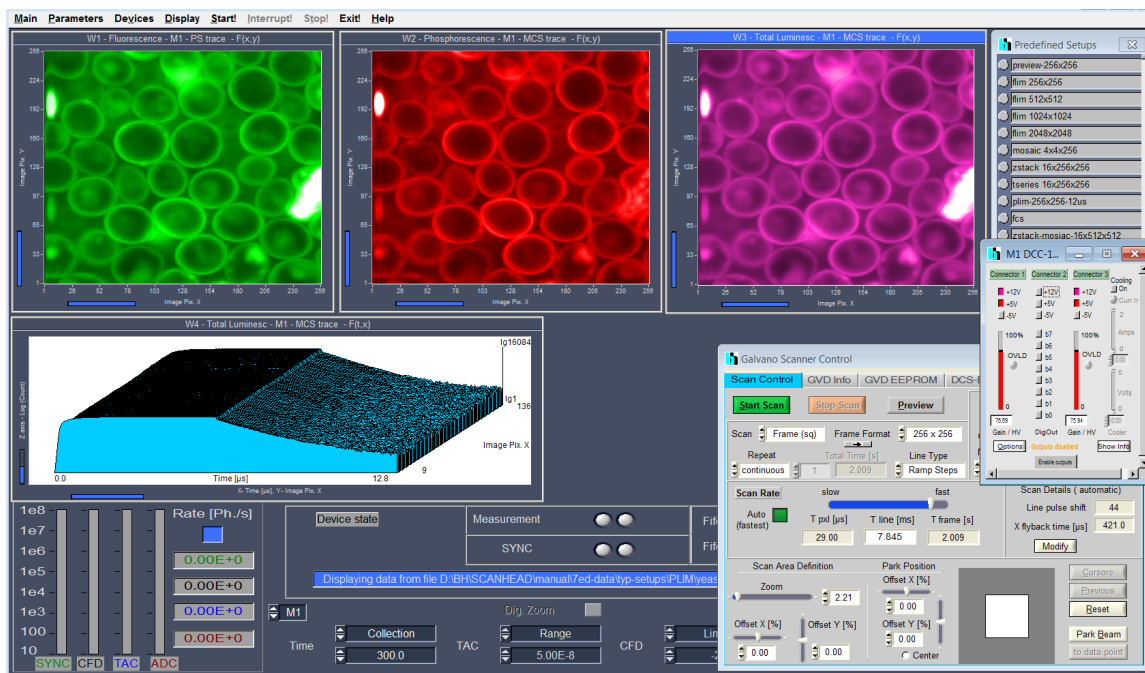


Fig. 307: Example of SPCM main panel in FLIM / PLIM configuration

PLIM Procedure

To run a PLIM measurement load a PLIM setup with the desired number of pixels from the Predefined Setup panel. Place the sample under the microscope, and run a preview to get the sample in the correct position and in the correct focal plane. (The Preview runs at high scan rate, independently of the scan rate defined in the PLIM setup.) Stop the Preview when the sample position and the focus are right.

In principle, you could start the PLIM recording now. However, there may be a problem. Unlike fluorescence decay times which are usually in the range from a few 100 ps to a few ns phosphorescence decay times can vary over orders of magnitude. It can therefore happen that you get PLIM data at a time scale that is either too short or too long compared with the phosphorescence lifetimes in the sample. Unless you know the approximate phosphorescence life-

time and the setup loaded before has the correct time scale you may have to change the MCS time scale and the scan rate, see Fig. 301 and Fig. 303. Otherwise, start the PLIM measurement and take a look at the phosphorescence decay curves in the middle left part of the main panel. If the curves are displayed at a reasonable scale let the measurement run. If the time scale is wrong abort the measurement (Click the Stop Scan button twice) and change the MCS time scale and the scan rate.

Examples

Fluorescence lifetime images of yeast cells stained with tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate. FLIM / PLIM data were acquired at 473 nm excitation wavelength, and with the parameters shown in the section above. FLIM and PLIM images are shown in Fig. 308, left and right. The FLIM image mainly shows the autofluorescence of the cells. The PLIM images shows the phosphorescence of the ruthenium dye.

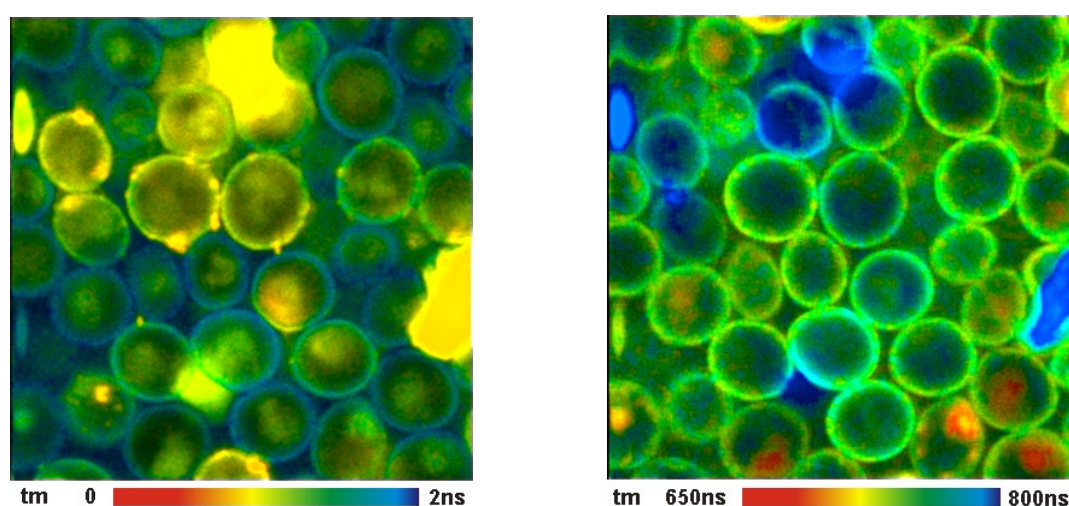


Fig. 308: FLIM and PLIM images of yeast cells stained with (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate. Excitation 473 nm, 256x256 pixels.

Phosphorescence is also obtained from various inorganic samples. Fig. 309 was obtained from an Autumit crystal (a uranium mineral). The phosphorescence lifetimes vary from about 100 μ s to 400 μ s. The lifetime image is shown on the left, decay curves of two selected spots on the right. The pixel time was 3.6 ms, the laser-on time 200 μ s. The excitation wavelength was 405 nm, a 435 nm long pass filter was used in the emission path. The Autumit phosphorescence was so strong that the laser power had to be reduced to the absolute minimum.

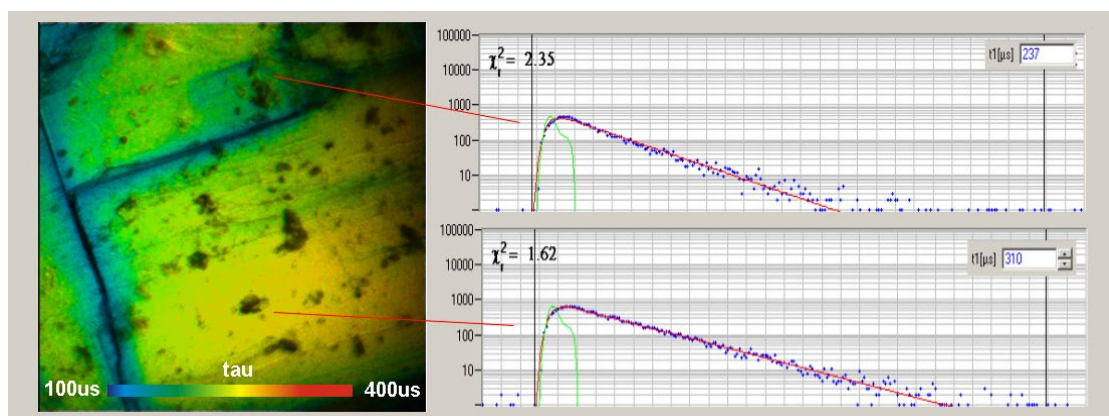


Fig. 309: PLIM image of a uranium mineral. Decay curves if two arbitrary selected spots are shown on the right. 256x256 pixels, 256 time channels, pixel time 3.6 ms, excitation 405 nm, emission filter long pass 435 nm.

FCS Measurements

Principle

FCS, or Fluorescence correlation spectroscopy, is based on exciting a small number of molecules in a femtoliter volume, detecting the fluorescence photons, and correlating the fluctuations of the fluorescence intensity. The fluctuations are caused by diffusion, rotation, intersystem crossing, conformational changes, or other random effects. The technique dates back to a work of Magde, Elson and Webb published in 1972 [382]. Theory and basic applications of FCS are described in [470, 520].

The required femtoliter detection can only be achieved in the focus of a high-NA microscope lens. That means FCS measurements must be performed in a microscope or a similar optical setup. Excitation and detection from a sufficiently small volume can be achieved by confocal detection or by two-photon excitation. FCS measurements can therefore be performed in a laser scanning microscope.

The general correlation procedure for parameter-tagged photon data is illustrated in Fig. 310. In typical TCSPC data, the time-channel width, T , is shorter than the dead time of the detector/photon counter combination. Therefore only one photon can be recorded in a particular time channel. Consequently, $N(t)$ and $N(t + \tau)$ can only be 0 or 1. The calculation of the autocorrelation function therefore becomes a simple shift, compare, and histogramming procedure. The times of the individual photons are consecutively shifted by one time-channel interval, T , and compared with the original detection times. The coincidences found between the shifted and the unshifted data are transferred into a histogram of the number of coincidences, G , versus the shift time, τ . The obtained $G(\tau)$ is the (un-normalised) autocorrelation function.

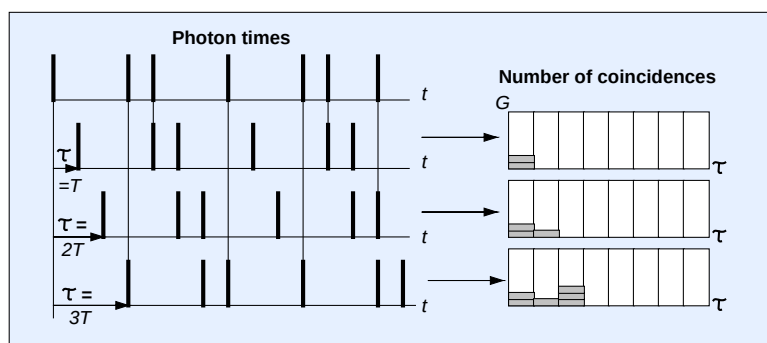


Fig. 310: Calculation of the autocorrelation function from TCSPC time-tag data

The algorithm is known as 'Linear Tau' algorithm. The equidistant τ channels result in an easily predictable statistics. This is an advantage if a model function is to be fitted to the correlation data. Unfortunately, for long correlation times the linear-Tau algorithm leads to an extremely large number of τ channels in $G(\tau)$, and to intolerably long calculation times. Therefore, in practice a 'Multi-Tau' algorithm is used which successively applies binning steps to the photon data for increasing τ values. Details of the Multi-Tau algorithm are described in [77].

System Parameters for FCS Measurement

FCS measurements are performed in the ‘FIFO’ mode of the SPC module in combination with the ‘Beam Park’ mode of the DCS-120 scanner [77, 68, 219]. Typical system setup parameters for FCS are shown in Fig. 311 through Fig. 322.

The measurement control part of the System Parameters is shown in Fig. 311. ‘Operation Mode’ is set to ‘FIFO’. The settings shown on the left calculate and display decay curves, FCS curves, and an MCS (intensity) trace online. The time-tag data are *not* saved. The settings on the right calculate the curves online, but simultaneously save the time-tag data into a file ‘fluorescein-a.spc’. The file size is limited to 100 MB. The measurement stops when this file size is reached. The measurement can also be stopped after a defined acquisition time or by an operator command.

‘Collection time’ defines the acquisition time for the ‘Stop T’ option. ‘Display Time’ is the update interval for the online display.

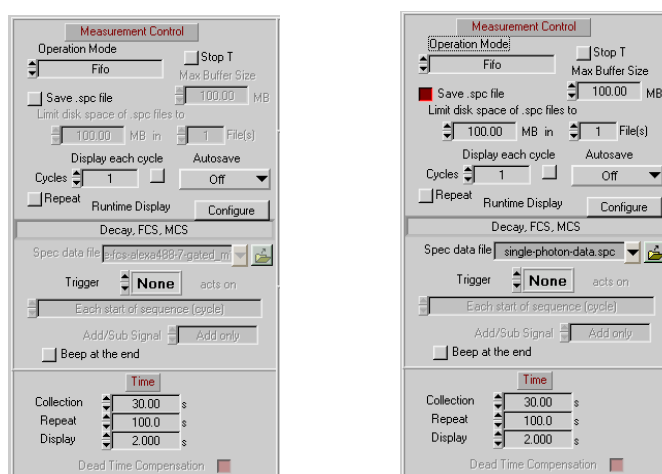


Fig. 311: FCS: Measurement control part of the ‘System Parameters’ panel. Left without, right with saving of the time-tag data.

Configure Panel

FCS calculation and online display is configured via the configure panel shown in Fig. 312. With the settings shown, decay curves, FCS curves, and an MCS (intensity) trace are displayed. FCS curves are calculated by a multi-tau algorithm, and within a correlation time window of 10 ms. K_{τ} defines the binning used in the Multi-Tau algorithm. It is the number of τ steps after which a binning step is inserted. Large K_{τ} means less binning and high resolution of the calculated FCS curve, low K_{τ} means more aggressive binning and, consequently, low resolution. The settings shown calculate FCS with $K_{\tau} = 33$. The MCS trace is calculated at a resolution of 1 ms per point.

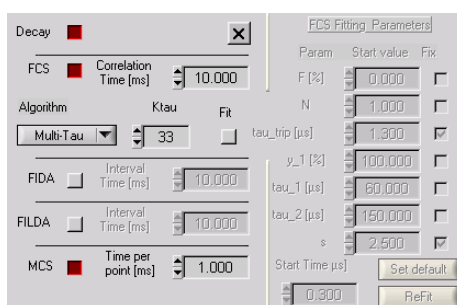


Fig. 312: Configure Panel, definitions for online calculations

TCSPC System Parameters

The TCSPC parameter section of the system parameters is shown in Fig. 313. Definitions for the SPC-180N (and for older SPC-150 or SPC-150N modules) are shown on the left, definitions for the SPC-QC-104 on the right. The TAC parameters for the SPC-180NX and the Timing Parameters for the SPC-QC-104 are the same as in the FLIM modes. The number of time channels (ADC Resolution) in the FIFO mode is always 4096 time channels. ‘ADC Resolution’ is therefore disabled.

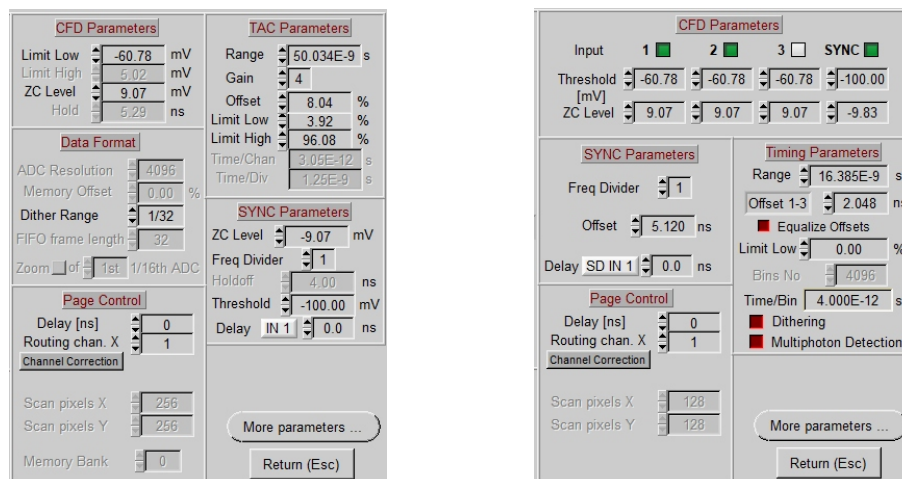


Fig. 313: TCSPC parameter section of the system parameters. Left: SPC-180N family modules. Right: SPC-QC-104

Macro Time Source (SPC-180NX)

For FCS experiments with pulsed lasers it is recommended to use the SYNC frequency from the laser as a macro time clock. This avoids artefacts by aliasing of the laser repetition frequency with the internal macro time clock. The macro time source is defined under ‘More Parameters’ in the System Parameters panel, see Fig. 314.

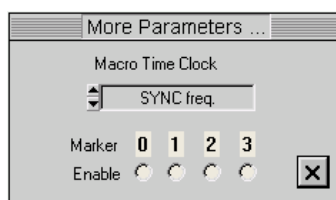


Fig. 314: Definition of the laser frequency as a macro time source

Synchronous Start of Several TCSPC Modules (SPC-180N)

For cross-correlation of photons recorded in the two SPC-180NX (or SPC-150N or SPC-150) modules of a dual-channel FLIM system it is required that these not only work on the same macro-time clock period but also start their recordings synchronously. Identical macro time clock periods are achieved by using the laser Sync as a source of the macro time clock, see Fig. 314. The simultaneous start of several TCSPC modules is achieved by declaring one module a ‘Trigger master’, see Fig. 315.

For the SPC-QC-104 a definition of synchronous start is not necessary. The detection channels are on the same TCSPC board and thus automatically start simultaneously.

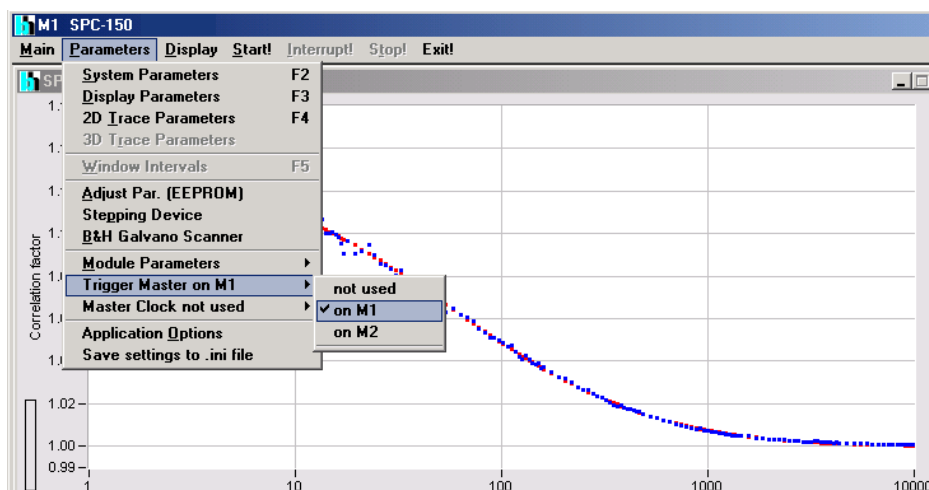


Fig. 315: Left: Synchronous start of several SPC-180N, -150N, or -150 modules via the trigger master function

Scanner Parameters

The scanner parameters for FCS are shown in Fig. 316. The scanner is in the 'Beam Park' mode, with the laser beam parked in the centre of the field. 'Beam blanking' is off to keep the laser running without an active scan.

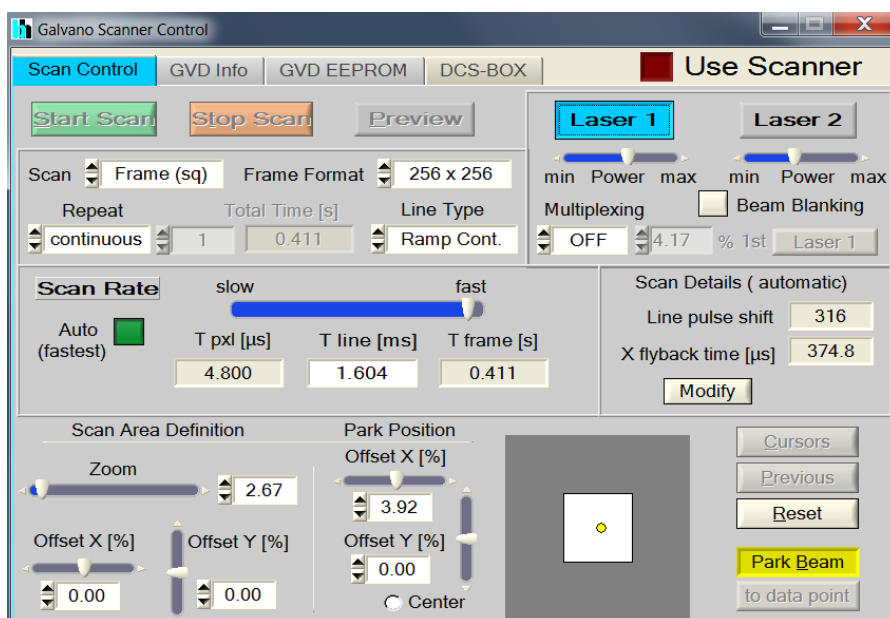


Fig. 316: Scanner parameters for FCS. Beam blanking is OFF, the beam is parked in the middle of the field

FCS Fit

The SPCM software is able to run a fit on the calculated FCS data. The corresponding definitions are shown in Fig. 317. The model function contains two diffusion times, τ_1 and τ_2 , and a triplet time, τ_{trip} . F is the relative triplet population, N the number of molecules in the detection volume, and y_1 the relative amplitude of the slow diffusion component. For the online fit we recommend to use a simple model, preferably one diffusion time and a triplet time. The start values of the fit procedure are defined on the right of the 'Configure' panel.

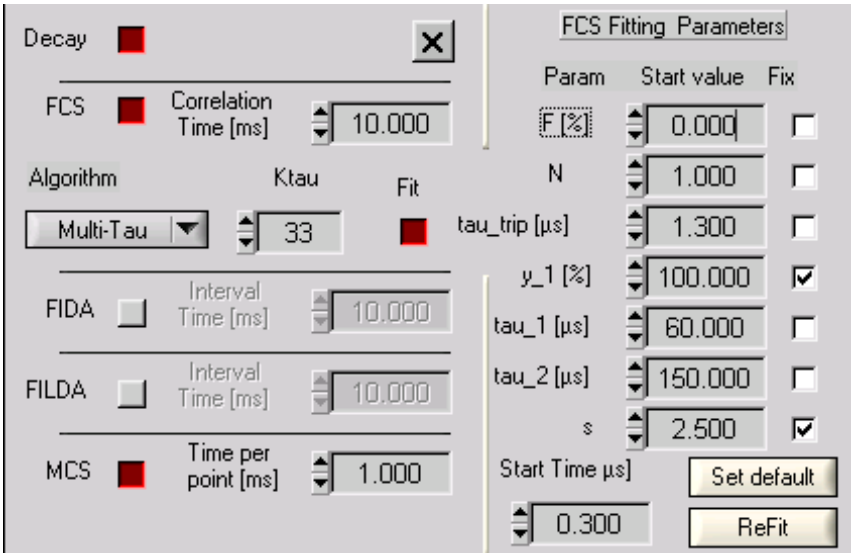


Fig. 317: Configuration of FCS online calculation with FCS fit.

The fit results are displayed in the 'Trace Statistics' Window, Fig. 318. This window opens by a click on the 'Trace Statistics' button in the SPCM main window. In Fig. 318 the trace statistics has been placed below the FCS display panel. The fit results correspond to the red curve.



Fig. 318: Display of the FCS fit results.

User Interface

With the online display configured for decay, FCS, and MCS the main panel of the SPCM software assumes a configuration as shown in Fig. 319.

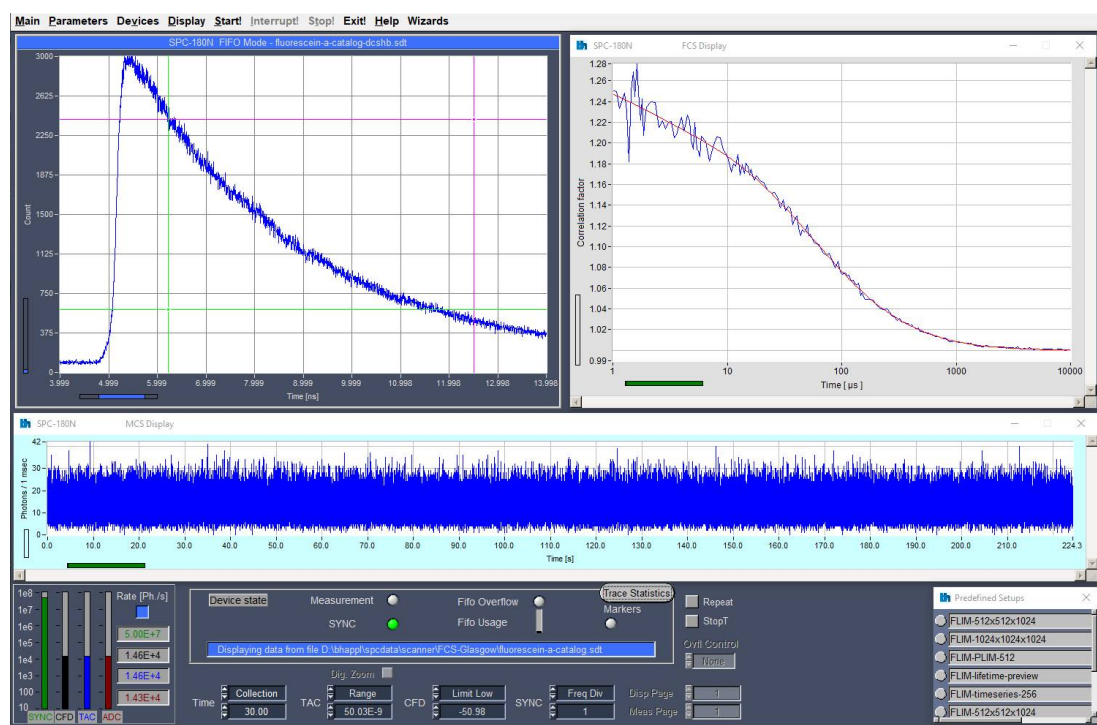


Fig. 319: Main panel with decay curve, FCS, and MCS trace windows. Fluorescein in water, Filter 530 ± 25 nm, 475 nm laser. Scanner, detector, and predefined setup panels not shown.

Trace Parameters

The curves to be displayed in the three curve windows are defined under ‘Trace Parameters’. Each curve window has its own trace parameters. To open the trace parameters, click on ‘Display’, ‘Trace Parameters’. A click into one of the curve windows selects the corresponding trace parameter set. The panels for the decay curve window and the FCS window are shown in Fig. 320. The panels show the simple case that the decay curve of one detector, and the autocorrelation and the fit for the same detector are to be displayed.

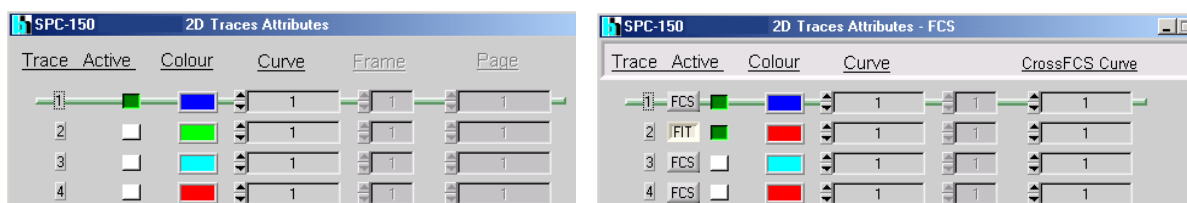


Fig. 320: Trace parameters. Left for decay window, right for FCS window. One detector channel, autocorrelation.

The FCS trace parameters for cross-correlation are shown in Fig. 321. The left setup is typical of an SPC-152 system. It has two parallel TCSPC modules. The trace parameters define correlation of the data of Module 1 with those of Module 1, and correlation of the data of Module 2 with those of Module 2. These are the autocorrelation curves. The third curve is correlation of Module 1 versus Module 2. This is the cross-correlation. The fit is run on the cross-correlation data.

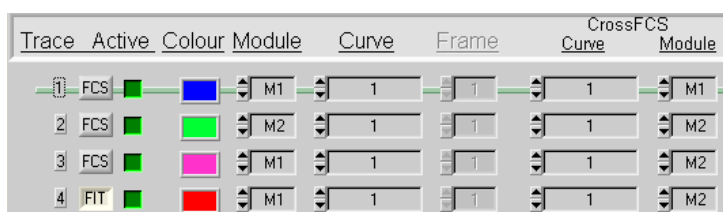


Fig. 321: Trace parameters for dual-channel system. Dual-module system. Trace 1 and 2: Autocorrelation of both channels, Trace 3: cross-correlation between both channels, Trace 4 a fit of the cross-correlation data.

Display Parameters

The display scale (linear or logarithmic) and the curve style (curves, points, connected points) are defined in the 'Display parameters'. As for the trace parameters, every display window has its own display parameters. The display parameter panels for the decay and the FCS window of Fig. 319 are shown in Fig. 322.

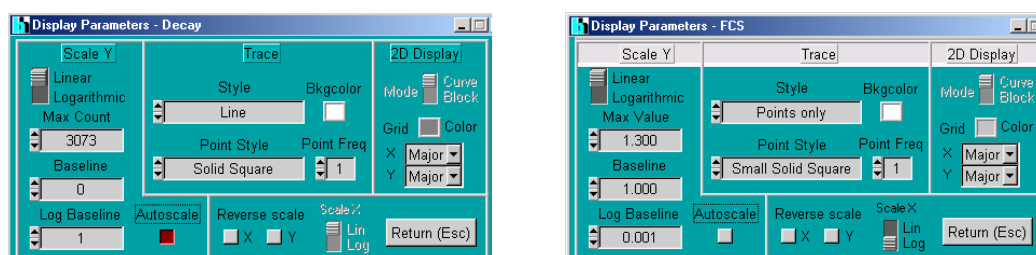


Fig. 322: Display parameters. Left for decay window, right for FCS window

Gated FCS

It can happen that the FCS signal is contaminated by Raman emission of the solvent. If Raman emission cannot be suppressed by filters it can be rejected from the recorded data by time-gating. An example is shown in Fig. 323.

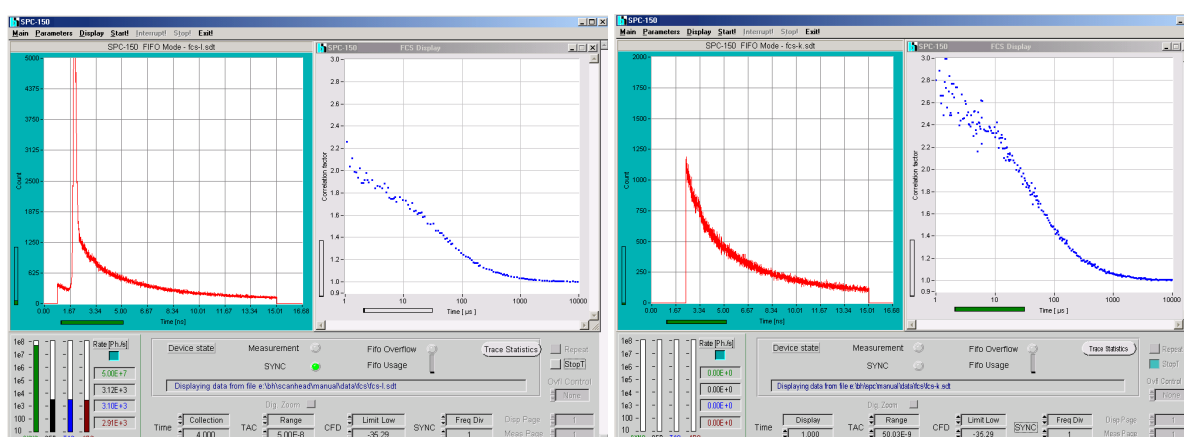


Fig. 323: Left: Ungated FCS recording. The sharp peak in the decay curve is caused by Raman scattering. Right: Gated FCS recording. The Raman peak is suppressed, with a corresponding increase of the correlation coefficient. Rhodamine 110 in water, 475 nm laser, 485 nm long-pass filter

Fig. 323, left, shows the ungated signal. The Raman signal is visible as a sharp peak at the beginning of the fluorescence decay. In Fig. 323, right, the Raman peak was gated off. The correlation amplitude is clearly higher than in the ungated case.

In the SPC-180N gating is achieved by the 'TAC Limit High' parameter, in the SPC-QC-104 by the 'Limit Low' parameter. (The difference comes from the fact that the modules use dif-

ferent timing principles) We recommend to use the parameter input options in the main window, and adjust the limits online. Please see Fig. 324.

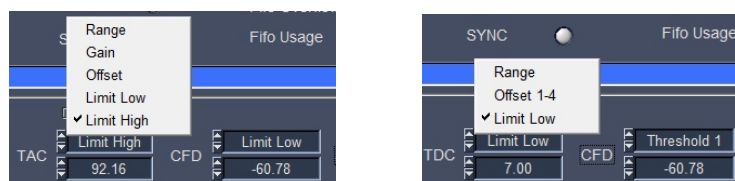


Fig. 324: Adjusting the 'Limit' parameter for suppression of a Raman peak. Left: SPC-180N, SPC-150N. Right: SPC-QC-104

FCS with CW Excitation

FCS does not necessarily need a pulsed laser, it works also with CW excitation. The advantage of CW excitation is that higher laser power is available. The disadvantage is that no decay curve is obtained. That means you cannot see whether the recorded signal is indeed fluorescence, or possibly laser background, detector background or Raman light from the solvent.

FCS with CW excitation is easy in DCS systems with SPC-QC modules. Switch the laser to CW mode, change the operation mode to 'Absolute Time', and disable Input 4 (the Sync Input). In the Configure panel, enable only 'FCS'.

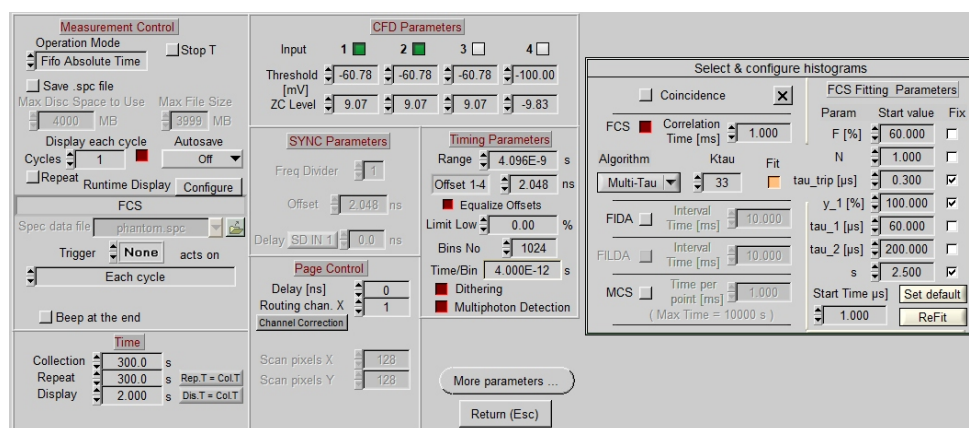


Fig. 325: System parameters for FCS with CW laser. SPC-QC-104 module.

With these settings, the system will record FCS in both detector channels and display the results in an FCS window. There will also be a 'Coincidence' window. Data in this window have no meaning for FCS but can be used for anti-bunching experiments [77].

In DCS systems with SPC-180 Modules, switch the laser you want to use to CW. Leave the other laser in the pulsed mode, but turn its intensity fully down. Turn also the optical attenuator fully down. Switch on both lasers. The first laser (running in CW mode) will provide the excitation light, the second laser a synchronisation signal for the SPC modules. When the measurement is started it will record FCS the usual way. Of course, no decay curve will show up in the decay window.

Selecting a Position for FCS from an Image

The beam park position for FCS can be selected from an image. Record an Image in the 'FIFO Imaging' mode. Turn off 'Scan Rate - Auto' and select a scan rate about 10 times slower than the 'Auto' scan rate. This avoids an offset of the beam park position by the dynamic lag of the

scanner. Enable the cursors in the image, and turn on 'Cursor settings, see Fig. 326. Place the 'Data Point' on the spot of interest for FCS, as shown in Fig. 326.

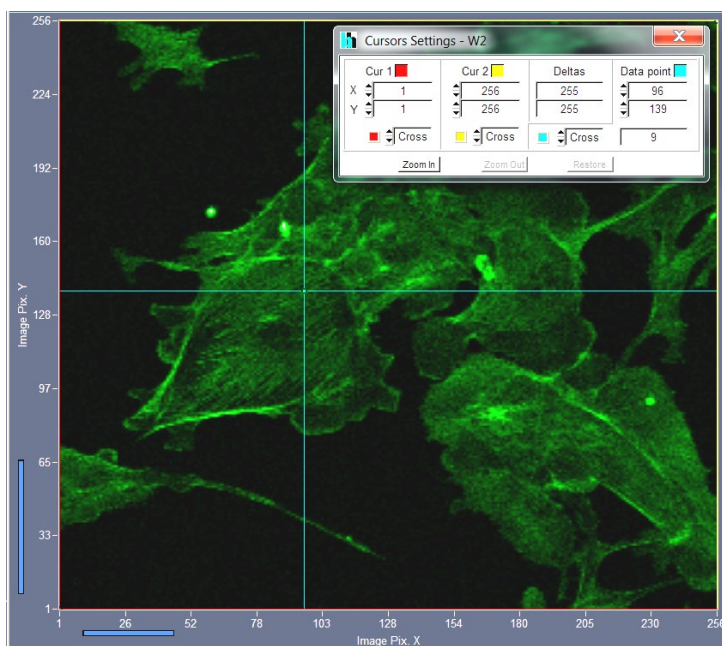


Fig. 326: Selecting the position of FCS from a previously recorded image

Then put the scanner in the Beam Park mode, see Fig. 327. A click on 'To Data Point' moves the beam into the position selected in the image. When this is done switch to 'FIFO' mode or select an FCS setup from the panel of predefined setups, and start the FCS measurement.

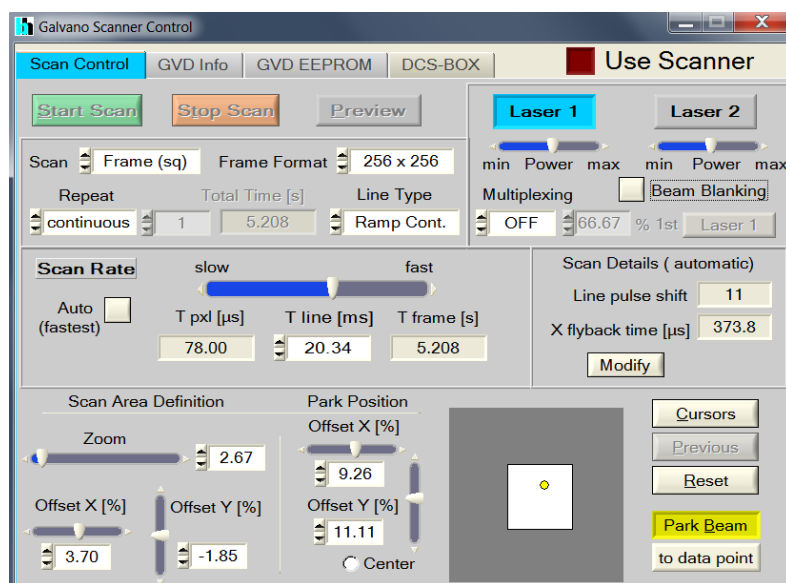


Fig. 327: Scanner panel, beam-park position defined by 'to data point' button.

Optical System

It should be noted here that FCS recording requires a near-perfect optical system. FCS works only if the detection volume is smaller than about one femtoliter. A focal volume this small is obtained only with a high NA objective lens, correct matching of the refractive indices, and perfect alignment of the scanner. Because FCS is done in aqueous solutions or living cells normally a water immersion lens is required. The NA should be 1.2 or higher. At the scanner, a pinhole size of 1 or 0.5 should be selected. For best results, we recommend to run a pinhole

alignment procedure before a series of FCS measurements is started. Please see 'Electronic Pinhole Alignment', page 50.

This is especially important for FCS measurements in cells. The fluorophore concentration in cells is often much higher than the optimum value of 10^{-9} mol/l. Although this has no direct influence on the correlation time the correlation coefficient (and thus the signal-to noise ratio of the FCS result) is usually much lower than for measurements in solution [68].

Even with these precautions, success of FCS measurement is not always guaranteed. Typical sources of failure are

- Concentration too high. In this case, a fluorescence decay function is displayed but there is no correlation in the FCS data. An indication of this problem is that the count rate is unexpectedly high. Typical FCS count rates are 10,000 counts per second and below.
- Contamination of the sample solvent with a fluorescent dye. Indications are high count rate, possibly wrong decay time, and the fact that the count rate does not decrease when the sample is diluted.
- Contamination of the immersion fluid with a fluorescent dye. An indication of this problem is that the count rate remains high when the sample is removed but goes down when the immersion fluid is cleaned off.
- Detector background or environment light. FCS reacts extremely sensitive to counting background. The background must be lower than 800 count per second to see FCS at all. For good results, the background should be 150 counts per second or less. Keep environment light out of the system. Keep the room temperature low to reduce the detector background. For best results, use cooled HPM detectors, such as the HPM-100-40-C.

Metabolic FLIM

Principle

Metabolic FLIM is based on the recording of lifetime images of NAD(P)H (nicotinamide adenine (pyridine) dinucleotide) and FAD (flavin adenine dinucleotide). In biological systems both NADH and FAD exist in a protein-bound and in a free form. Bound and free forms have different decay times. For NAD(P)H the lifetime is long (a few ns) when it is bound and short (a few 100 ps) when it is free. For FAD the bound component has a short lifetime and the free component a long one. The resulting decay functions are double exponential, with the fast components originating from unbound NADH and from bound FAD. The fluorescence behaviour of NADH and FAD is illustrated in Fig. 328.

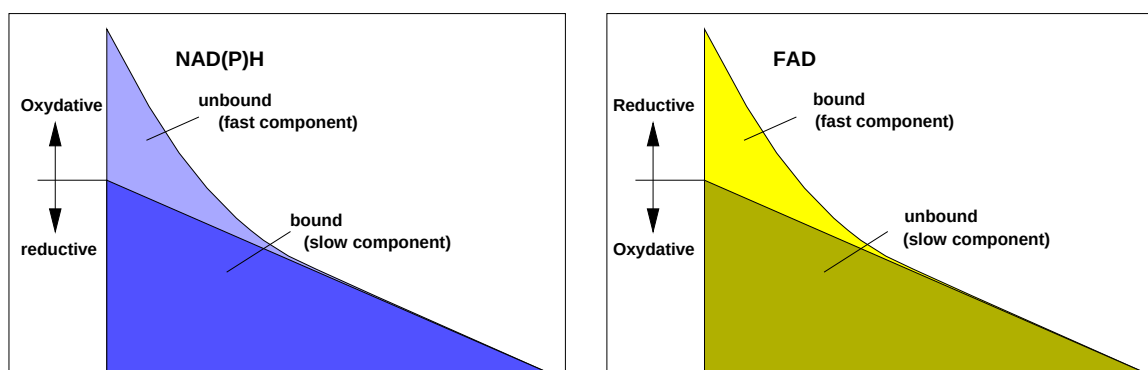


Fig. 328: Fluorescence decay behaviour of NAD(P)H and FAD

The composition of the NAD(P)H and FAD decay curves depends on the metabolic state. When the metabolism becomes more oxidative the amounts of bound NADH and of bound FAD increase, resulting in an increase in the amplitude of the slow decay component of NADH and of the fast decay component of FAD. When the metabolism becomes reductive the fast component of NADH and the slow component of FAD increase. The metabolic state of the cells can thus be derived from the amplitudes of the decay components. These are obtained by double-exponential decay analysis. Importantly, the component amplitudes depend only on the metabolic state, not on other parameters, such as pH, viscosity, or ion concentrations. The component amplitudes therefore deliver quantitative information on the metabolism of the cells. Details are described in chapter 'Autofluorescence FLIM of Cells and Tissue'.

To record lifetime images of NAD(P)H and FAD the signals from both compounds have to be separated. Approximate excitation and emission spectra of NAD(P)H and FAD are shown in Fig. 401. The spectra were taken from [360] and [512]. The figure shows that NAD(P)H needs an excitation wavelength shorter than 380 nm. FAD can be excited from 350 nm all the way up to 475 nm. That means, in principle, NAD(P)H and FAD could be excited at a single wavelength, in the range from 350 to 380 nm. However, a look at the emission spectra shows that the emission signals cannot be separated in this case. A clean NAD(P)H signal can be detected only from about 425 to 475 nm. Above 475 nm the NAD(P)H signal is overlaid by emission from FAD. A similar problem exists for the FAD. The FAD emission occurs from about 480 nm to more than 600 nm. However, there is a strong overlap from the NAD(P)H emission. A clean FAD signal can therefore only be detected if an excitation wavelength above 400 nm is used, where the excitation of NAD(P)H is weak.

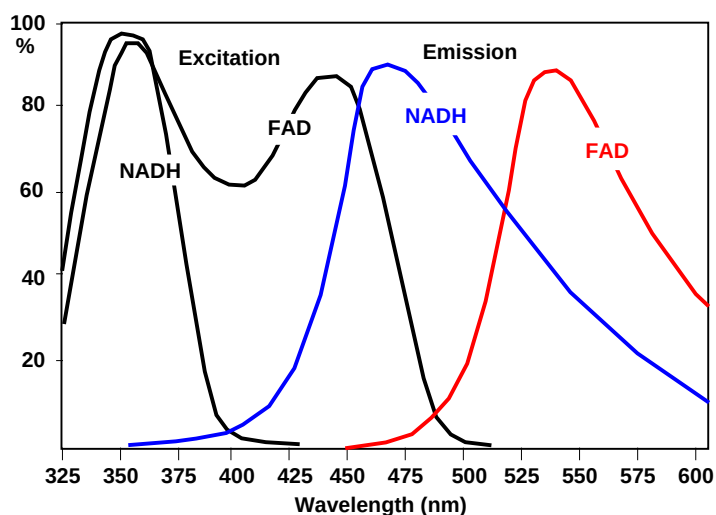


Fig. 329: Excitation and emission spectra of NADH and FAD

Suitable (one-photon) excitation wavelengths and detection wavelengths are shown in Fig. 330. Excitation of NADH can be obtained from a 375 nm laser, excitation of FAD from a 405 nm or 445 nm laser. Detection wavelengths are from 420 nm to 470 nm for NADPH, and from 490 nm to about 600 nm for FAD.

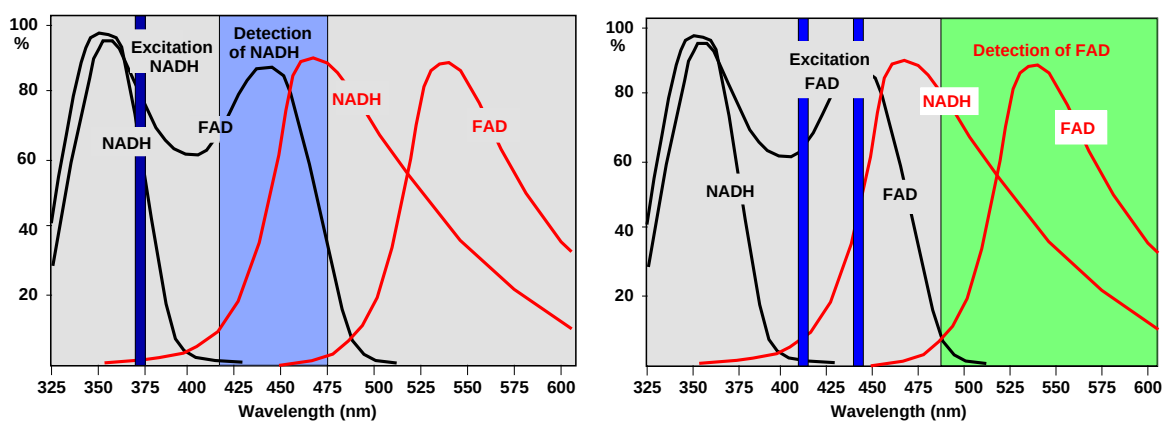


Fig. 330: Excitation wavelength and detection wavelength intervals for metabolic FLIM

TCSPC Parameters for Metabolic FLIM

TCSPC parameters for metabolic FLIM are shown in Fig. 331. The number of time channels is 1024, the number of pixels 512 x 512. Do not attempt to improve the signal-to-noise ratio by reducing the number of time channels or the number of pixels. The signal-to-noise ratio is independent of the number of time channels, and a smaller number of pixels prevents you from using the advantages of the 'intelligent binning' function of SPCImage.

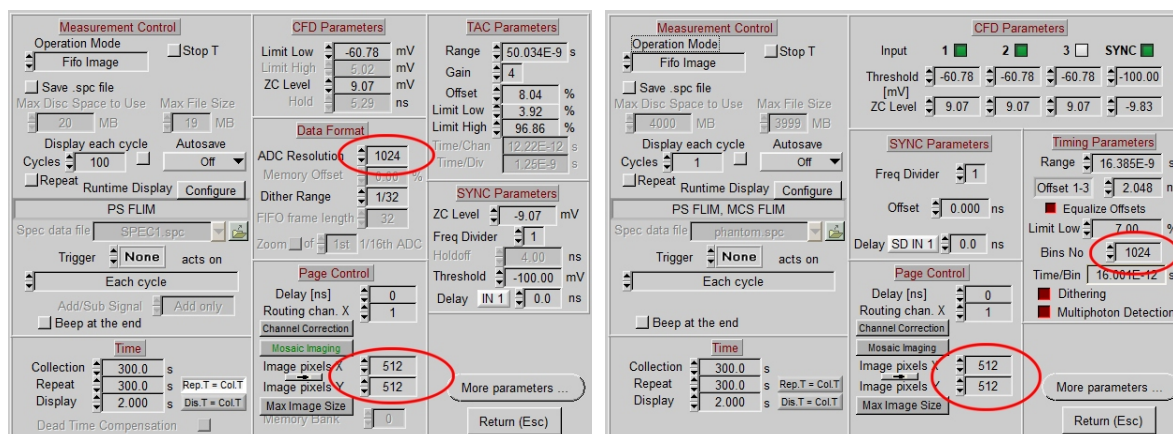


Fig. 331: TCSPC system parameters for metabolic FLIM. Left: SPC-180N. Right: SPC-QC-104

Metabolic FLIM by Recording NAD(P)H or FAD Data Separately

Metabolic FLIM by NAD(P)H

Good results can be obtained from NAD(P)H FLIM data alone. Pure NAD(P)H imaging may therefore be an option if you have only one laser or only one detection channel. In that case, use the standard FLIM setup described under 'Parameter Setup for Basic FLIM Experiments' page 123. Use an excitation wavelength of 375 nm, and an emission filter from 420 to 475 nm. Use a high-NA lens to collect as many photons as possible.

Metabolic FLIM by FAD

Metabolic FLIM results can also be obtained from FAD. In that case, use 445 nm for excitation and an emission filter that transmits from 480 nm to 600 nm. A simple long-pass filter may do the job. However, be aware that FAD imaging is more complicated than NADH imaging. In the FAD channel you detect also FMN. The lifetime of FMN is about 5 ns, and does not change with the metabolic state. That means triple-exponential analysis has to be used to obtain quantitative results. The best (and probably the only) way to split the contributions from FAD and FMN is to use the 'Global Fit' option of SPCImage NG.

Sequential Recording of NAD(P)H and FAD

Sequential recording of NAD(P)H and FAD data may be required if you use a tuneable excitation source, such as the Ti:Sa laser of the DCS-120 Super MPC system or the SHG from this laser. For NAD(P)H, use 750 nm for two-photon excitation and 350 to 370 nm for one-photon excitation. For FAD, use 880 to 920 nm for two-photon excitation, and 405 to 410 nm for one-photon excitation. The one-photon excitation wavelength should be below the detection-wavelength interval for NAD(P)H. This avoids the need of changing the main dichroic between the two measurements. On the detection side, use both channels of the DCS system, one detecting in the range of 420 to 475, the other in the range of 490 to 600 nm. Use a secondary dichroic with a transition wavelength of 480 nm. This way, you need not change filters when you switch the laser wavelengths.

Multiplexed Recording of NAD(P)H and FAD

The best way to record the NAD(P)H and the FAD signals is by laser-wavelength multiplexing and multiplexed TCSPC. The principle is described in section 'Excitation Wavelength Multiplexing', page 137.

The TCSPC system architecture for NAD(P) / FAD FLIM is shown in Fig. 332. The FLIM system uses the two parallel TCSPC channels of the DCS-120 system. One channel detects in the spectral range of NAD(P)H, the other in the range of FAD. The two ps diode lasers of the DCS-system (375 nm and 405 nm) are multiplexed, and fluorescence photons excited by the two lasers are recorded into different data blocks in the memory [77]. Multiplexing of the lasers is controlled by the multiplexing functions of the GVD-120 scan controller of the DCS-120 system. For user interface, parameter setup, and other technical details please see section 'Excitation Wavelength Multiplexing', page 137.

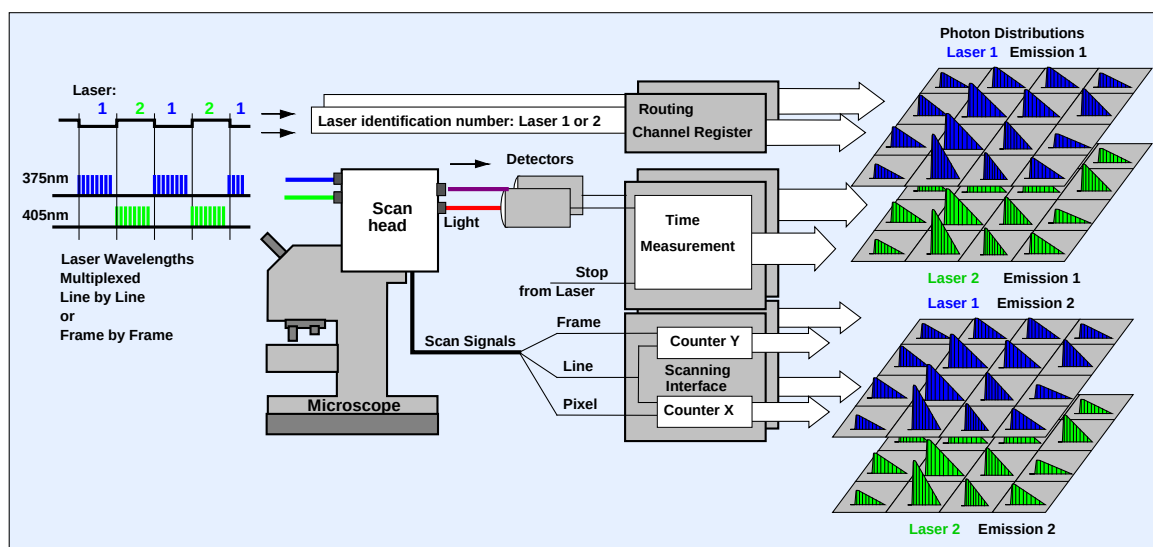


Fig. 332: Principle of simultaneous NADH / FAD FLIM

The recording process generates lifetime images for four combinations of excitation and detection wavelengths:

	Excitation	Emission	Signal detected
Image 1	375 nm	420 to 470 nm	NAD(P)H
Image 2	375 nm	490 to 600 nm	FAD + NAD(P)H, not used
Image 3	405 nm	420 to 470 nm	not used
Image 4	405 nm	490 to 600 nm	FAD

The data of interest are the ones in Image 1 (NAD(P)H) and in Image 4 (FAD). Please see [88] for more technical details.

Typical Results

Images for NADH and FAD are shown in Fig. 333, left and right. The tumor cell (to which the cursor is set) has a larger a_1 than the rest of the image in the NAD(P)H image, and a smaller a_1 in the FAD image. More results are presented in [88] and in chapter 'Autofluorescence FLIM of Cells and Tissue'.

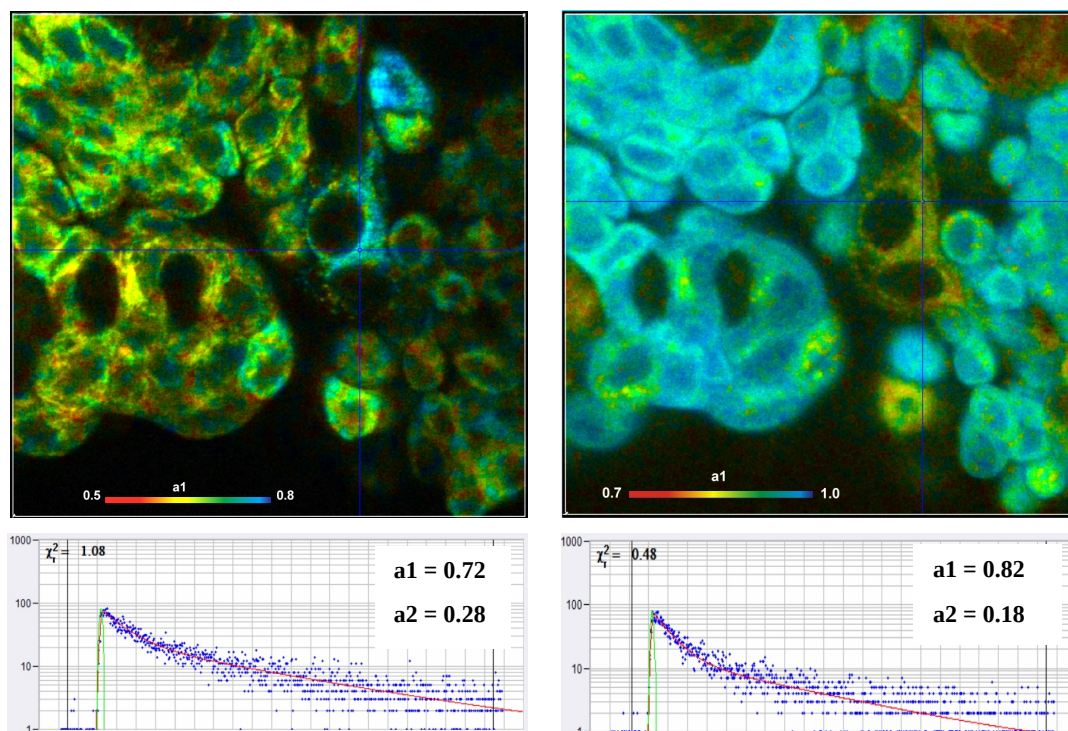


Fig. 333: NADH FLIM image (left) and FAD FLIM image recorded simultaneously by excitation wavelength multiplexing and dual-channel recording. Data analysis by SPCImage NG, MLE fit. Colour coding shows the amplitude of the fast decay component, a_1 . Bottom: Decay curves at the cursor position.

Metabolic FLIM with the DCS-120 MACRO System

Metabolic FLIM of macroscopic objects can be recorded by the DCS-120 MACRO system, see page 8. As described above, the system needs to be equipped with a 375 nm laser and a 405 nm laser. The parameter setup is the same as for systems with microscopes. Images for NAD(P)H and FAD can be recorded separately or simultaneously by laser multiplexing.

It should be noted, however, that the effective NA of the MACRO system is considerably smaller than that of a DCS system with a microscope. The low NA is an unavoidable consequence of the large image area. As a result, the count rate detected for a given laser power is smaller than in a microscope. However, a DCS MACRO system can be operated at higher excitation power. This is facilitated by directly coupled lasers, avoiding loss in excitation fibres. Because the excitation is spread over a large there is no risk of photodamage or photobleaching. Therefore, do not hesitate to use the highest available laser power. Pull the laser attenuator at the scan head up to the maximum. In the scanner panel, pull the power sliders up to about 60%. This gives high power but still yields a clean laser pulse shape without tails or afterpulses. Under these conditions the count rate from unlabelled samples should be on the order of a few 100,000 counts per second. Examples of a metabolic FLIM images taken with the DCS-120 MACRO are shown in Fig. 334 and Fig. 335. More applications are described in [99, 381, 352, 353, 532, 533, 534, 541, 644].

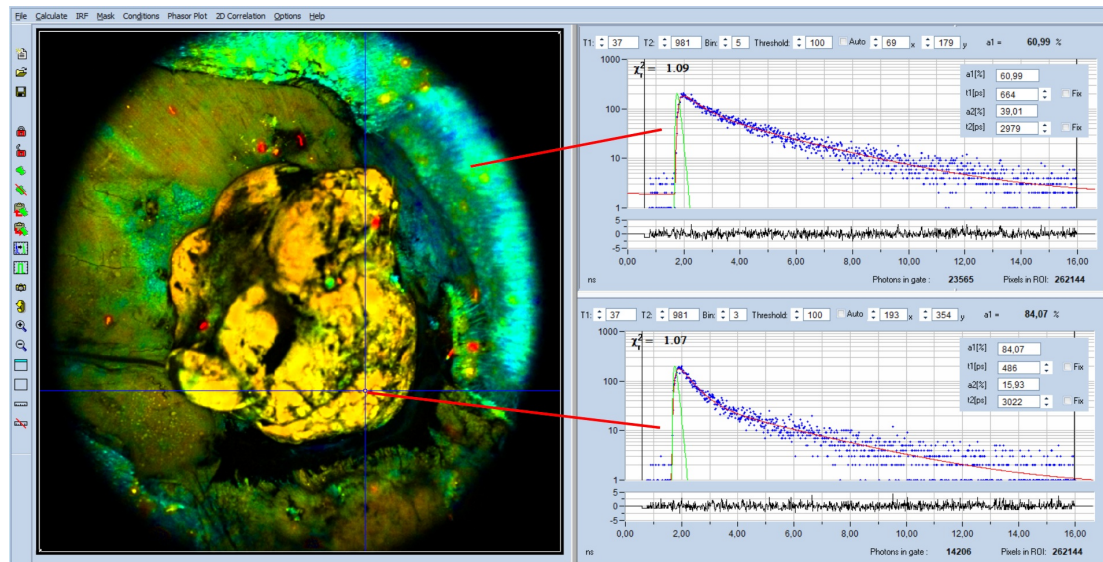


Fig. 334: MACRO image of an open tumor in a mouse. Recorded by DCS-120 MACRO, excitation 375 nm, emission 420 to 480 nm. Analysis by SPCImage NG, MLE fit, a1 image. a1 in the tumor is 84 %, compared to 61 % in the surrounding tissue. The difference in the decay profiles is clearly seen in the curves on the right.

Fig. 335 shows a similar image of a freshly excised rat brain with a glioblastoma. The acquisition time for both images was about 100 seconds, the data were analysed by the maximum-likelihood fit of SPCImage NG.

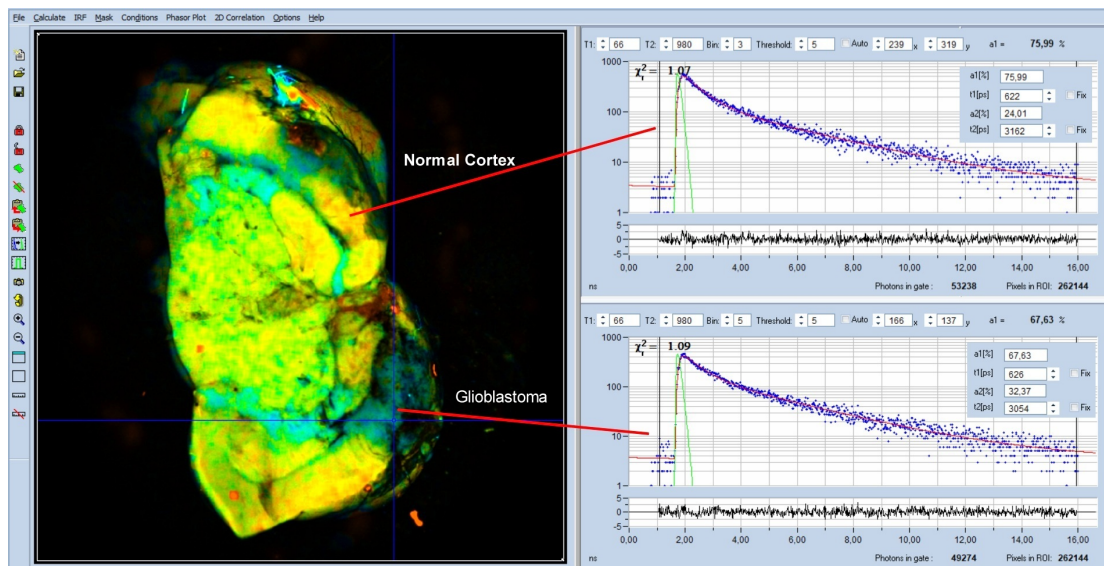


Fig. 335: Rat brain with glioblastoma (right). Autofluorescence, NADH image, excitation 375 nm, detection 430 to 475 nm. Data courtesy of Marina Shirmanova and Vladislav Shcheslavskiy, University Nishni Novgorod.

Near-Infrared FLIM

Near infrared FLIM is often considered a way to avoid autofluorescence in FLIM data recorded with exogenous fluorophores. It is correct that autofluorescence is much weaker at NIR wavelengths than in the visible range. However, at reasonable labelling or expression levels autofluorescence is rarely a problem. More important is the fact that red or near-infrared excitation goes deeper into tissue than blue or visible light. Also, fluorescence emitted in the deep red or near-infrared leaves tissue more efficiently than fluorescence in the visible-range. Near-infrared FLIM is therefore a way to perform FLIM in tissue without the need of a mul-

tiphoton system. Moreover, near-infrared FLIM is also used to evaluate contrast agents and fluorophores in diffuse optical imaging applications [129, 314, 362, 371]. In any case, it is important whether the lifetimes of NIR fluorophores change with their binding targets [84, 106] or whether they bear biologically relevant information [14, 238].

Technical Solution

Near-infrared FLIM requires a laser with a wavelength in the red or near-infrared region, and a detector that is sensitive in the spectral range above 700 nm. Moreover, the main beamsplitter of the scanner must reflect at laser wavelength, and transmit at the detection wavelength. A normal confocal scanner does not have the correct dichroic beamsplitters for these wavelengths. NIR FLIM therefore poses a number of problems to commonly used laser scanning microscopes [77, 83]. In some cases these problems can be solved by using the Ti:Sa laser of a multiphoton microscope as a one-photon excitation source, or by using two-photon excitation by an OPO [34, 77].

The DCS-120 system solves these problems by a ps diode laser of suitable wavelength and a replaceable beamsplitter, see Fig. 97, page 54 and Fig. 83, page 47. Most NIR dyes absorb strongly around 780 nm. A BDS-SM laser of this wavelength is available and compatible with the DCS-120 system. However, 780 nm excitation can create a problem. The Stokes shifts of NIR dyes is very small. With 780 nm excitation wavelength it can be difficult to split the emission from the excitation. We therefore recommend a laser with 640 nm instead of one with 780 nm. Virtually all NIR dyes have enough absorption at 640 nm. Excitation at 640 nm leaves enough room between the excitation and the emission. The 640 nm laser can be implemented in the system by replacing the second laser of a basic DCS-120 system with a BDS-SM-640nm laser or using a BDS-SM-640nm in the Laser Hub of a BDS-120-CMI system.

For detection above 700 nm the standard HPM-100-40 detector in the long wavelength detection channel has to be replaced with an HPM-100-50 [77]. A cooled version (HPM-100-50-C) is recommended though not absolutely necessary. All in all, this gives a system that excites the commonly used NIR dyes, and detect the fluorescence efficiently up to 900 nm.

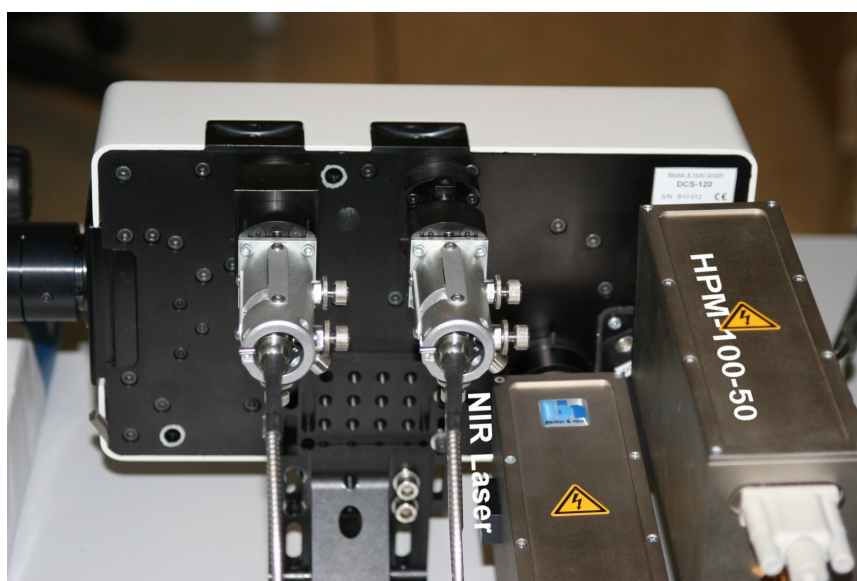


Fig. 336: Back view of DCS-120 scanner, showing input fibre for NIR laser and NIR detector

Examples

Indocyanin Green

Fig. 344 shows pig skin stained with Indocyanin Green (ICG). Indocyanine Green has an absorption maximum around 780 nm. At concentrations higher than about 50 $\mu\text{m/l}$ aggregates form which have an absorption maximum at 690 nm. The fluorescence is emitted around 820 nm [180]. The small stokes shift between the absorption of the monomers and the emission makes it difficult to suppress scattered laser light and wideband spectral background of the diode laser. Fortunately, ICG has sufficient absorption down to 630 nm. We therefore used a 640 nm BDL-SMC ps diode laser for the experiments. This provided convenient spacing between the excitation and emission wavelengths, and solved any filter leakage problems.

The image shown in Fig. 337, left is from a focal plane about 20 μm below the top of the skin. The image on the right was taken from the back of the skin sample. Both images show the intensity-weighted lifetime of a double-exponential fit (t_i in SPCImage, see page 384 of this handbook).

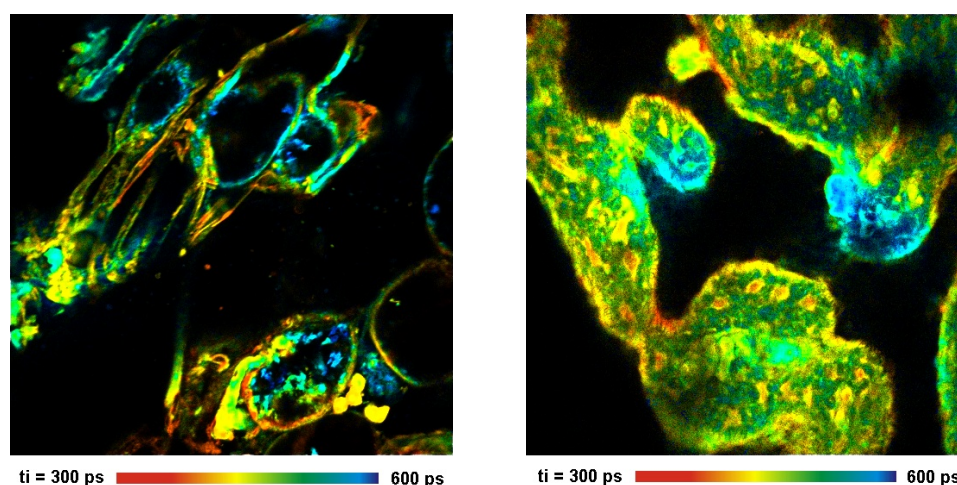


Fig. 337: Pig skin sample stained with ICG. Left: Image from the top of the skin, about 20 μm from the highest structures. Right: Image taken from the back of the skin sample. Fit with double-exponential model, intensity-weighted lifetime. DCS-120 WB system, HPM-100-50 detector.

There are surprisingly large changes in the fluorescence lifetime throughout the sample. It is known that ICG has a lifetime of about 200 ps in water, and about 600 ps when bound to serum albumin. Typical fluorescence decay functions and the corresponding decay parameters are shown in Fig. 338.

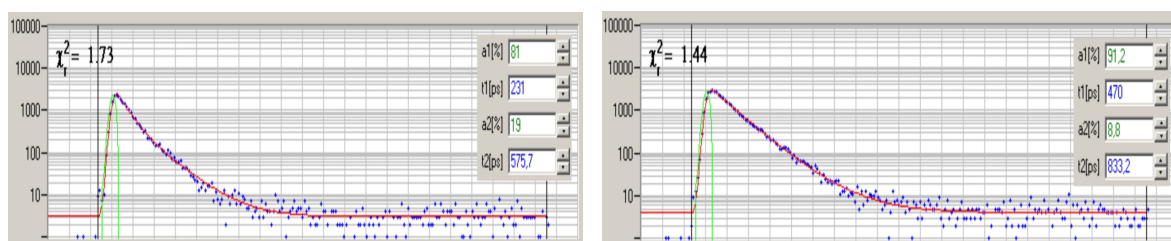


Fig. 338: Decay functions from selected 15x15 pixel areas of Fig. 337. Left: Short-lifetime areas (yellow or orange). Right: Long-lifetime areas (green)

In the short-lifetime areas (yellow-orange in the images) the lifetimes of the decay components are 231 ps and 576 ps. This is marginally compatible with a mixture of bound and unbound ICG. In the long-lifetime areas (green in the images) the fit delivers two decay components, with lifetimes of 470 ps and 833 ps. This is clearly incompatible with a simple mixture of bound and unbound ICG.

The results show that the assumption of an essentially invariable fluorescence lifetime (and thus quantum efficiency) of ICG in biological systems is not correct. The variability may have an impact on the reconstruction of tissue parameters in diffuse optical imaging experiments.

3,3'-Diethylthiatricarbocyanine

The spectral properties of 3,3'-diethylthiatricarbocyanine are very similar to those of ICG. There is an absorption peak at 760 nm, and a blue-shifted one at 670 nm. The intensity of the peaks depends on the environment, in particular on the binding to biological targets [155]. A pig skin sample stained with 3,3'-diethylthiatricarbocyanine is shown in Fig. 339. The figure shows that there is not only excellent contrast in the image, but also significantly different fluorescence lifetime in different tissue structures. Decay curves in selected pixels are shown on the right. A double-exponential fit delivered amplitude-weighted lifetimes from about 960 ps to 1530 ps, with component lifetimes from 780 ps to 2620 ps.

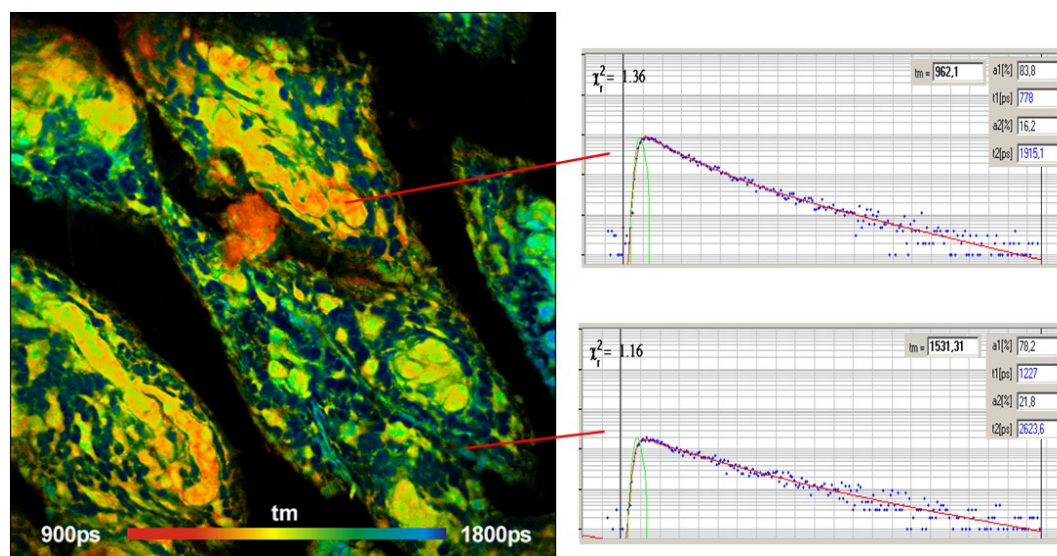


Fig. 339: Pig skin sample stained with 3,3'-diethylthiatricarbocyanine. Left: Lifetime image. Right: Decay curves in selected spots of the image. Excitation with BDL-SMC-650nm, detection 780 nm to 900 nm, HPM-100-50 detector.

Methylene Blue

Methylene Blue has an absorption band from 550 to 690 nm, with a maximum at 660 nm. Fluorescence is emitted from 650 nm to 750 nm, with a maximum at 680 nm. Methylene Blue is a biomedically interesting compound. It has anti-viral and anti-bacterial effects, and it has been used as a drug against malaria [500]. It has been used to induce apoptosis in cancer cells [630], and to treat psoriatic skin lesions by photodynamic therapy [487]. The use to treat Alzheimer disease is under clinical trial [393]. Fig. 340, left, shows a pig skin sample stained with Methylene Blue. As can be seen from the lifetime image and from the decay curves (Fig. 340, right) Methylene blue delivers distinctly different decay times depending on the tissue structures it binds to. Both the lifetimes of the decay components and the amplitudes change. The exact mechanism of the lifetime changes is not known. Methylene blue shows changes in its absorption spectrum on pH variation. It can be expected that these are accompanied by lifetime changes. More important, it is known that Methylene Blue is a redox indicator. It is thus possible that the changes originate from variation in the redox potential of the proteins. If this speculation is correct Methylene Blue could be a highly potent marker for the tissue state.

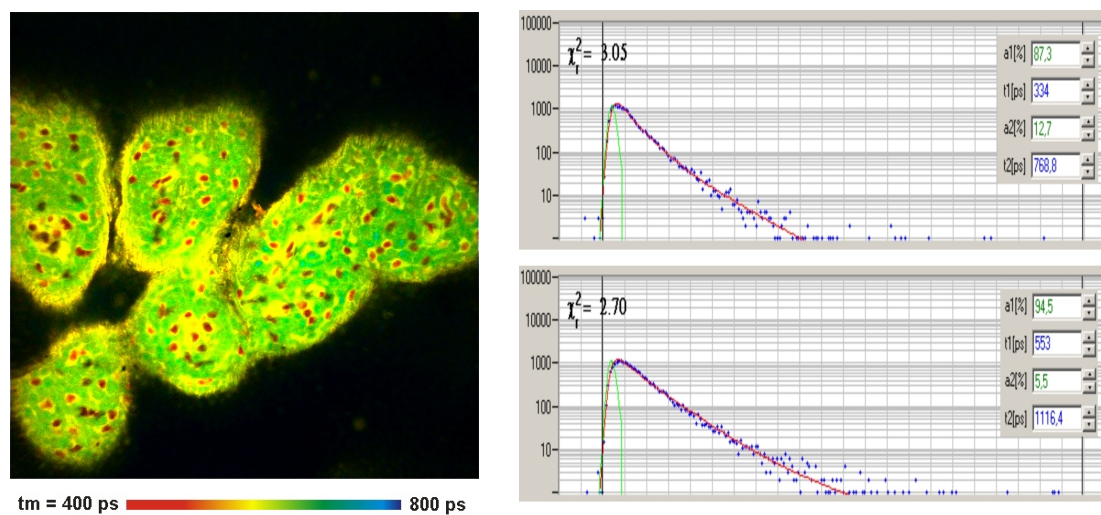


Fig. 340: Pig skin stained with Methylene Blue. Left: Lifetime image, double-exponential model, amplitude-weighted lifetime. Right: Decay curves in 3x3 pixel areas. Top: From red spots (fast decay). Bottom: From green areas (slow decay). DCS-120 system, 640 nm ps diode laser.

DCS-120 Multiphoton-Excitation Systems

Multiphoton FLIM excites fluorophores by simultaneous absorption of several (typically two) photons of the excitation light. To get the multiphoton process working, extremely high power density is necessary. It therefore works only with femtosecond laser pulses focused into the sample by a high numerical-aperture microscope lens.

A multiphoton FLIM system has a number of advantages over a one-photon system. The excitation wavelength is in the NIR. Absorption and scattering in the NIR is lower than in the visible range. Multiphoton excitation therefore penetrates much deeper into the sample. The non-linearity of the excitation process further has the effect that fluorescence is only excited in the focus of the microscope lens. This has the advantage that no out-of-focus suppression by a pinhole is needed. Photons scattered on the way out of the sample can thus be efficiently detected. There are also other advantages, such as the absence of photobleaching in out-of-focus planes, and easy excitation of fluorophores with absorption wavelengths in the UV. Moreover, the short pulse width of the laser provides the additional benefit that the FLIM system is able to fully exploit the time resolution of ultra-fast hybrid detectors [52, 53, 93, 94].

DCS-120 MP System

The DCS-120 MP system uses a femtosecond fibre laser for excitation. It consists of a normal microscope, the DCS-120 scan head, two non-descanned detectors with the associated optics, the TCSPC-FLIM data acquisition electronics, and the SPCM data acquisition and control software. As usual, data analysis is performed by SPCImage NG.

The DCS-120 MP is simple and easy to use. Nevertheless, it has the typical features of a multiphoton microscope: It delivers FLIM from sample layers as deep as 100 μm , excites fluorophores with absorption wavelengths in the ultraviolet region, and it delivers IRF widths down to less than 20 ps FWHM. The fibre laser does not need warm-up time so that the system is ready for use immediately after turn-on. The biggest advantage of the DCS-120 MP is its low price. By avoiding the need of an expensive Ti:Sa laser, it is the least expensive two-photon system on this planet.

The standard laser of the DCS-120 MP is the Toptica Femto-Fibre Smart. The excitation wavelength is 780 nm. The wavelength is ideal for exciting fluorescence of endogenous fluorophores, most importantly NAD(P)H. The DCS-120 MP is thus able to run NADH-based metabolic-FLIM experiments in cells and in tissue. Beside this, it works for most of the commonly used exogenous fluorophores, such as DAPI, ALEXA 488, and fluorescent proteins, such as CFP, Cerulean, and GFP. Admittedly, 780 nm is not the optimal excitation wavelength for these fluorophores. However, two-photon excitation spectra are wide, and there is usually enough absorption below the maximum of the absorption spectrum to obtain good FLIM data.

DCS-120 MP system with Ti:Sa Laser

For users who feel uneasy with a single excitation wavelength the DCS-120 MP can also be delivered with a Ti:Sa laser. Ti:Sa-based systems are available either with a Coherent or Spectra Physics lasers. Ti:Sa systems usually also contain an acousto-optical modulator (AOM). The AOM is not absolutely required for the function of the DCS-120 MP system. Its use is, however, strongly recommended. There are several reasons for that. The most obvious one is that it allows the user to maintain reproducible excitation intensities. The AOM settings are stored in the FLIM setup and data files so that a particular experiment can exactly be re-

produced by loading the .sdt file. The AOM is also used for PLIM. PLIM requires the laser to be on/off modulated at the time scale of less than a microsecond. Different than in a diode laser, there is no way of fast internal modulation in a titanium-sapphire laser. Finally, the AOM has a spectral cleaning function. Ti:Sa lasers emit wide-band background light in the spectral range where fluorescence is to be detected. This is usually not a problem as long as only thin samples (single cells) are imaged. However, if the system is used for tissue imaging the spectral background is scattered back in the detection path and causes severe loss in image contrast and lifetime accuracy.

A motorised sample stage can be added on demand. Both the Ti:Sa laser and the sample stage are controlled by SPCM data acquisition software. With these extensions the DCS-MP rises into the league of the 'big' multiphoton microscopes. Unavoidable, the price rises correspondingly.

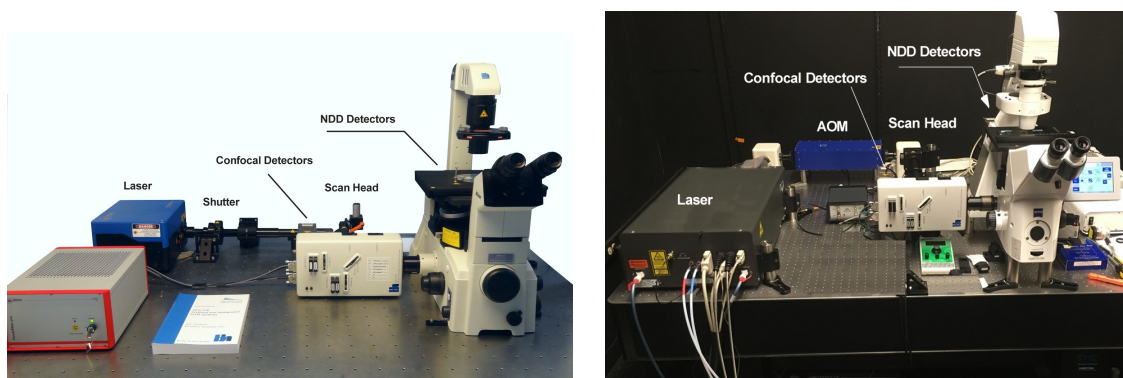


Fig. 341: Left Basic DCS-120 MP system. Right: DCS-120 MP system with Ti:Sa laser and AOM.

DCS-120 DMP system

The DCS-120 DMP uses a Toptica Femto Fibre Ultra laser system with two synchronised femtosecond fibre lasers of 785 nm and 920 nm. The lasers can be multiplexed for simultaneous FLIM of NAD(P)H and FAD and on/off modulated for simultaneous FIM/PLIM. With these capabilities, the DCS-120 DMP is the ideal multiphoton metabolic FLIM system. It can also be used for classic FLIM measurement with the commonly used fluorophores and fluorescent proteins, for FRET measurements, and for FLIM of carotenoids, melanin, and other ultra-short lifetime fluorophores. As for the DCS-120 MP system, the IRF width with the ultra-fast HPM-100-06 detectors is better than 20 ps fwhm.

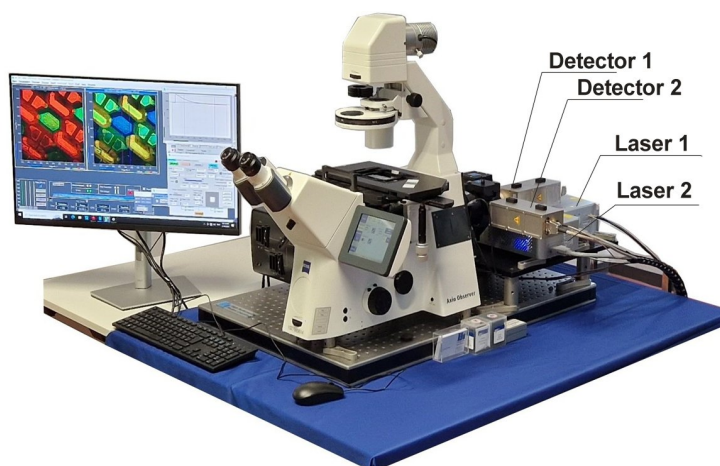


Fig. 342: DCS-120 DMP Dual Multiphoton excitation system

DCS-120 Super MPC System

The DCS-120 Super MPC system combines tuneable multiphoton FLIM with tuneable femto-second-excitation confocal FLIM.

The Super MPC system uses a Ti:Sa laser or an OPO for tuneable multiphoton excitation. In addition, the SHG of the Ti:Sa laser is available for tuneable excitation in the visible range. Multiphoton FLIM is performed via a NDD (non-descanned detection) beam path, one-photon excitation via a confocal beam path. The DCS-120 Super MPC can be used for deep-tissue FLIM and single-cell imaging as well. Because the excitation pulses are derived from the pulses of the Ti:Sa laser the time resolution of the DCS-120 Super MPC system extremely high not only in the multiphoton mode but also in the confocal mode. The system is able to resolve lifetimes down to 10 ps for excitation in the visible range. No other FLIM system has these capabilities. The DCS-Super-MPC system is shown in Fig. 343.

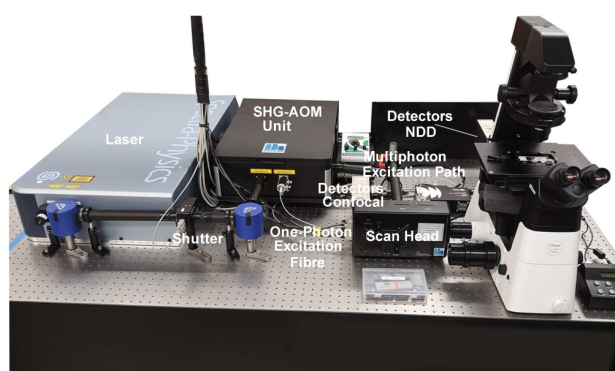


Fig. 343: DCS-120 Super MPC system. Multiphoton FLIM by Ti:Sa laser combined with tuneable confocal FLIM via SHG of Ti:Sa laser

General System Architecture

Excitation Beam Path

Fibre coupling, as it is used for the visible lasers of the DCS -120 system, cannot be used for the femtosecond-laser beam. The spectral dispersion in the glass would increase the pulse width so much that efficient multiphoton excitation would not be obtained. The laser beam must therefore be free-beam coupled into the scanner. In the DCS-120 multiphoton systems 'Laser Input 2', i.e. the input for the laser of longer wavelength, is used for the femtosecond laser, see Fig. 344.



Fig. 344: Input of a Ti:Sa laser into the DCS-120 scanner

Alignment of the laser beam with respect to the scanner is critical. It is therefore mandatory to place the laser, the scanner, and the microscope on a solid optical table. The scan head must be fixed to the table by a metal bracket. The laser beam is fed into the scanner via several mirrors. For laser safety reasons the entire laser beam path is covered by tubes and mirror housings. Please do not remove these elements. They also protect the mirrors from dust. Dust particles can damage the mirrors when they are hit by the laser beam.

AOM

In DCS-120 MP systems with Ti:Sa lasers an AOM (Acousto-Optical Modulator) is used for controlling the laser power. In principle, the laser power could also be controlled by the attenuator wheel at the laser input of the scan head. However, using an AOM has the advantage that the laser power is controlled electronically via the user interface of SPCM, see Fig. 356, page 208. When measurement data are saved the selected power is stored together with the FLIM data. That means the measurement conditions can later be reproduced by loading this file.

The principle of the AOM is illustrated in Fig. 345, right. A high-frequency signal induces a grating in the AOM crystal. The grating diffracts away a part of the laser beam. How much it diffracts depends on the power of the signal, the angle on the frequency of the signal and the wavelength. Due to the wavelength dependence the AOM has a spectral cleaning function. Spectral background, as it often appears in Ti:Sa lasers is substantially suppressed. The spectral cleaning function is another reason to use an AOM in Ti:Sa systems.

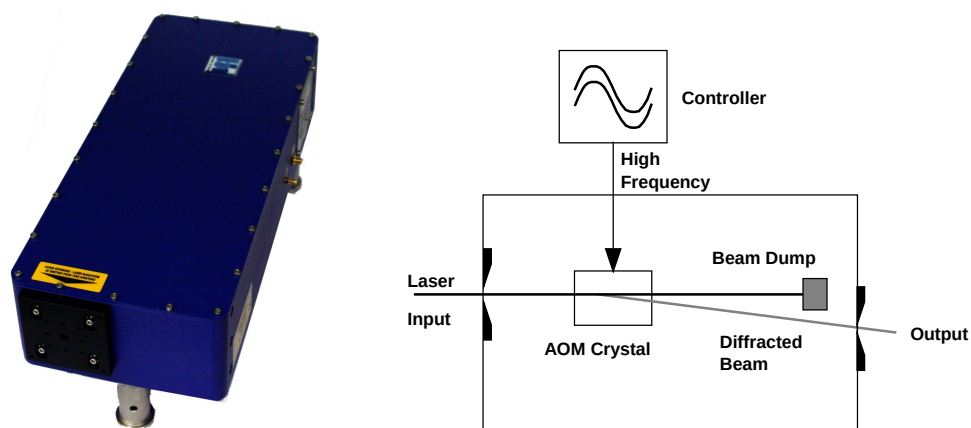


Fig. 345: Left: AOM for DCS-120 MP Ti:Sa systems. Right: Principle of AOM function

Beam Splitter in the Microscope Filter Carousel

For multiphoton imaging, a dichroic beamsplitter is inserted in the microscope filter carousel, see Fig. 346. The beam splitter sits in a filter cube which is specific of the microscopes of the different manufacturers. The splitter separates the fluorescence signal from the laser beam,. Usually it also contains the first transfer lens of the NDD beam path, see Fig. 348. For visual inspection of the sample or for confocal imaging with diode lasers the beamsplitter has to be rotated out of the beam path.

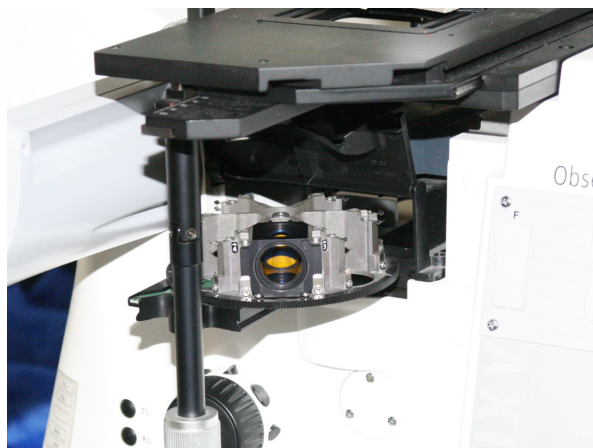


Fig. 346: NDD Beamsplitter in the filter carousel of an Axio Observer microscope

Main Dichroic Beamsplitter in the Scan Head

The main dichroic beamsplitter (MBS) of the scan head directs the laser beam down to the galvanometer mirrors. In confocal systems the MBS splits the fluorescence beam off the excitation beam and sends it into the detection beam path. For microscopes which are used solely for multiphoton FLIM the fluorescence does not go back through the scanner. Multiphoton versions of the DCS-120 therefore use a mirror in place of the MBS. Only systems with an additional diode laser use a dichroic beamsplitter. A typical transmission/reflection characteristic for an MBS for an NIR fs laser and a 405 nm diode laser is shown in Fig. 347. Suitable beamsplitter assemblies are available from bh.

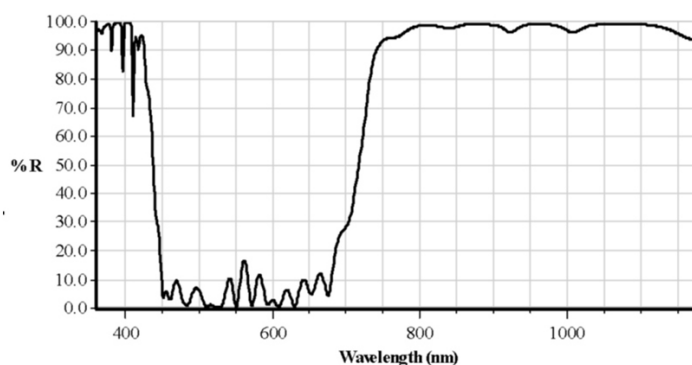


Fig. 347: MBS for multiphoton excitation and excitation by a 405 nm ps diode laser

NDD Beam Path

The NDD beam path transfers the fluorescence light emitted in the sample to the detectors. On the way to the detectors it has to pass the NDD beam splitter, a shutter, a laser blocking filter, and a secondary beam splitter that splits the light into two spectral components which are further cleaned by emission filters, see Fig. 348. These optical elements set a limit to the diameter of the fluorescence beam. Efficient NDD optics has to feed the fluorescence light through the limited diameter of the optical path without losing photons which are not perfectly collimated by the microscope lens. The NDD optics of the DCS multiphoton systems solves this problem by a periscopic design. The principle is shown in Fig. 348. A lens, L1, in the main NDD beamsplitter cube projects an image of the back aperture of the microscope lens on a second lens, L2. L2 projects an image of the first lens on the lenses L3 and L4. L3

and L4 project an image of L2 on the detectors. This way, even uncollimated light from deep sample layers is able to pass the narrow NDD beam path and reach the detectors.

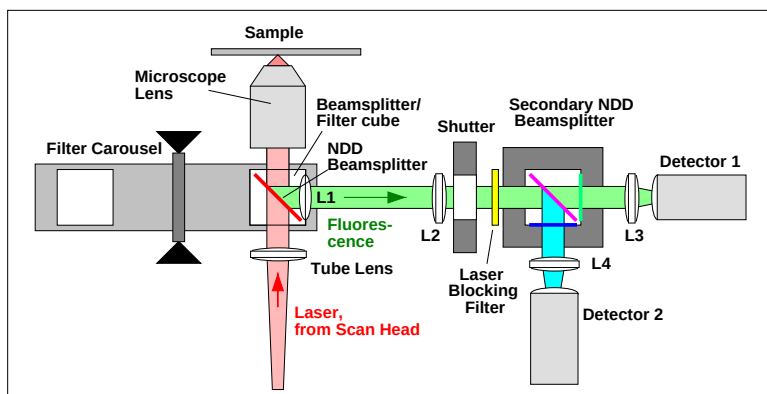


Fig. 348: Left: Principle of non-descanned detection optics (inverted microscope).

Secondary Beamsplitter and Filters in the NDD Beam path

In the DCS-120 multiphoton systems the fluorescence light is split on two detectors by a secondary dichroic beamsplitter in the NDD light path. The beamsplitter module together with the detectors is shown in Fig. 349.



Fig. 349: NDD detector / beamsplitter modules. Left: Two HPM-100 detectors. Right: One HPM-100 detector

For easy replacement of filters the beamsplitter module contains a plug-in module that holds a standard Zeiss type microscope filter cube, see Fig. 350. The plug-in module can be pulled out and the filter cube replaced. Several microscope filter cubes can be configured with different dichroic mirrors and filters, and inserted when needed. For systems with only one detector the microscope cube contains just the emission filter, and no dichroic mirror.



Fig. 350: Parts of the NDD beamsplitter module. The module contains a slider (shown middle) that holds a standard Zeiss type microscope filter cube (shown right).

Connection of NDD Optics to the Microscope

For the Zeiss Axiovert and Axio Observer microscopes the NDD optics module is attached to the epi-fluorescence (or ‘reflected light’) port at the back of the microscope body. The NDD optics module of a dual-channel system connected to the back of a Zeiss Axio Observer are shown in Fig. 351 the NDD module connected to a NIKON inverted microscope in Fig. 352.

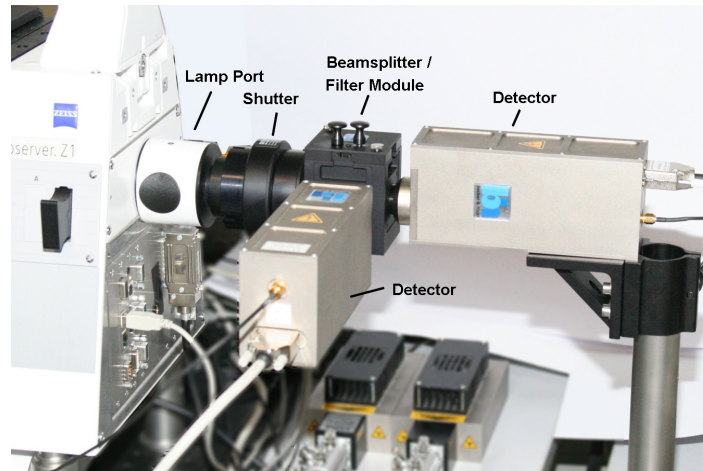


Fig. 351: Non-descanned detectors of the DCS-120 MP attached to the lamp port of a Zeiss Axio Observer microscope. Shutter, secondary NDD beamsplitter module, and two HPM-100 hybrid detectors. Additional diode lasers for confocal FLIM are seen at the bottom.

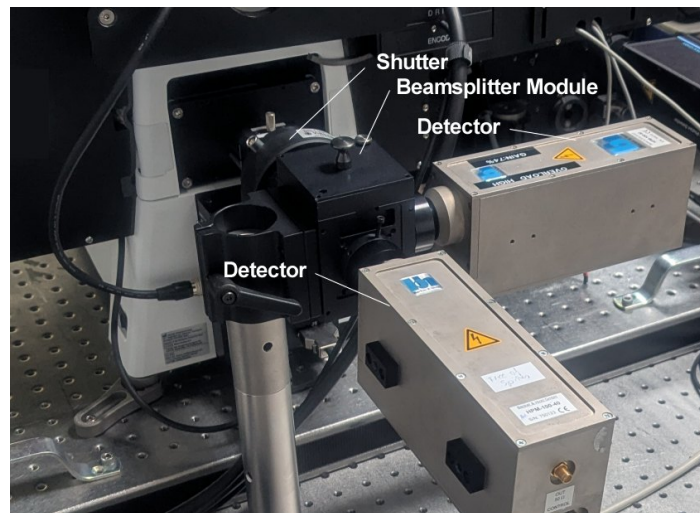


Fig. 352: Non-descanned detectors attached to a NIKON microscope

Peculiarities of Non-Descanned Detection

Users of confocal FLIM are sometimes surprised by the behaviour of a multiphoton system with non-descanned detection. A confocal system collects photons only from the excited volume of the sample. Photons from outside this volume are suppressed by the pinhole. Non-descanned detection is different. NDD is designed to detect photons from deep sample layers, which are scattered on the way to the surface of the sample. To detect these photons NDD collects photons from a large area of the sample surface. However, large-area detection comes at a price: Different than in a confocal system there is nothing that suppresses background photons in the NDD beam path. The situation is illustrated in Fig. 353.

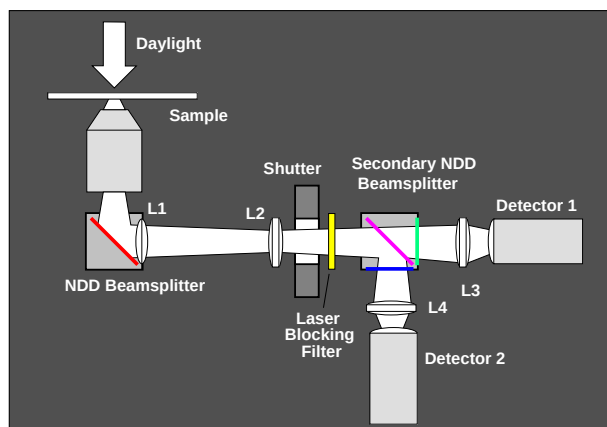


Fig. 353: Daylight leaking into a non-descanned detection path reaches the detectors unsuppressed by a pinhole

The only way to prevent daylight from reaching the detectors is to prevent it from getting into the system at all. Most background light gets into the detection path through the sample. Therefore, cover the back of the sample with a light-tight cover. Cover the entire microscope with black cloth or use the incubator box shown in Fig. 15, page 9. Even this may be not enough. Check the detector count rates when the shutter is open and the laser is off. The ideal background count rate for HPM-100-40 detectors should be below 800 count per second. To reach this value you may have to turn off the room lights and work in darkness. This may look inconvenient but it pays off: The accuracy of FLIM results is impaired dramatically if the data are not free of background [82].

Another frequent problem of NDD is distortion of the decay signals by reflections. Same as background, reflections are more problematic for NDD than for confocal detection. In a confocal setup reflections are suppressed by the pinhole, in an NDD setup they are not. The biggest source of reflections is, surprisingly, the microscope lamp. Fluorescence in the sample is emitted isotropically, i.e. also through the back of the sample and up into the microscope lamp. From the mirror behind the lamp it is reflected back to the sample, through the sample, and in the NDD detection path. It arrives at the detectors one or two ns after the real signal. The solution is easy: Put a black cover over the back of the sample.

Reflections can also occur in a correctly designed and correctly used NDD system. The NDD system is designed to transfer all photons from the sample to the detector. That means it also transfers photons from the detector back toward the sample. Since photocathodes are partially reflective, a noticeable amount of light is reflected back into the beam path. It can be reflected a second time at another optical surface and reach the detector a second time. The NDD path is designed in a way that such reflections are dispersed out of the normal beam path. However, it is not possible to avoid reflections completely without compromising the efficiency of the NDD beam path. Light reflected at the detectors can even get all the way back to the sample, and from there a second time to the detectors. The relative amplitude of these reflections is less than 10^{-3} and rarely objectionable in normal FLIM results.

Control of Detectors and Shutter

The NDD detectors and the shutter of the DCS-120 multiphoton systems are controlled by a DCC-100 detector controller card or a DCU-400 detector controller module. The control panel for a system with two NDD detectors is shown in Fig. 354. The gain of the detectors is set via the 'Gain Det 1' and 'Gain Det 2' sliders, the shutter is controlled by the DigOut button marked 'Shutter'. Please note that the gain sliders are used to set the optimum detector gain

for single-photon detection. The detector gain cannot be used to change the sensitivity of the detectors. Please see section ‘Detector Control’, page 116 for details of detector control.

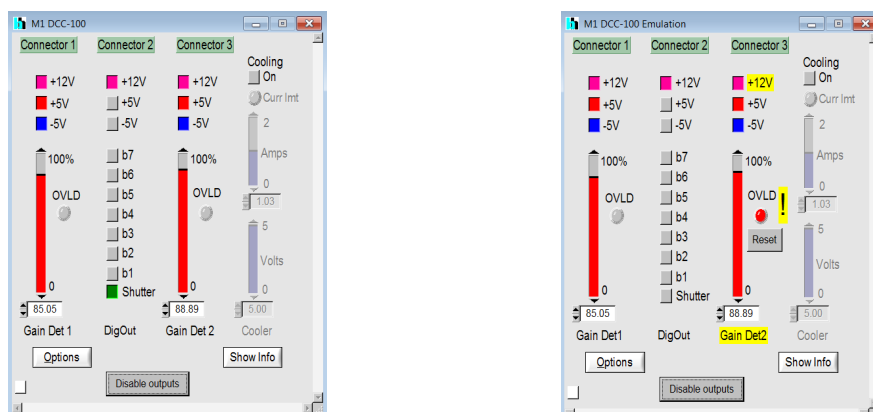


Fig. 354: Detector-control panel for a DCS-120 MP system with two NDD detectors. Left: Detectors active, shutter open. Right: After overload shutdown of Detector2, shutter closed

Fig. 354, left, shows the detector control panel with the detectors enabled and the shutter opened. Fig. 354, right shows the panel after an overload shutdown has occurred. In that case, the DCC-100 shuts down the detector and closes the shutter. Overload is usually caused by room light, light from the microscope lamp, or high laser power. If an overload has occurred, find and remove the source of the overload, click on the ‘Reset’ button, and open the shutter again.

A DCS-120 MP or DCS-120 DMP system may have additional diode lasers and confocal detectors. A system like this has four detectors. Two are non-descanned detectors, two are confocal detectors. Systems with four detectors either have two DCC-100 detector controller modules or a DCU-400 detector controller. When two DCC modules are used the non-descanned detectors are controlled by DCC Module 1, the confocal detectors by DCC Module 2, see Fig. 355. The unused pair of detectors must be turned off by clicking the 'Disable Outputs' button.

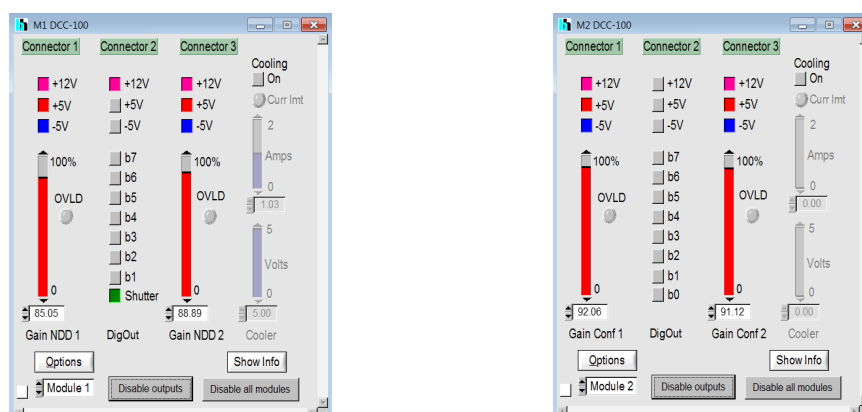


Fig. 355: Control of NDD detectors (left) and confocal detectors (right) in a combined multiphoton / Confocal DCS system.

Control of Ti:Sa Laser and AOM

The Ti:Sa laser is controlled via the panel shown in Fig. 356. It controls both the laser and the AOM. This guarantees that both the laser and the AOM are set to the same wavelength. If you are using a laser which is not supported by the SPCM software make sure that the AOM is set to the correct wavelength, see lower right of the AOM section of the panel control panel. If

the laser is running at a different wavelength than the AOM is set to the laser beam at the AOM output points into a wrong direction. The result can be that the beam does not arrive at the microscope lens, or that it arrives off centre. In the first case the system records no image at all, in the second case it records images with poor spatial resolution. Please see page 113 for details of AOM function.

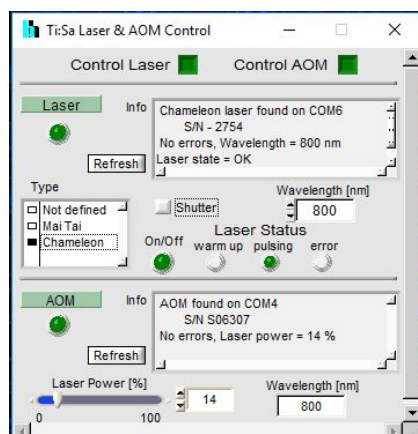


Fig. 356: Laser and AOM control panel

The Laser/AOM control features shown in Fig. 356 work in combination with the laser control features of the GVD-120 scan controller of the DCS system. The scanner control panel for the DCS-120 MP system is shown in Fig. 357.

The laser beam is turned on and off via the left ‘Laser’ button of the GVD panel, marked ‘Ti:Sa’ is Fig. 357. Beam Blanking should be enabled, as shown in Fig. 357. The function turns off the laser beam in the flyback phases of the scanning, and when the scanning is not running. ‘Multiplexing’ is set to ‘OFF’ for normal FLIM, and to ‘Pixel’ for simultaneous FLIM/PLIM operation. The other scanner control functions are the same as for the confocal DCS-120 systems.

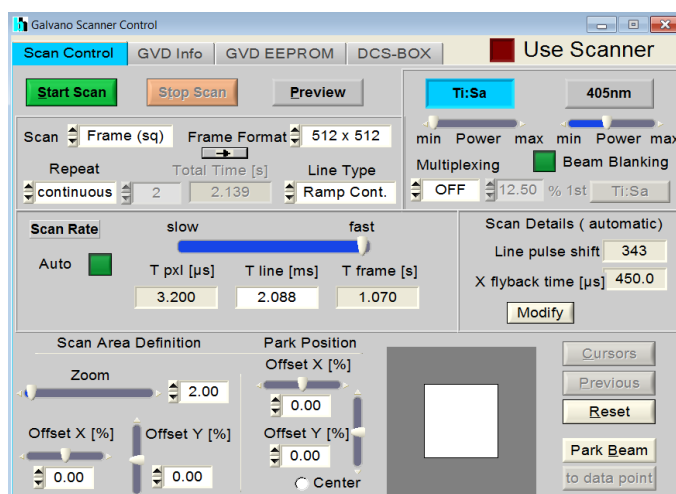


Fig. 357: Scanner control panel of the DCS-120 MP system. The Ti:Sa laser beam is switched on and off by the left Laser button, marked ‘Ti:Sa’.

Multiphoton FLIM Procedure

The general procedure of recording multiphoton FLIM is the same as for one-photon FLIM. Put the sample in the microscope. Switch the microscope beam path to the eyepieces. Rotate

the filter carousel to move the two-photon dichroic out of the beam path. Get the sample into focus and move it in the desired position. Then rotate the carousel in the position with the multiphoton dichroic in. Switch the microscope beam path to the scanner. Start the 'Preview' mode. Note that the focus depth for the Ti:Sa laser wavelength may be not exactly the same as for visible light. Focus into the desired sample plane, and zoom into the desired image area. Adjust the laser power. Then close the preview and start a FLIM recording. Let the acquisition run until you have reached the desired signal-to-noise ratio. The individual steps of the procedure are outlined below.

1. Focus through the Eyepieces

Put the sample under the microscope. Switch the microscope beam path to the eyepieces (Fig. 358, left). Turn on the microscope lamp in the transmission beam path (Fig. 358, left). Rotate the multiphoton beam splitter in the filter carousel out of the beam path (Fig. 358, middle). Look at the sample through the eyepieces, focus, and shift the sample into the desired position (Fig. 358, right).

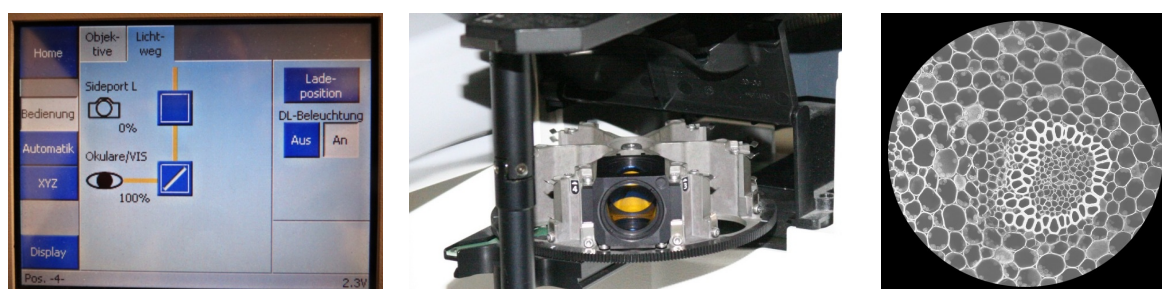


Fig. 358: Left: Switch beam path to 'Eyepieces' and turn on TL lamp (Zeiss Axio Observer). Middle: Rotate MP beamsplitter in filter carousel out of beam path. Right: Focus on sample and shift sample in desired position.

Be aware that the focus position in the eyepieces may not be exactly the same as for the femtosecond laser. Therefore, some fine focusing in the Preview mode may be required later. When you are working with thick samples you may have problems to find a correct focus position at all. Through the eyepieces you cannot see into the depth of the sample, and all you see is a blurry image from a layer close to the surface. In that case, do your best to get close to the surface of the sample, and do the final focusing in the Preview mode.

2. Set the Correct Microscope Beam Path

Rotate the NDD splitter into the beam path (Fig. 359, left). Turn off the microscope lamp (TL Illumination OFF, see Fig. 359, right). Switch the microscope beam path to 'Left Side Port'. For some microscope 'RL Illumination' (Reflected-Light Illumination) must be ON. These microscopes have a shutter in the reflected-light path. The shutter must be open to send the fluorescence light to the NDD detectors.

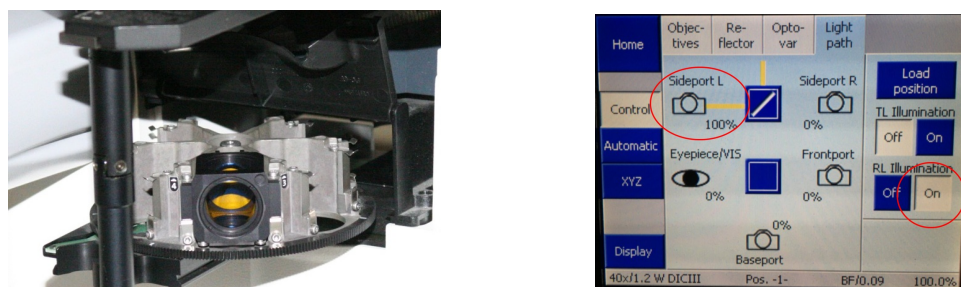


Fig. 359: Left: Rotate MP beam splitter into the light path. Right: Switch to left side port, turn TL illumination off, turn RL light path on.

3. Activate the Femtosecond Laser

When using a Ti:Sa laser, open the laser shutter in the Ti:Sa / AOM control panel, and select a laser power of 5 to 10%. When using a femtosecond fibre laser, turn on the laser in the GVD panel. See Fig. 360.

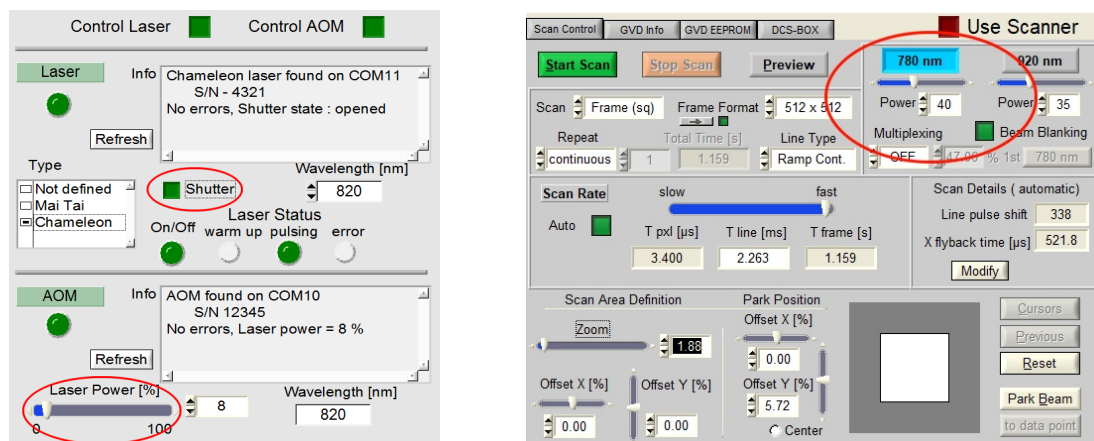


Fig. 360: Left: Ti:Sa laser, Shutter and power control. Right: Fibre laser, Laser on/off in the GVD panel

4. Activate Detectors

Turn off the room light or otherwise keep ambient light away from the microscope. Enable the NDD detectors and open the NDD shutter in the detector control panel as shown in Fig. 361. The CFD count rates should remain in the sub-1000-cps range, if possible even below 500 cps (counts per second). If the rates are higher, the reason is leakage of ambient light into the detection path. Cover the microscope, cover the back of the sample as described at page 206.

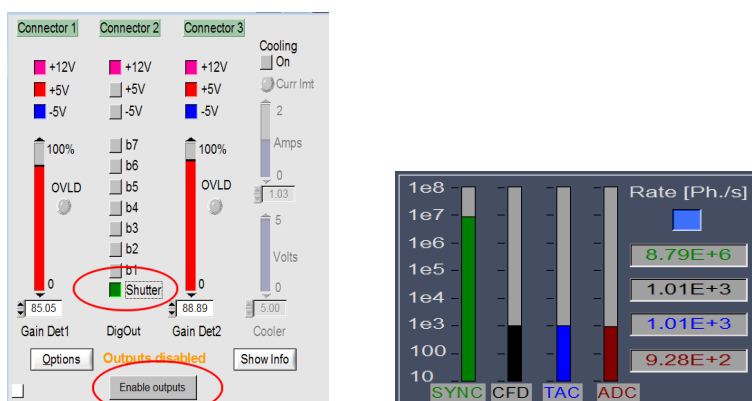


Fig. 361: Left: Enable NDD detectors and open NDD shutter. Right: The CFD count rate should remain in the sub-1000-cps range.

5. Run a Preview

Turn on the femtosecond laser in the scanner control panel and start the Preview mode. Looking at the images displayed by SPCM, fine-adjust the focus, fine-adjust the sample position, and select an appropriate Zoom in the GVD panel. Adjust the laser power. If the sample allows, try to reach a count rate on the order of 0.5 to 1 MHz. Note that this is the average count rate over the entire image. If large parts of the image are dark the actual count rate may be higher than displayed.

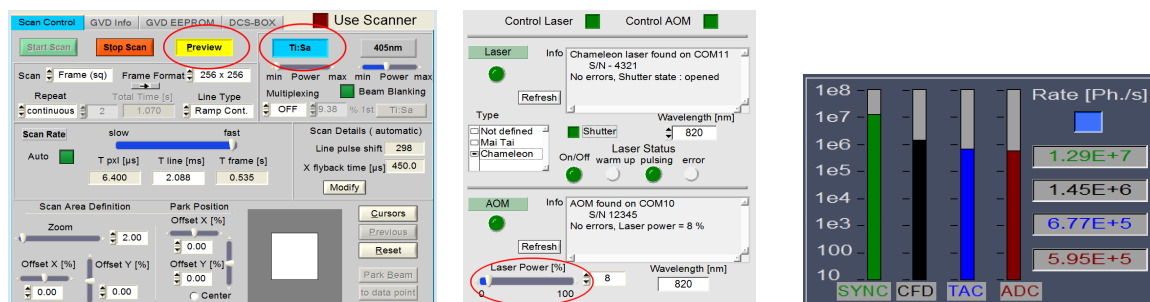


Fig. 362: Left: Preview mode with Ti:Sa laser beam turned on. Middle: Adjusting the laser power. Right: If the sample allows, try to reach a CFD count rate of about 1 MHz.

6. Record the FLIM image

Finish the Preview mode. If you want to change the pixel number of the final recording, this is the last chance to do so: Change it in the scanner panel and click on  to transfer the new pixel number into the SPCM system parameters. Start the FLIM recording. Watch how the images build up. Record until you have images with sufficient signal-to-noise ratio. FLIM experiments often aim on subtle effects in the fluorescence lifetime. This requires substantially more photons than simple imaging of the spatial structure of a sample. If in doubt, better record for a longer period of time. The FLIM accuracy increases with the square root of the number of photons recorded.

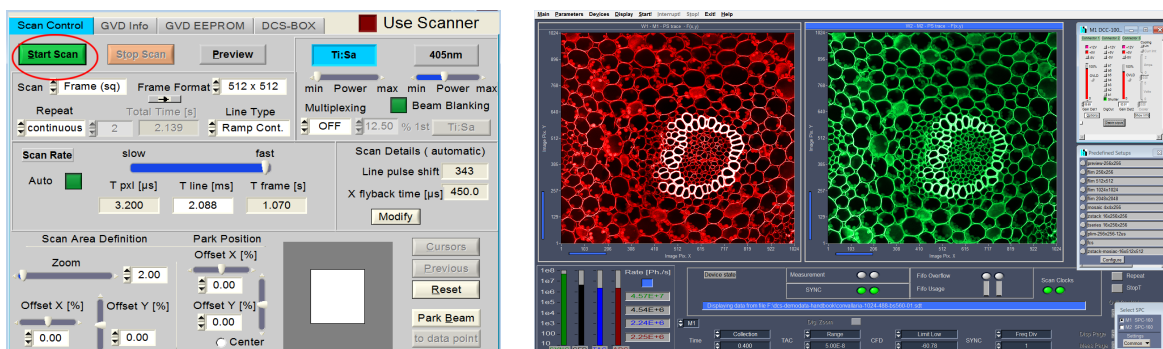


Fig. 363: Left: Start the FLIM recording. Right: Record until you have images with good signal-to-noise ratio

7. Save the Data

Save the .sdt data in the SPCM software. Saving .sdt data is important because only these contain the complete set of instrument setup parameters.

8. Inspect the Data with SPCImage

Inspect the data with SPCImage. Make sure that the data are good. For best lifetime accuracy, the background in the decay curves should be no higher than 1% of the peak count, the curves should be placed correctly within the observation time interval, and there should be a sufficient number of photons in the pixels.

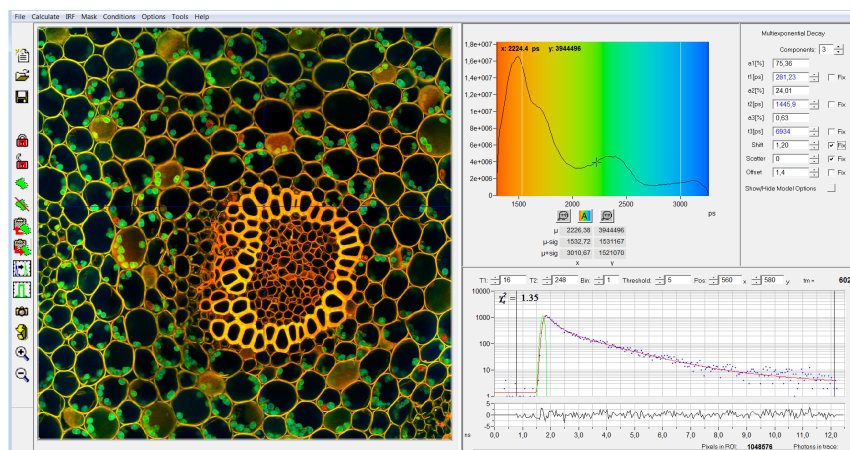


Fig. 364: Check of the data with SPCImage

For recording further images from the same sample or from a similar sample you do not have to go through all steps of this procedure again. Just follow the condensed procedure described at page 41 of this handbook.

Failure to Record Images?

Immediate overload shutdown: Make sure the microscope lamp is turned off. Turn off room light, cover the sample and the microscope. A non-descanned detector collects light from a much larger area than a confocal detector, and therefore picks up more ambient light.

No photons at all: Check that the detectors are turned on, see Fig. 361. Check that the Ti:Sa laser is warmed up and pulsing. The laser shutter must be open. See laser control panel, Fig. 360.

A few photons, no images: Check that the MP beamsplitter in the filter carousel is in the beam path. Check that the NDD shutter and the laser shutter are open. Check that the Ti:Sa is enabled in the scan control panel. In systems with four detectors, make sure you have turned on the correct pair of detectors. The NDD detectors are on DCC M1. Check the microscope beam path configuration, Fig. 359. 'RL illumination' must be on.

Many photons but no images: Out of focus? Move the focal plane up and down. The plane for 2p imaging is not exactly the same as for the eyepieces. Oil and water immersion objectives: Check that the immersion fluid is present. Make sure the sample is inserted in the correct orientation. The cover slip must point to the objective lens. Check the Ti:Sa laser button in the scanner panel. The laser may be OFF, and what you are recording is room light.

No image in one channel: Make sure that there is the right beamsplitter cube in NDD beamsplitter module. Make sure there is no overload shutdown, and both detectors are turned on.

Examples of Two-Photon FLIM

Deep-Tissue FLIM

Fig. 365 demonstrates the deep-tissue FLIM capability of the DCS-120 MP system. A pig-skin sample was excited at 780 nm, and the fluorescence detected in the wavelength interval from 450 to 600 nm. Several images were taken in different depth in the sample. The laser power was adjusted to obtain a count rate of about 10^6 photons per second. To obtain high-

quality data, an acquisition time of 2 minutes per image was used. The count rate remained stable over the entire acquisition time, indicating that photobleaching was negligible.

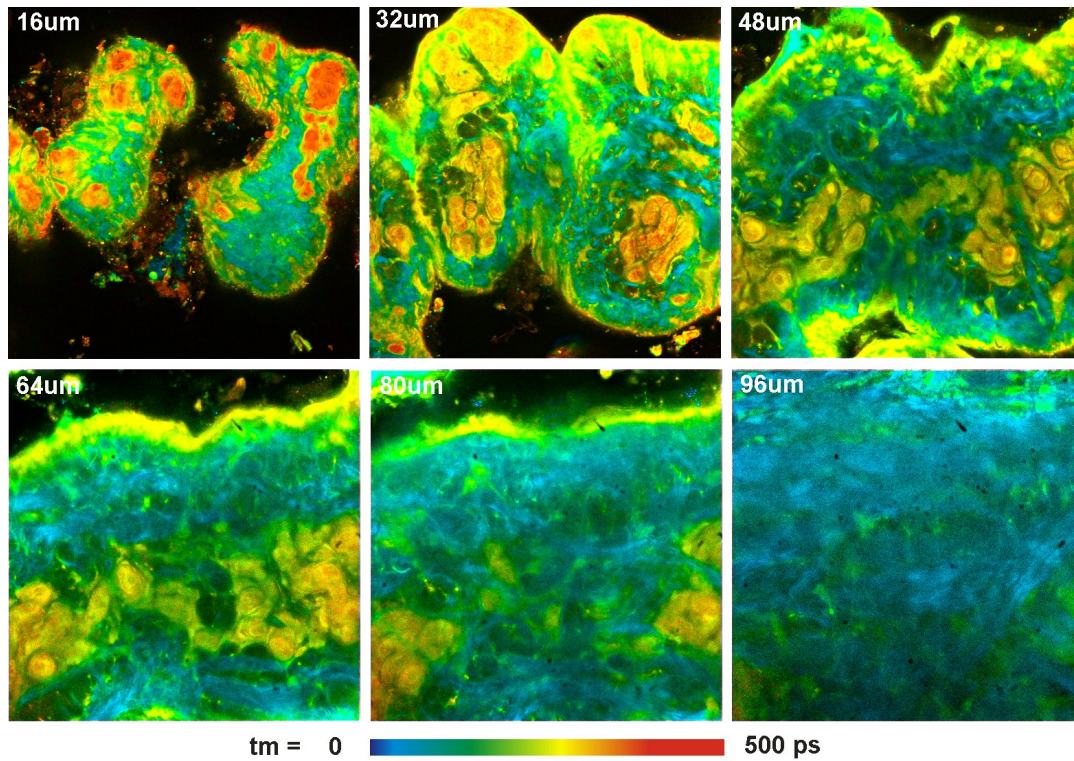


Fig. 365: Pig skin, autofluorescence, lifetime images in different depth in the sample. Amplitude-weighted lifetime of triple-exponential decay model. Excitation 805 nm, 512x512 pixels, 256 time channels. Zeiss Axio Observer Z1, Water C apochromate NA=1.2, detection through lamp port, HPM-100-40 hybrid detector.

The quality of the data shown in Fig. 365 is so good that colour-coded images of individual parameters of a triple-exponential decay model can be generated. Of particular interest are the contribution of the ultra-fast decay component, q_1 , and the ratio of the amplitudes of the medium and slow component, a_2/a_3 . The fast component is dominated by SHG. SHG is generated by collagen. q_1 therefore shows the amount of this tissue constituent. The slow and medium decay component are dominated by bound and unbound NADH and by bound and unbound FAD. The corresponding images are shown in Fig. 366.

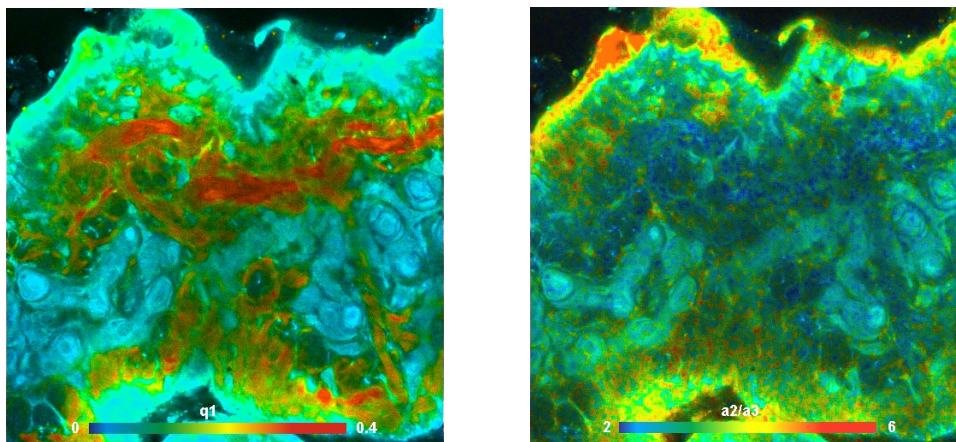


Fig. 366: Images of individual decay parameters of a triple-exponential fit to the data. Left: Relative intensity contribution, q_1 , of the fastest decay component. Right: Amplitude ratio, a_2/a_3 , of the medium and slow decay component. Depth 48 μm below the surface.

Two-Photon Metabolic FLIM

The high penetration depth of two-photon excitation is a highly desirable feature for metabolic FLIM of tissue. Moreover, two-photon excitation navigates around the complication that the best (one-photon) excitation wavelength for FAD is within the detection wavelength interval of NADH. Another benefit is the fast IRF of a two-photon system.

Two-photon metabolic FLIM can be efficiently performed with the DCS-120 DMP dual-excitation multiphoton system. With its two laser wavelengths and fast multiplexing capability it delivers NAD(P)H and FAD FLIM data simultaneously. The DCS-120 DMP uses the Toptica Femto Fibre Ultra laser system. It contains two synchronised lasers with 780 nm and 920 nm, individual AOMs, and about 500 mW of power per laser channel. The control of the laser is fully integrated in the SPCM FLIM data acquisition software via the scan control panel, see Fig. 367. The setup shown multiplexes the two lasers frame by frame. The TCSPC system parameters, 3D Trace Parameters, and Display parameters are shown in Fig. 368. They are the same as for multiplexed FLIM with confocal systems. For details please see 'Excitation Wavelength Multiplexing', page 137.

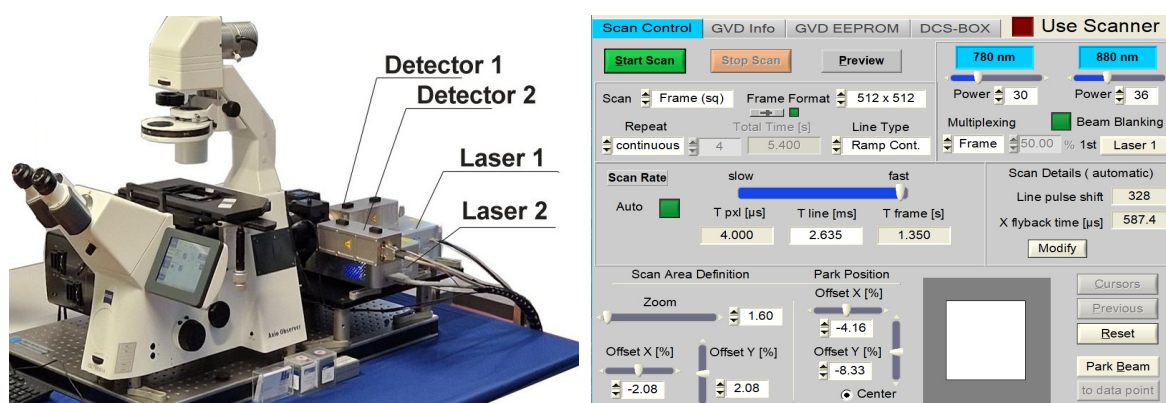


Fig. 367: Left: DCS-120 DMP dual-excitation metabolic FLIM System. Right: Scan control panel.

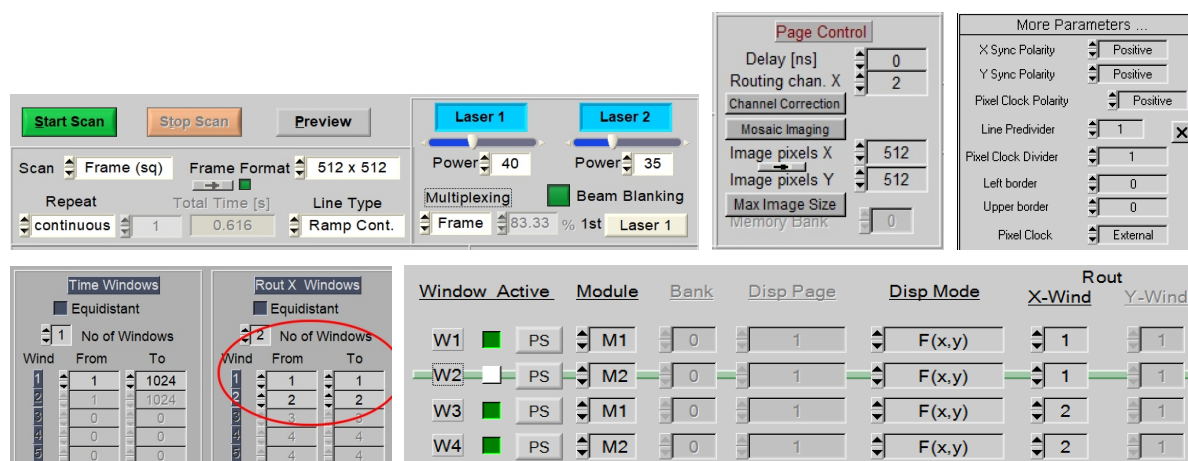


Fig. 368: Scan parameters, TCSPC system parameters, window intervals, and 3D trace parameters for multiplexed two-photon metabolic FLIM

The images generated by the setup are:

	Excitation	Detection	Signal detected
Image 1	780 nm	420 to 470 nm	NAD(P)H
Image 2	780 nm	490 to 600 nm	NAD(P)H + FAD, not used
Image 3	920 nm	420 to 470 nm	Collagen, SHG of 920 nm laser
Image 4	920 nm	490 to 600 nm	FAD

Image 1 and image 4 are the desired NADH and FAD images. Image 3 contains the SHG signal from collagen, excited at 920 nm. The SHG image provides supplementary information about connective-tissue structure, vascularisation, or skin ageing.

Typical Results

Typical results are shown in Fig. 369 through Fig. 372. Fig. 369 shows NAD(P)H and FAD images of yeast cells recorded by the DCS-120 DMP FLIM system. Fluorescence decay curves in a selected spot of 5x5 pixels are shown below the images.

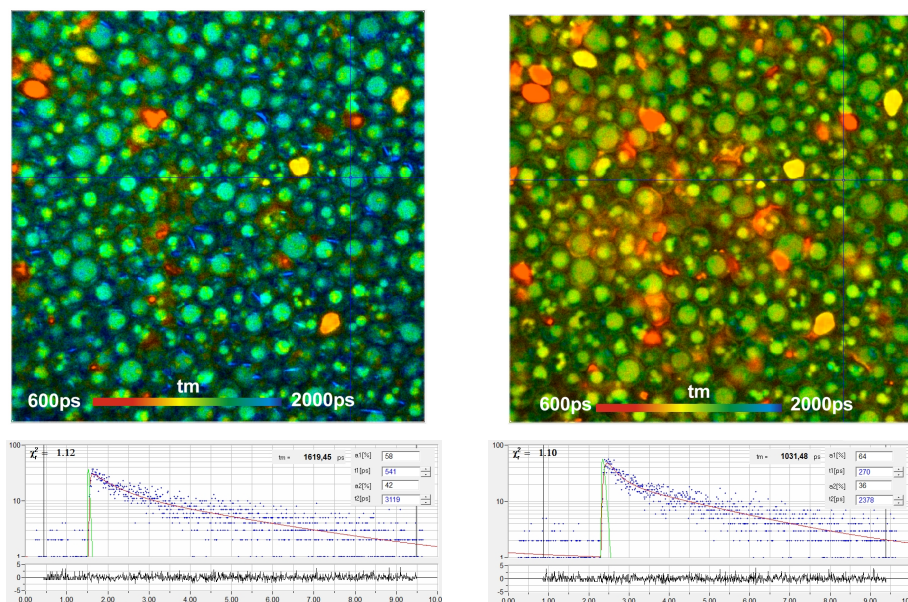


Fig. 369: 2-Photon metabolic FLIM of yeast cells, Images and decay curves at cursor position. Left: NADH. Right: FAD. Data analysis by SPCImage NG.

The amplitude-weighted lifetimes, t_m , for the NAD(P)H and the FAD are 1619 ps and 1031 ps, respectively. As expected, the decay components for NAD(P)H have lifetimes of 512 ps and 3119 ps. With 58%, the amplitude of the fast component is in the normal range (the cells were imaged under atmospheric pO_2). The components for FAD are 270 ps and 2378 ps, with amplitudes of 64% and 36 %, respectively.

Fig. 370 to Fig. 372 show 2-photon metabolic-FLIM images obtained from pig skin. Fig. 372 is the image from the NADH channel. The decay curve shown lower right is from a 5x5 pixel area around the cursor position. The decay function contains 67% of 432 ps and 33% of 2820 ps, compatible with the known lifetimes of unbound and bound fractions of NADH. Fig. 371 shows the image in the FAD channel. The decay components at the cursor position are 62% of 431 ps and 38% of 2490 ps. These values are generally compatible with FAD. It should, however, be noted that other fluorophores, such as FMN (Flavinmononucleotid), melanin, or porphyrines may contribute to the decay function. Fig. 372 is the image in the SHG channel. The signal mainly comes from collagen, and thus marks the connective-tissue structures. SHG is an ultra-fast process, therefore the 'lifetime' of the SHG signal should be zero. However, a small contribution from fluorescence is present, visible as a low-amplitude tail in the decay curve. The effective t_m averaged over the image is therefore not exactly zero, see lifetime histogram in the upper right.

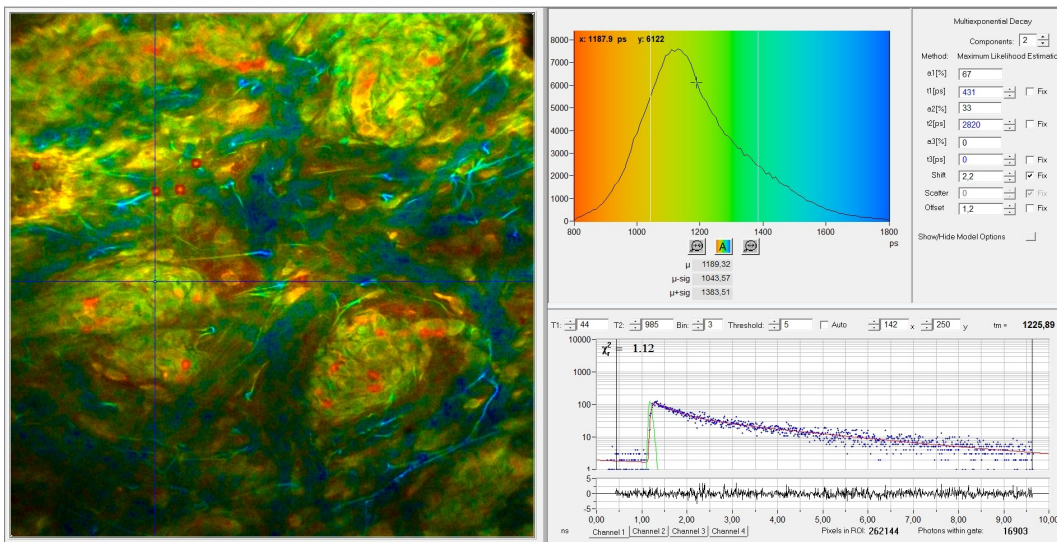


Fig. 370: 2p metabolic FLIM of Pig skin, tm image in the NADH channel

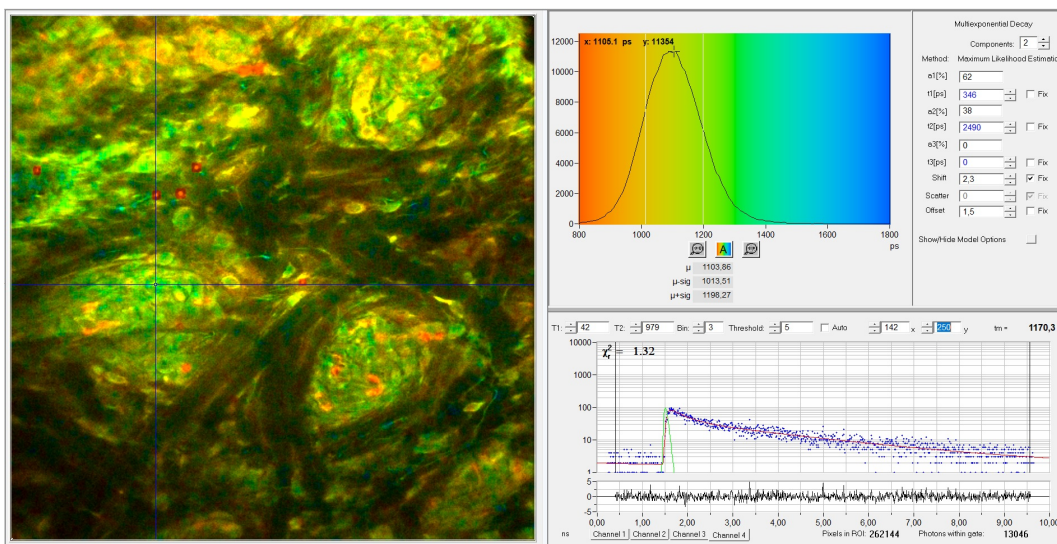


Fig. 371: 2p metabolic FLIM of Pig skin, tm image in the FAD channel

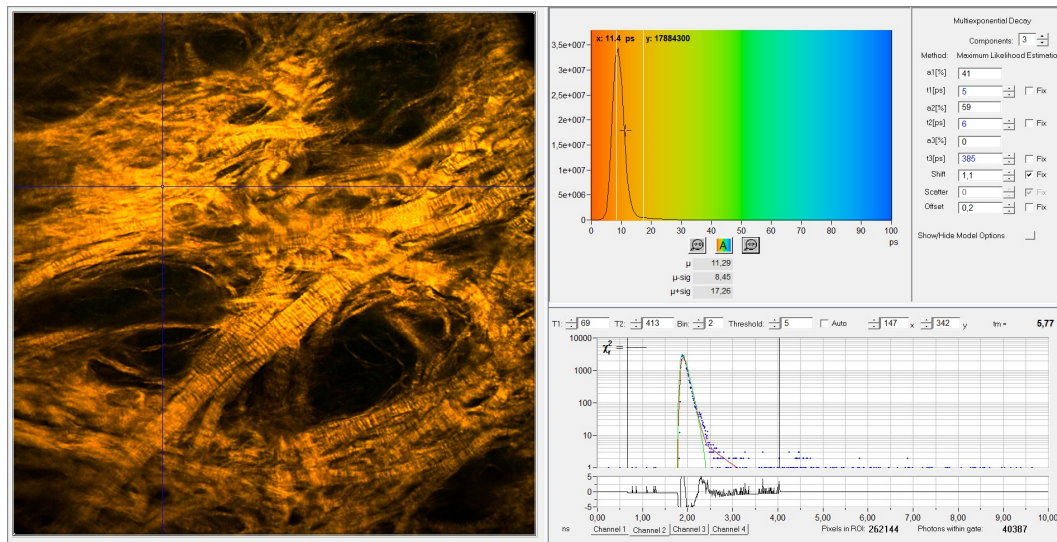


Fig. 372: 2p metabolic FLIM of Pig skin, image in the SHG channel

More about metabolic FLIM can be found in section 'Metabolic FLIM', page 189 and in chapter 'Autofluorescence FLIM of Cells and Tissue', page, 248.

NADH FLIM with Ultra-Fast Detectors

Another application of multiphoton FLIM is the excitation of fluorophores with absorption spectra in the UV. The most promising application is multiphoton FLIM of NADH, see chapter 'Autofluorescence FLIM of Cells and Tissues'. NADH has two decay components, a fast one for unbound NADH and a slow one for bound NADH. The unbound/bound ratio bears information on the metabolic state of the cell. The task of NADH FLIM is thus to separate the components of the decay function, and determine the amplitude ratio of the components, a_1/a_2 , and the component lifetimes, τ_1 and τ_2 .

For recording the data shown in this section we used a DCS-120 MP system with a Ti:Sa laser and an ultra-fast HPM-100-06 detector [77] attached to the NDD beamsplitter at the back port of a Zeiss Axio Examiner microscope. The excitation wavelength was 750 nm, the photons were detected in the wavelength interval from 440 to 470 nm. The data were recorded with 512 x 512 pixels and 1024 time channels per pixel. The time channel width was 12 ps.

Fig. 373 shows FLIM data of live cells. The data were analysed with SPCImage. A lifetime image of the amplitude-weighted lifetime of a double-exponential fit is shown in Fig. 373, left. To obtain enough photons for double-exponential decay analysis a binning factor of 2 was used in SPCImage.

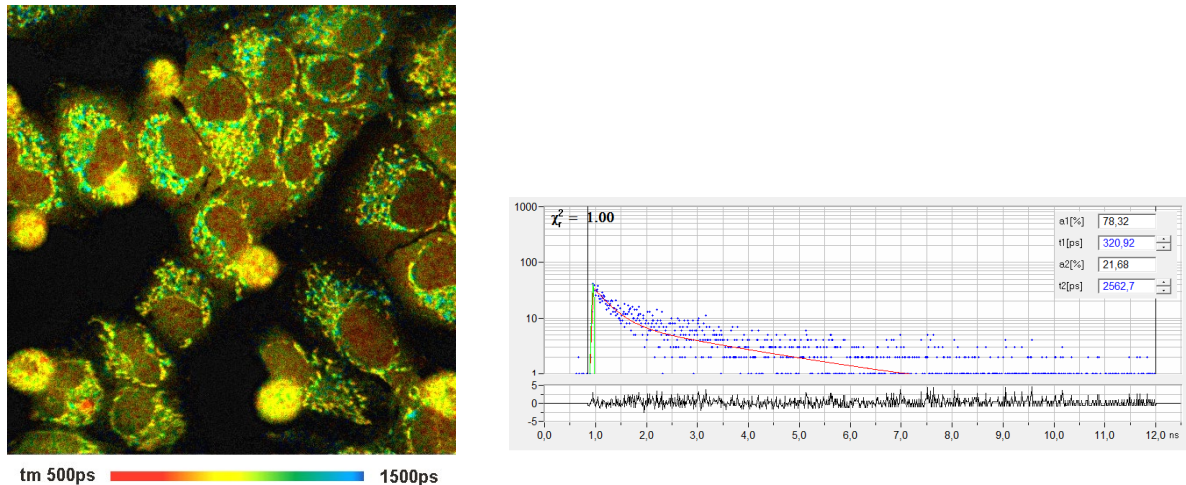


Fig. 373: Left: NADH Lifetime image, amplitude-weighted lifetime of double-exponential fit. Right: Decay curve in selected spot, 9x9 pixel area. FLIM data format 512x512 pixels, 1024 time channels. Time-channel width 12ps.

As expected, the time resolution of the detection system is excellent. The rise of the fluorescence occurs over less than two time channels, indicating that the IRF is indeed shorter than 24 ps. Compared with slower detectors, the high time resolution results in a substantially improved fit stability in the lifetime analysis.

Images of the amplitude ratio, a_1/a_2 (unbound/bound ratio), and of the fast (τ_1 , unbound NADH) and the slow decay component (τ_2 , bound NADH) are shown in Fig. 374. Such images are normally noisy, and visibly impaired by fitting artefacts. Not so in the data recorded with the fast HPM detector and the Ti:Sa laser. The component decay times and the a_1/a_2 ratio

are obtained at almost the same quality as the t_m image, without visible fitting artefacts. One reason is the MLE fit of SPCImage NG which gives a better accuracy than the traditional weighted least-square fit. The other reason is that the IRF is so fast that it does no longer interfere with the decay time of the fast fluorescence component. The iterative deconvolution process of the data analysis therefore virtually becomes a simple curve fitting, with a sharper χ^2 minimum and a better fit reliability.

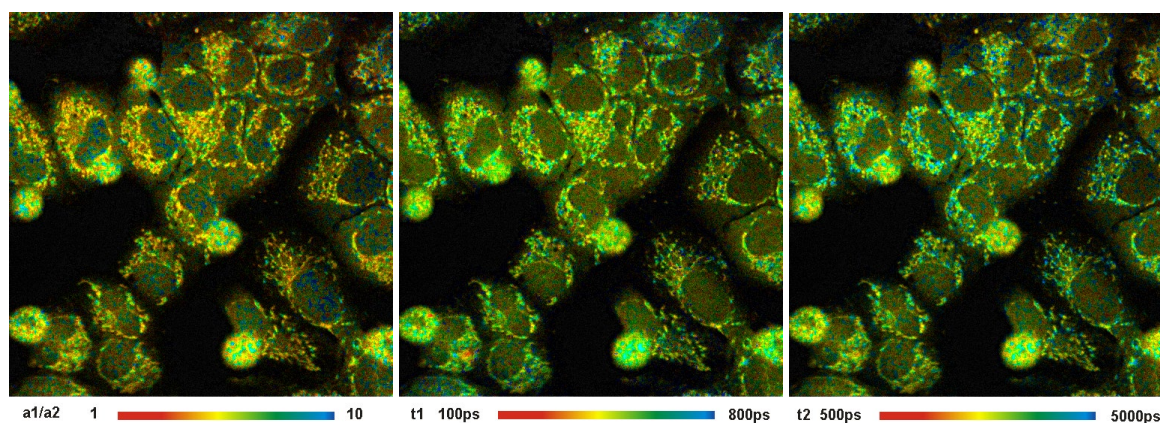


Fig. 374: Left to right: Images of the amplitude ratio, a_1/a_2 (unbound/bound ratio), and of the fast (t_1 , unbound NADH) and the slow decay component (t_2 , bound NADH). FLIM data format 512x512 pixels, 1024 time channels. Time-channel width 12ps.

Ultra-Fast FLIM of Biological Material

The high time resolution of the bh multiphoton FLIM systems makes fluorescence-decay components visible which have never been detected before. Fig. 375 shows FLIM data of mushroom spores. From left to right, images of the fast decay component, t_1 , of the mean lifetime, t_m , and of the amplitude of the fast component, a_1 , are shown. A decay curve at the cursor position is shown on the right. t_1 at the cursor position is 11 ps, and a_1 is in the range from 98 to 100%. Please see [93] for more data.

Fig. 376. shows FLIM of Pollen grains. The FLIM system detects a component with 10 ps lifetime [94], see Fig. 376. The data shown here were detected with a DCS-120 MP system using a Femto Fibre Pro Toptica femtosecond fibre laser, an HPM-100-06 detector and SPC-150NX TCSPC modules. Please see [87] for details.

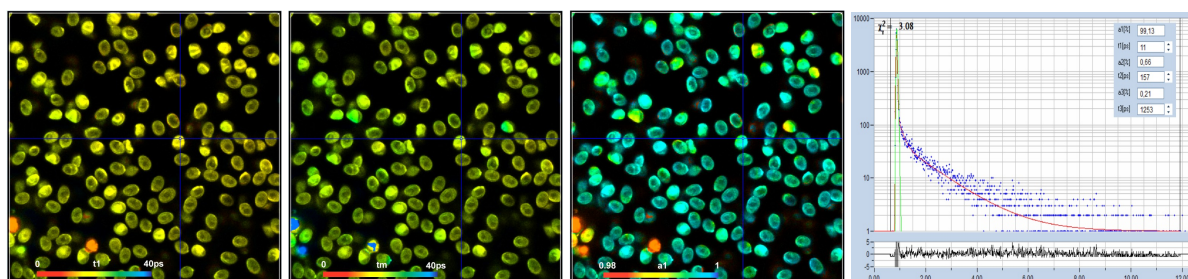


Fig. 375: 2p FLIM of Mushroom Spores of *Agaricus campestris*. The fast component has a lifetime of $t_1 = 11$ ps

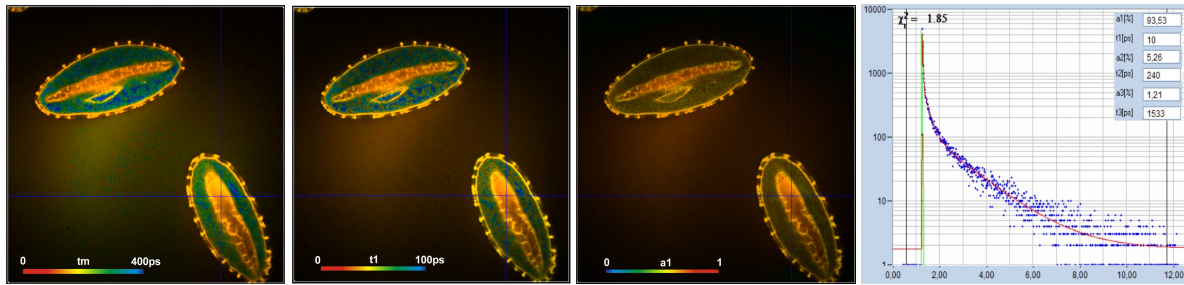


Fig. 376: 2p FLIM of Pollen Grains. The fast component has a lifetime of $t_1 = 10$ ps

More ultra-fast FLIM results of pollen and spores are described in [93, 94]. Ultra-fast FLIM of human hair is described in [96], ultra-fast FLIM of malignant melanoma in [97].

For resolving ultra-fast decay components the standard setting of the TCSPC timing parameters may be not optimal. As a rule of thumb, the time-channel width should be 10 times shorter than the decay time of the fast decay component. With component lifetimes of 10 to 20 ps a time channel with of 1 to 2 ps should be used.

Some examples are shown in Fig. 377 and Fig. 378. The easiest way of reducing the time channel width is to increase the number of time channels (the 'ADC Resolution'). With the standard settings used for 'normal' FLIM measurements this reduces the time channel width to 2.44 ps, see Fig. 377. The advantage is that no change in the TAC offset or the Sync delay is necessary.

Further reduction of the time-channel width is possible by decreasing the TAC range to 25 ns (possible in the NX modules) and/or increasing the TAC gain, see Fig. 378. For these settings you may have to change the TAC Offset or the delay in the Sync channel. For easy adjustment of the Sync delay we recommend to use a Sync Delay Box (see page 61) in the Sync channel for such high-resolution measurements. FLIM at a time-channel width of 300 femtoseconds is described in [98].

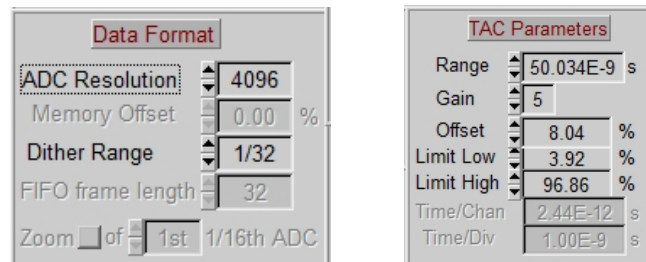


Fig. 377: Reduction of time channel width by increasing the ADC resolution. With standard TAC settings and ADC Resolution 4096 the time-channel width becomes 2.44 ps.

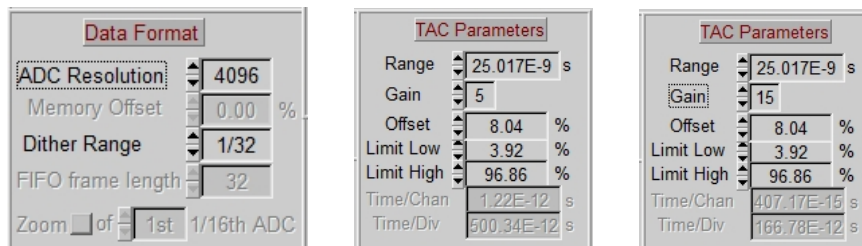


Fig. 378: Further reduction of time-channel width by decreasing the TAC Range (middle) and/or increasing the TAC gain (right). The setting on the right yields a time-channel width of 407 femtoseconds.

Tryptophan FLIM

Tryptophan is an essential amino acid. It is, together with other amino acids, a constituent of most proteins. Tryptophan is fluorescent, and its fluorescence is the dominating part of protein fluorescence. Tryptophane fluorescence is dependent on biological parameters, such as protein constitution, protein folding, and presence of other amino acids in the closer molecular environment. This dependence makes tryptophan a potential endogenous fluorescence marker for molecular parameters in biological systems.

The problem of tryptophane (TRP) fluorescence imaging is that the excitation and emission wavelengths are in the UV. The excitation maximum is around 280 nm, the emission band extends from about 300 nm up to 400 nm [360]. In a normal microscope direct excitation of tryptophan is not possible because the required excitation wavelength does not pass the objective lens and the tube lens. In a laser scanning microscope the problem is even greater because the excitation has to pass also the scanner optics.

Tryptophan can be excited by three-photon excitation in the range of 740 nm to 840 nm. However, three-photon excitation needs high power. The result is strong excitation of NADH and FAD by two-photon excitation with potentially destructive effects to the cells.

Another possibility is to excite tryptophan by two-photon (2p) excitation. The optimum excitation wavelength for tryptophan would then be in the range from 530 to 560 nm. There is no significant absorption of NADH or FAD at this wavelength. It can therefore be expected that photodamage will remain at an acceptable level. A suitable excitation wavelength can be obtained from the new 540 nm VALO Aalto laser with SHG option [599]. The laser can be added as a second laser to a DCS-120 MP system. Details are described in [77] and [101].

Fig. 379 shows a tryptophan lifetime image of live *Escherichia coli* (*E. coli*) bacteria recorded with this system. The image shown was recorded with an acquisition time of 90 seconds. The entire image contains about 10 million photons. The image was analysed with a double-exponential decay model. The decay components at the cursor positions are 62.7 % of 1573 ps and 37.3 % of 4740 ps. The amplitude-weighted lifetime is 2762 ps.

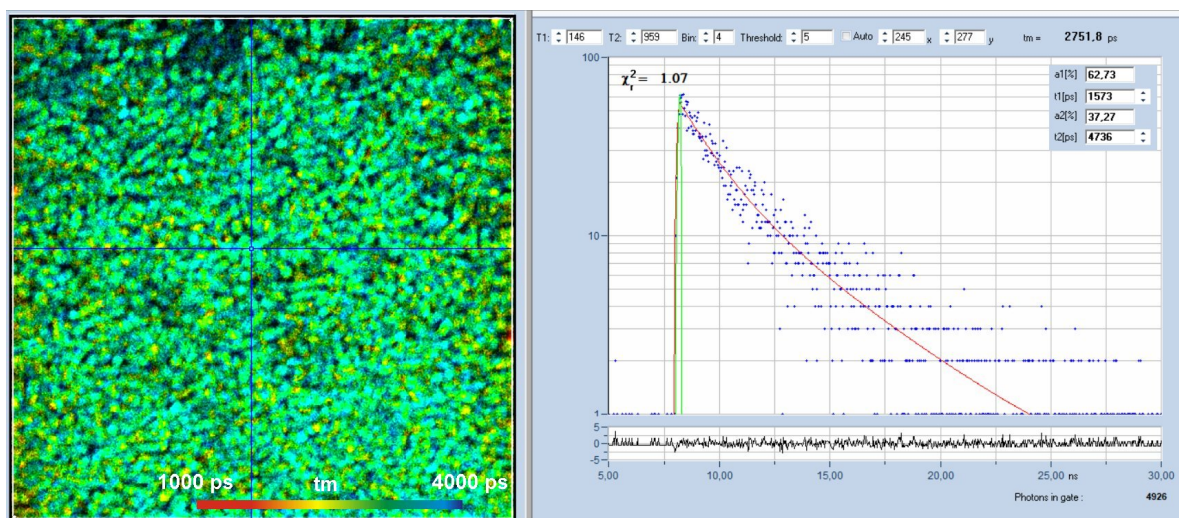


Fig. 379: Tryptophan FLIM of *E. coli* bacteria. Left: Lifetime image. Amplitude-weighted lifetime of double-exponential decay. Right: Decay function at cursor position. 512 x 512 pixels, 1024 time channels, two-photon excitation at 540 nm, lifetime range from 1000 ps to 4000 ps. Analysis by bh SPCImage NG 9.0.

Laser Safety

bh DCS-120 multiphoton systems are considered safe in terms of laser safety. Nevertheless, please obey to the usual laser safety rules. In particular, do not take out microscope lenses when the laser is on. Do not look into on open position of a microscope lens when the laser is on.

Caution is especially indicated when modifications to the system are made by the user, especially when confocal systems are upgraded with user-specific lasers and beam configuration components. In particular, make sure that laser light cannot be reflected back to the eyepieces. That means that microscopes with beamsplitters between the eyepiece and the scanner port should not be used, or the beamsplitter position be blocked. Moreover, make sure that laser light is not reflected from optical surfaces into the environment. Light reflected off filters or ND wheels at the laser inputs of the DCS-120 scan head should be blocked by a suitable beam stop. For Ti:Sa laser, we recommend to attenuate the laser power down to about 20% directly behind the laser output. Do not use an absorptive filter. It could break by the heat of the absorbed power. Better use a beamsplitter that reflects off 80 % of the power. The reflected beam should be blocked by a beam stop.

Applications

Why Use FLIM

Microscope users often consider the fluorescence lifetime just an additional parameter to separate the signals of different fluorophores. It is certainly correct that different fluorophores usually have different fluorescence lifetimes, and their signals can thus be separated by FLIM. However, reducing the fluorescence lifetime to a mere separation parameter means missing the point of FLIM. FLIM applications are not primarily aiming at separating signals of different fluorophores. Instead, FLIM is used to discriminate *different fractions of the same fluorophore* in different states of interaction with its environment. This is achieved by using a basic property of the fluorescence lifetime: The fluorescence lifetime of a fluorophore depends on its molecular environment but, within reasonable limits, not on its concentration. Molecular effects can thus be investigated independently of the unknown and usually variable fluorophore concentration [75, 105, 120, 360, 476, 535, 568].

Ion concentrations

An excited molecule can dissipate the absorbed energy by interaction with other molecules. The effect is called fluorescence quenching. The fluorescence lifetime, τ , then becomes shorter than the normally observed fluorescence lifetime, τ_0 , see Fig. 380, left. One quenching mechanism is ‘collisional’ quenching, i.e. the collision of the excited molecule with another molecule, atom, or ion. Typical quenchers are oxygen, halogens, heavy metal ions, and a variety of organic molecules. For fluorescence lifetime microscopy it is important that the rate constant of fluorescence quenching depends linearly on the concentration of the quencher. The concentration of the quencher can therefore be directly obtained from the decrease in the fluorescence lifetime. The classic example is quinine, the lifetime of which follows the reciprocal Cl^- concentration over two orders of magnitude. A practical example is shown in Fig. 380, right. A comprehensive overview about quenching by various ions can be found in [360].

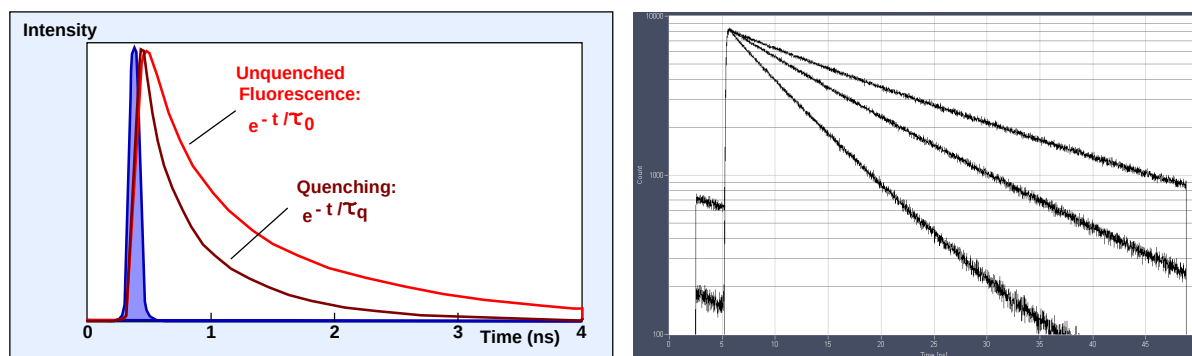


Fig. 380: Left: Fluorescence quenching. Right: Change of the fluorescence lifetime of quinine sulphate with the Cl^- concentration.

Of particular interest to cell biology are Ca^{++} and Cl^- , both of which are important to the function of the neuronal system. Applications of fluorescence-lifetime recording and FLIM to the measurement of Ca^{++} and Cl^- concentrations can be found in [76, 342, 628] and [233, 242, 252, 307], respectively.

Oxygen

Oxygen is an efficient fluorescence quencher for a large number of fluorophores [360], especially those of longer fluorescence lifetime. Strong effects are observed for pyrene, anthracene, and coronene. Unfortunately these compounds absorb and emit in the UV. They are

therefore of limited usefulness in microscopy. Oxygen sensors are therefore mainly based on phosphorescence. Frequently used oxygen sensors are mostly based on organic ruthenium, europium, platinum and palladium chelates [245, 360, 364, 434, 435] but also a few purely organic compounds are used [197]. Unfortunately the phosphorescence lifetimes are so long - from hundreds of nanoseconds to many microseconds - that they are difficult to record in combination with the high laser pulse-repetition rates and fast scan rates used in modern laser scanning microscopes. We have therefore developed a phosphorescence lifetime imaging techniques based on laser modulation and dual-time-base recording [73], see 'Simultaneous FLIM / PLIM', page 172.

Oxygen Effect on NADH and FAD fluorescence

Oxygen has a strong effect on the fluorescence of the endogenous fluorophores NADH and FAD. Chance et al. defined a 'redox ratio' that is a direct indicator of the amount of oxygen used in the mitochondria of the cells [146, 147]. Indirectly, oxygen has an influence on the ratio of bound and unbound NADH [249, 156, 159]. Effects of oxygen probably exist also for other endogenous fluorophores [510, 512]. Oxygen-induced changes of the decay profiles of endogenous fluorophores can be expected to become important with the introduction of FLIM into clinical applications.

Binding to Proteins, Protein Configuration

Many fluorescent molecules, including endogenous fluorophores, form complexes with other molecules, in particular proteins. The fluorescence spectra of these different conformations can be virtually identical, but the fluorescence lifetimes are often different. The exact mechanism of the lifetime changes is not always clear. The most likely mechanisms are fluorescence quenching and a change in the configuration of the fluorophore which, in turn, changes the rate of internal non-radiative decay. In practice, for almost all dyes the fluorescence lifetime depends more or less on the binding to proteins, or lipids [319, 360, 480]. When a fluorophore is bound to a protein also the protein configuration may have an influence on the fluorescence lifetime [104]. Fluorescence lifetimes of flavinols in plant cells recorded by TCSPC FLIM were found to be as short as 45 ps, probably due to extremely efficient quenching [419]. Kirchberg et al. [317] found changes in the decay profile of Lucifer Yellow depending on the conformation of the protein to which it was bound. A glucose sensor based on this mechanism has been described in [497]. An effect of target protein configuration has even been found for EGFP which had long been believed to be insensitive to its environment [250]. Lifetime changes of diphenylhexatriene (DPH) depending on the cholesterol concentration were used in [267]. Davey et al. found an increase of the fluorescence lifetime of dil-C18 and Alexa-488-labelled IgE in local membrane structures [172]. Garcia et al. used a fluorescently labelled ATP analog to detect actomyosin states in muscle sarcomeres [239]. An application of environment-dependent lifetime variation to the analysis of histological samples has been described in [215]. Environment-dependent changes of fluorescence parameters have even been observed for quantum dots and nano-particles [144, 167, 244, 648]. There is an enormous amount of fluorophores used in microscopy today [274, 568], and only a few of them have been tested for target-specific lifetime changes. The most promising ones are those of medium and low quantum efficiency. These are most likely to be influenced by the local molecular environment. FLIM users are encouraged to keep eyes on lifetime changes that may be suitable as probe functions, see also section 'Near-Infrared FLIM' of this handbook.

Binding to DNA and RNA

It has been shown by van Zandvoort et al. that the fluorescence lifetime of SYTO13 is different when bound to DNA or RNA [601]. The effect has been used to measure the RNA:DNA ratio in living bacteria cells and bacteria colonies [612]. Douma et al. used the environment-

dependent lifetime of SYTO13 and SYTO41 to differentiate between different cells and cell structures in blood vessels [198].

Endogenous Fluorophores, Effect of Metabolic Activity

Most endogenous fluorophores are mixtures of slightly different compounds, with almost identical spectra but different fluorescence lifetimes [512, 514, 515]. The composition of the mixture may vary, and thus induce a change in the amplitudes of the decay components and in the apparent lifetime. Moreover, the lifetimes depend on the binding to proteins, on the oxygen concentration [510], or other metabolic parameters. The most common endogenous fluorophores are NADH and FAD which both exist in a bound and in an unbound form. The lifetime differences between the forms are substantial, and bound and unbound fractions can be easily distinguished by FLIM [358, 440]. Both the fluorescence lifetimes of the decay components and the relative amplitudes have been found sensitive to the metabolic state of cells and tissue [113, 156, 158, 159, 249, 548, 549].

Local Viscosity

Local viscosity can be sensed by ‘molecular rotors’. These are fluorophores that have a high degree of internal flexibility. Rotation inside the fluorophore provides for a radiationless decay path. The non-radiative decay rate changes with the viscosity of the solvent. Viscosity measurement by FLIM with a Bodipy-based molecular rotor has been demonstrated in [286, 345, 369,]. In general, the fluorescence lifetime of any fluorophore of high molecular flexibility can be expected to depend on the viscosity of the environment. Lifetime variation of dialkyl-indocarbocyanines due to micro-viscosity changes in cell membranes has been used in [577].

Influence of Local Refractive Index

The radiative decay rate of a fluorophore depends on the local refractive index [559, 566, 567]. The refractive index varies within biological cells and tissue. The lifetime changes induced by these variations can be used to gain structural information about biological specimens and to study membrane effects, e.g. the transition of a bacterium or a virus through a cell membrane [591, 592].

Proximity to metal surfaces, binding to nanoparticles

Extremely strong effects on the decay rates must also be expected if dye molecules are bound to metal surfaces, especially to metallic nano-particles [137, 240, 385, 459, 474]. Proximity to metal surfaces changes both the radiative and non-radiative decay rates. It can happen that the radiative rate increases dramatically. The result is a decrease in the fluorescence lifetime without a decrease (or even with an increase) in intensity, accompanied by an increase in photostability. Similar, yet less dramatic effects have been reported also for non-metallic nanoparticles. They are interesting when such particles are used as carriers of drug delivery. TCSPC measurements on Cy3 encapsulated in calcium phosphate nanoparticles have shown reduced non-radiative and increase radiative decay rates, resulting in a 20 fold increase in brightness [414, 417]. The use of cyanine-loaded lipid nanoparticles was demonstrated in [582], and improved fluorescence efficiency was verified by TCSPC. Changes of the fluorescence lifetime depending on the substrate a protein construct is resting on were exploited in [288].

Solvent Polarity

The molecular conformation of a fluorophore and thus its fluorescence behaviour is also influenced by the solvent properties, especially the solvent polarity [106, 360]. Therefore, the fluorescence lifetimes of many fluorophores depend more or less on the solvent polarity

[266]. Moreover, when a molecule enters the excited state the solvent molecules around it rearrange. Consequently, energy is transferred to the solvent, with the result that the emission spectrum is red-shifted. Solvent (or spectral) relaxation in water happens on the time scale of a few ps. However, the relaxation times in viscous solvents and in dye-protein constructs can be of the same order as the fluorescence lifetime. The effects can be measured by TCSPC [457]; applications of spectral relaxation to cell imaging have not been reported yet.

pH Sensors

Many fluorescent molecules have a protonated and a deprotonated form. The equilibrium between both depends on the pH. If the protonated and deprotonated form have different lifetimes the apparent lifetime is an indicator of the pH. A typical representative of the pH-sensitive dyes is 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) [10, 270, 274, 360, 494]. An example is shown in the application part of this chapter. Medintz et al. presented a pH sensor based on a quantum-dot/dopamine conjugate [396]. They determined the mechanism of the lifetime change to be electron transfer within the conjugate. Kundu et al. used 2,2'-bipyridine-3,3'-diol as a pH sensor [347].

Ion Concentration

There are fluorescence sensors for various ions. The sensors either undergo collisional quenching by the ions, or perform conformational changes which lead to a change in the fluorescence quantum efficiency. Most of the changes are accompanied by a corresponding change in the fluorescence lifetime. Please see [120, 360, 568] for an overview. The use of Ca^{2+} and Cl^- sensors is described in section 'Measurement of Molecular Environment Parameters', page 227.

Redox Potential

There are fluorophores that change their fluorescence behaviour depending on the redox potential of the local environment. Sensors based on GFP variants are described in [271]. An improved redox sensor based on a modified GFP (roGFPiE) was developed by Avezov et al. Test results obtained in various cells by TCSPC FLIM are described in [16].

Membrane Potential

Cells have concentration gradients of ions across their membranes. These gradients produce a voltage over the membrane which is characteristic of a number of fundamental cellular processes. Membrane potentials can be measured by FLIM with a voltage-sensitive dye. An application is described in [95].

Aggregation of Fluorophores

The radiative and non-radiative decay rates depend also on possible aggregation of the dye molecules. The electron systems of the individual molecules in aggregates are strongly coupled. Therefore the fluorescence behaviour of aggregates differs from that of the free molecules. Aggregation is influenced by the local environment; the associated lifetime changes can be used as a probe function. Lifetime heterogeneity due to aggregation was reported for E-Bodipy fluorophores, see [182]. Aggregation has also been used to observe the internalisation of dyes into cells [312]. Lifetime effects may also occur when dye-labelled proteins aggregate. This has been used to observe HIV-1 internalisation into cells [261], and to observe oligomerisation of labelled proteins [272].

Förster Resonance Energy Transfer: FRET

FRET was first theoretically explained by Theodor Förster in 1946 [225, 226]. The effect is also called fluorescence resonance energy transfer or simply resonance energy transfer (RET).

Fluorescence resonance energy transfer is an interaction of two molecules in which the emission band of one molecule overlaps the absorption band of the other. In this case the energy from the first molecule, the donor, can transfer into the second one, the acceptor. FRET can result in an extremely efficient quenching of the donor fluorescence and, consequently, in a considerable decrease of the donor lifetime. The energy transfer rate from the donor to the acceptor decreases with the sixth power of the distance. Therefore it is noticeable only at distances shorter than 10 nm [360]. FRET is used as a tool to investigate protein-protein interaction. Different proteins are labelled with the donor and the acceptor, and FRET is used as an indicator of the binding between these proteins. FRET is the most frequent FLIM application. The number of FLIM-based FRET papers has literally exploded in the last couple of years. Please see 'Fluorescence Resonance Energy Transfer (FRET)', page 233.

Electron Transfer

At distances smaller than about 1 nm between an (electron) donor and acceptor photo-induced electron transfer can occur [227, 360]. The excited molecule can either be the electron donor or the electron acceptor. The direction of the electron transfer is determined by the oxidation potential of the donor and the reduction potential of the acceptor. Electron transfer delivers two radicals, both in the ground (S_0) state. Both excited singlet states and triplet states can undergo electron transfer at high rates. In solution, the effective transfer rate is usually limited by diffusion. However, in ordered systems (e.g. complexes or adsorbates) the electron transfer rate may become extremely high. Electron transfer is a basic step in photosynthesis. It is also important to the development of organic photovoltaic devices and artificial photosynthesis systems [555]. In biological imaging photo-induced electron transfer, and the radicals produced by it, are a source of photobleaching and photodamage. Electron transfer is a possible explanation of the fact that some fluorophores are highly photostable in solution, but rapidly photobleach when attached to proteins. It also explains why unstained cells survive high exposure to light, while the same cells stained with exogenous fluorophores may not. Although electron transfer may be the basic mechanism of a number of quenching effects it is not explicitly used as a probing function.

Second-Harmonic Generation

Second-harmonic generation (SHG) is an ultra-fast nonlinear process that converts two photons of the incident light into one emitted photon. The wavelength of the emitted photon is exactly one half of the wavelength of the excitation photons. Second-Harmonic generation has nothing to do with fluorescence but often shows up as an ultrafast component in decay curves measured in multiphoton microscopes. Second harmonic components in FLIM data give additional information about the constitution of the tissue under investigation [390]. SHG emission requires some global organisation of the molecules. In biological system it mainly comes from connective tissue, especially from collagen in animal tissue and from cellulose and starch in plant tissue. The ratio of SHG and autofluorescence intensity in mammalian skin is an indicator of skin ageing [321]. SHG may also become important to localise nanoparticles in human skin.

Measurement of Molecular Environment Parameters

pH Imaging

Microscopic pH imaging can be achieved by staining the sample with a pH-sensitive fluorescent probe. These probes usually have a protonated and a deprotonated form. There is an equilibrium between both forms that depends on the pH of the local environment. If both forms have different fluorescence lifetimes the average lifetime is a direct indicator of the pH [360]. A typical representative of the pH-sensitive dyes is 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) [274]. In aqueous solution the lifetimes of the protonated form and the deprotonated form have been found 2.75 ns and 3.90 ns, respectively [270]. In the pH range from 4.5 to 8.5 both forms exist, and the fluorescence decay function is a mixture of both decay components. Thus, the lifetime of a single-exponential fit can be used as an indicator of the pH. An example of pH imaging of skin tissue is shown in Fig. 381. A single-exponential lifetime image is shown left, decay curves from different areas of the image are shown right. In areas of low pH fluorescence components of both protonated and deprotonated BCECF are found. The decay profile is therefore double-exponential. In areas of high pH essentially deprotonated BCECF is found, and the decay profile is single-exponential. It should be noted that the lifetimes of free BCECF differ from the lifetimes of BCECF bound in the tissue. The lifetime-pH relation must therefore be calibrated for bound BCECF.

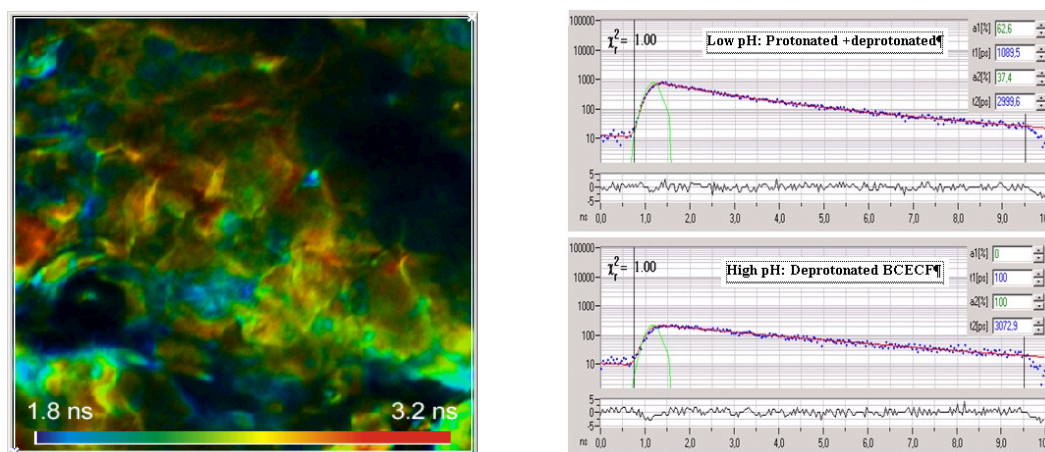


Fig. 381: Left: Lifetime image of skin tissue stained with BCECF. The lifetime is an indicator of the pH. Right: Fluorescence decay curves in an area of low pH (top) and high pH (bottom). LSM 510 NLO with SPC-830 FLIM module, courtesy of Thea Mauro, San Francisco

Calcium Imaging

Ca^{2+} ions are involved in a large number of cell functions, such as intracellular transport, membrane potential, muscle contraction, gene expression, and cell differentiation. There is a wide variety of Ca^{2+} sensors [359, 360, 408]. Most likely, the mechanism of the Ca^{2+} -dependent lifetime change is that the fluorophore has a Ca-bound and a Ca-unbound form of different fluorescence quantum efficiency and thus different fluorescence lifetime. The fluorescence lifetime of the bound form is higher than that of the unbound form. Consequently, the net fluorescence lifetime depends on the Ca^{2+} concentration. It can, however, happen that the fluorescence quantum efficiency of the unbound form is so low that the corresponding lifetime component is no longer observed. In that case, an intensity change but no lifetime change is observed [360]. This is the case for the Fluo sensors, as has been shown for Fluo-4 [76, 229]. However, the traditional Ca^{2+} dyes, such as calcium green and Oregon green, dis-

play large lifetime changes and work beautifully for lifetime-based Ca^{2+} measurement. An examples is shown in Fig. 382.

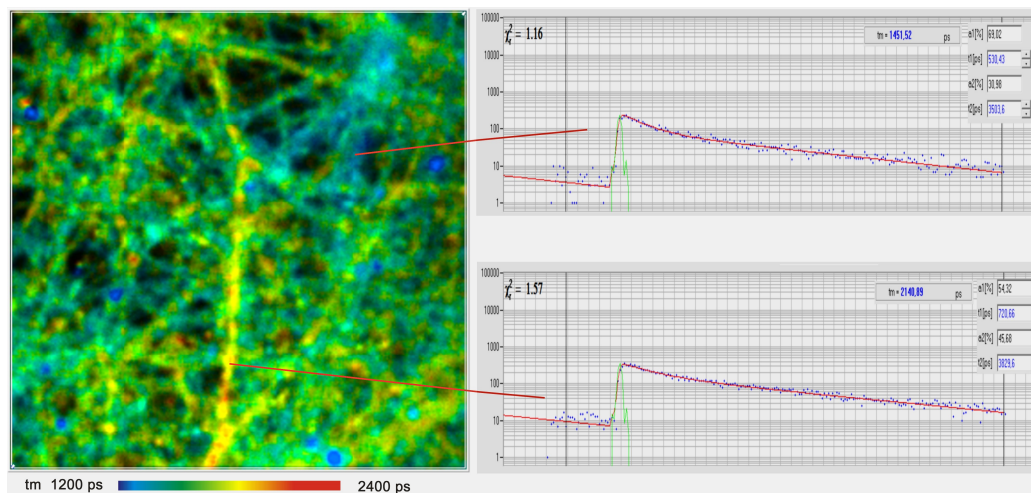


Fig. 382: FLIM image of cultured neurons stained with Oregon green OGB-1 AM. Colour range from $\tau_m = 1200$ ps (blue) to 2400 ps (red). Decay curves of regions with low Ca (top) and high Ca (bottom) shown on the right. Data courtesy of Inna Slutsky and Samuel Frere, Tel Aviv University, Sackler School of Medicine.

The advantage of FLIM over intensity-based Ca^{2+} imaging is that absolute values of the Ca^{2+} concentration are obtained. This has been used to quantify calcium concentrations in astrocytes of live mice with cortical plaques by multiphoton NDD FLIM [342].

Recording Ca^{++} Transients in Live Neurons

The Ca^{2+} concentration in cells can change within remarkably short periods of time. Recording these ' Ca^{2+} transients' requires a time resolution in the range of less than 50 ms. It is thus usually considered impossible to record Ca^{2+} transients by fluorescence lifetime detection. However, Ca^{2+} transients can easily be recorded by temporal Mosaic FLIM (see page 151) or FLITS (see page 164).

The application of FLITS to the recording of Ca^{2+} transients in live neurons on electrical stimulation has been described in [76] and [229]. A typical result is shown in Fig. 383. Hippocampal cultures were prepared from newborn rats and kept under physiological conditions for 12 to 18 days. The cultures were then loaded with OGB-1 AM, see [229] for details. A Zeiss LSM 7 MP multiphoton microscope with a normal Simple-Tau 150 FLIM system was used to run the FLITS experiments. The cells were stimulated periodically at a fraction of the line clock frequency. To run the experiments, an intensity image was taken by the LSM 7 MP, and an appropriate location for the line scan selected. Then the LSM 7 MP was switched into the line scanning mode. The data acquisition in the FLIM system, the scanning in the LSM 7 MP, and the stimulation were started. The stimulation pulses of 1 ms duration were applied to the cell culture in intervals of 3 seconds [229]. Data acquisition was continued over about 300 seconds, i.e. photons from about 100 stimulation periods were accumulated. The result is shown in Fig. 383, left.

To verify that the FLITS experiment did not cause cell damage or photobleaching a FLIM image was recorded after the FLITS experiment. It is shown in Fig. 383, right. It does not show any cell damage or photobleaching effects along the scanned line. It also shows that the Ca^{2+} concentration returned to the resting level, compare bottom of FLITS image on the left.

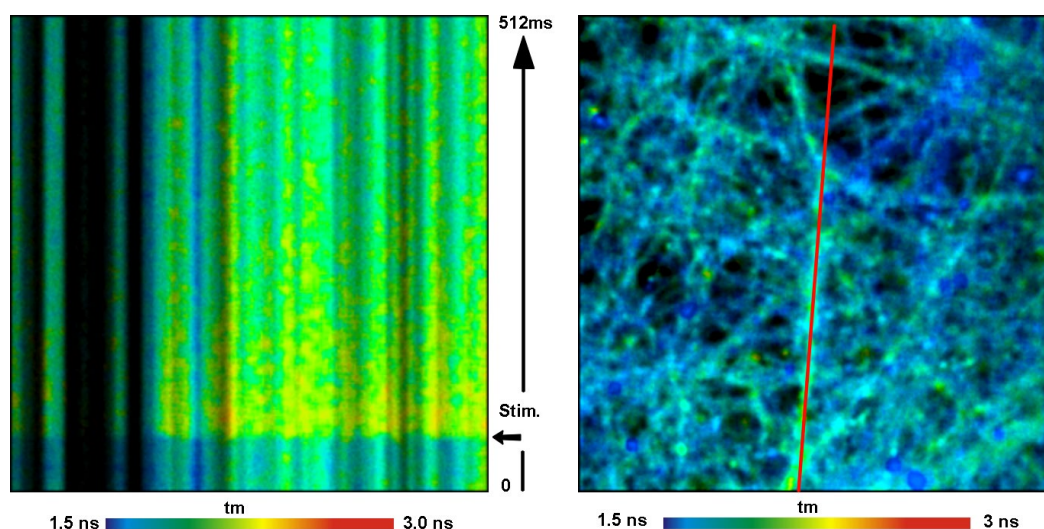


Fig. 383: FLITS of Ca^{2+} transients in live neurons. Left: FLITS image. Right: FLIM image taken after the FLITS recording. Red lines indicates position of FLITS scan. Data courtesy of Inna Slutsky and Samuel Frere, Tel Aviv University, Sackler School of Medicine.

Ca^{2+} recording by temporal mosaic FLIM is shown in Fig. 384. OGB-1 AM was used as a Calcium sensor. The sample was stimulated electrically, and 100 stimulation cycles were accumulated. A Zeiss LSM 7 MP was used for the experiment. With 64×64 pixels and a zoom factor of 5, the LSM 7 MP reaches a frame time of 38 ms. 150 milliseconds before every stimulation a recording through the entire 64-element mosaic was started. With the frame time of 38 ms, the acquisition thus runs through the entire mosaic in 2.43 seconds. The result shows clearly the increase in the fluorescence lifetime of the Ca^{2+} sensor in the mosaic elements 4 to 6, and a return to the resting state over the next 10 to 15 mosaic elements (380 to 570 ms).

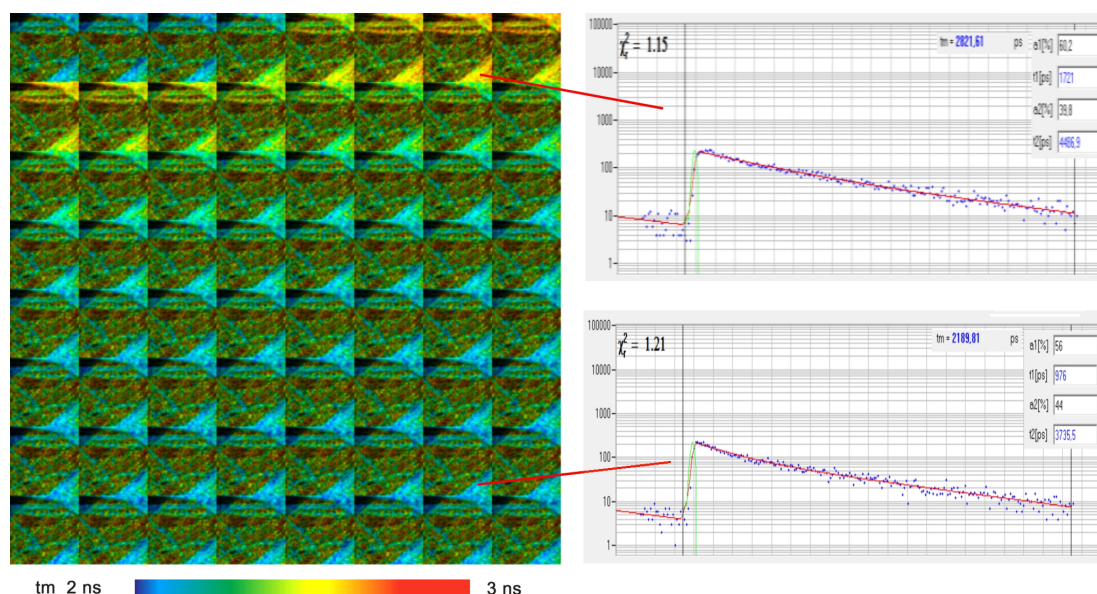


Fig. 384: Temporal mosaic FLIM of the Ca^{2+} transient in cultured neurons after stimulation with an electrical signal. The time per mosaic element is 38 milliseconds, the entire mosaic covers 2.43 seconds. Experiment time runs from upper left to lower right. Photons were accumulated over 100 stimulation periods. Recorded by Zeiss LSM 7 MP and bh SPC-150 TCSPC module. Data courtesy of Inna Slutsky and Samuel Frere, Tel Aviv University, Sackler Faculty of Medicine.

Chloride Imaging

The chloride concentration is important to the function of the neuronal system. An overview on Cl^- imaging is available in [120] and [242]. Measurements of the concentration of intracellular Cl^- in olfactory sensory neurons by TCSPC FLIM were described in [307]. MQAE was used as a fluorescent probe. MQAE is quenched by Cl^- , and the concentration can be calculated from the lifetime change via the Stern-Volmer equation. A bh SPC-730 module was used in conjunction with 2-photon excitation. Because 2-photon excitation does not cause photobleaching and photodamage outside the focal plane the authors were able to obtain z-stacks of the Cl^- concentration in dendrites over depth intervals up to 150 μm . Fig. 385 shows a spinal ganglion of a mouse stained with MQAE. Short lifetimes indicate high Cl^- concentration and vice versa.

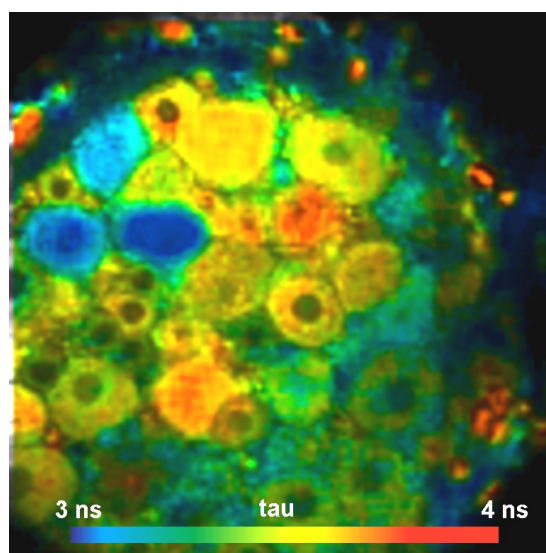


Fig. 385: Spinal ganglion of a mouse stained with MQAE. Short lifetime indicates high Cl^- concentration. Data courtesy of Thomas Gensch, Jülich Research Centre, Germany

The same technique was applied to somatosensory neurons in dorsal root ganglia of newborn mice [252]. It could be shown that the somatosensory neurons undergo a transition of Cl^- homeostasis during the first three postnatal weeks. The modulation of chloride homeostasis by inflammatory mediators was investigated in [233]. The influence of the Cl^- concentration (and of the pH) on the lifetime of GFP was studied in [13].

Local Viscosity

Local viscosity has a large influence on the rates of biochemical reactions in a cell. Changes in viscosity on the sub-cellular level have been shown to accompany a wide range of cell malfunctions. Changes in viscosity have been found for hematologic disorders, diabetes, Alzheimer disease, liver malfunction and cancer [540].

Viscosity on the molecular scale can be determined by ‘molecular rotors’. These are fluorophores which have two more or less rigid parts connected by a single bond. The two parts can rotate one against the other. Rotation is an additional relaxation pathway which competes with radiative relaxation. Ability of internal rotation therefore shortens the fluorescence lifetime. When the rotation is hindered by high viscosity of the molecular environment this relaxation pathway becomes less efficient, and the fluorescence lifetime increases. An example of viscosity measurement by a Bodipy-based sensor is shown in Fig. 386.

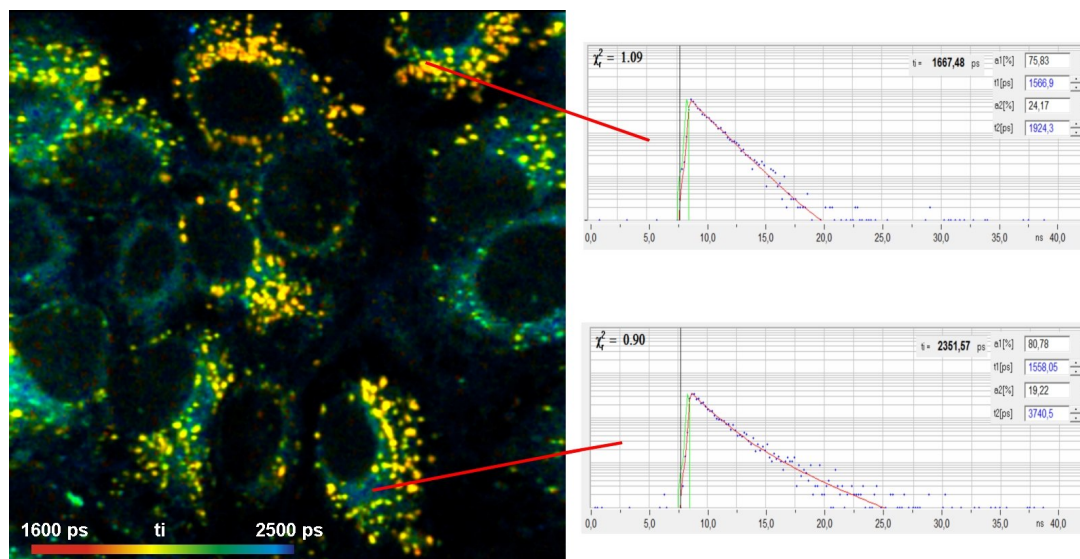


Fig. 386: Viscosity measurement by a Bodipy-based viscosity sensor. Long lifetime means high viscosity, and vice versa. Data courtesy of Marina Kuimova, Imperial College London.

There is an increasing number of publications about viscosity sensors, viscosity measurements in cells and tissues, and applications. Vysniauskas et al. used a molecular rotor to obtain spatially resolved viscosity maps of lipid photooxidation in giant unilamellar vesicles [608]. Sherin et al. obtained microviscosity maps of the membranes of porcine eye lens cells [538]. Mika et al. measured the viscosity of plasma membranes of *Escherichia coli* [404]. The results showed a high degree of lipid ordering within their liquid-phase plasma membranes. Ku-bánková et al. studied the assembly of proteins, including amyloid- β peptide from their mon-omeric forms into highly organised fibrillar states [340]. Shimolina et al. used viscosity-FLIM both on the single-cell level and in the connecting tissues of subcutaneous tumors in mice. They found viscosity imaging a potential tool of the progress of tumor treatment [540], and give a summary of possible applications.

Ponjavic et al. studied the effect of pressure on the molecular viscosity of a solvent. They took measurements of a thin liquid film between a glass substrate and a small ball pressed against it. The observed a dependence of the viscosity on the pressure and spatial heterogeneity within the fluid film [454].

The molecular rotors themselves and their reaction to viscosity, temperature, and other molecular parameters are subject of studies by A. Vyšniauskas et al. [609, 610]. Imaging of micro-bubbles and aerosol particles and their viscosity change upon hydration and chemical aging is described in [286, 287].

Other Environment Parameters and Conditions

FLIM of cellular Na^+ with CoroNaGreen has been demonstrated by Meyer et al. [400]. The authors found differences in the Na^+ concentration in the cytosol, the nucleus, and in the perinuclear region. Saxl et al. presented a glucose sensor based on Baudan bound to a glucose/galaktose-binding protein (GBP) [497]. It was concluded that Baudan fluoresces with short lifetime for the open form of the protein and with long lifetime for the closed form of the protein. The protein configuration changes at binding to glucose, and so does the average lifetime. Kirchberger et al. [317] found changes in the decay profile of Lucifer Yellow depending on the conformation of the protein to which it was bound. Levitt et al. [370] used the fluorescence lifetime of Nile Red for measurements of the intracellular polarity.

It has been shown by van Zandvoort et al. that the fluorescence lifetime of SYTO13 is different when bound to DNA or RNA [601]. The effect has been used to measure the RNA:DNA ratio in living bacteria cells and bacteria colonies [612]. Douma et al. used the environment-dependent lifetime of SYTO13 and SYTO41 to differentiate between different cells and cell structures in blood vessels [198]. Davey et al. found an increase of the fluorescence lifetime of dil-C18 and Alexa-488-labelled IgE in local membrane structures [172]. Garcia et al. used a fluorescently labelled ATP analog to detect actomyosin states in muscle sarcomeres [239]. An application of environment-dependent lifetime variation to the analysis of histological samples has been described in [166] and [215]. Imaging of other cell conditions, such as oxidative stress, GTP-ATP interactions, or self-organisation of porphyrines are described in [168, 178, 521].

Kuchlyan et al [344] describe an Hg^{2+} sensor based on a rhodamine-spirolactam derivate. Normally, Hg is an efficient fluorescence quencher. However, when Hg^{2+} binds to the rhodamine-based spirolactam the fluorescence intensity increases significantly, and the fluorescence lifetime increases. The authors recorded FCS and FLIM with a DCS-120 confocal scanning system to verify that the change is indeed an effect of binding to Hg^{2+} .

The exact mechanisms of the lifetime changes are not always known. The Cl^- sensitivity of MQAE is almost certainly due to classic collisional quenching, as it is known for quinine sulphate [360], see Fig. 380. In other cases the reason may be a change in the internal flexibility of the molecule, and a corresponding change in the non-radiative decay rate. This mechanism is known for ‘molecular rotors’, such as Bodipy, which respond to the viscosity of the solvent [345], see page 230. Also the polarity of the environment has an influence on the conformation of the fluorophore molecule and thus on its fluorescence lifetime [106, 105]. For dyes forming aggregates, such as ICG, the environment influences the degree of aggregation. This, in turn, changes the fluorescence lifetime. Whatever the lifetime changes are caused by, environment-dependent lifetime changes occur for a large number of fluorophores. Consequently, there is a large potential in finding lifetime-based reporters for biological environment parameters among commonly known fluorophores.

Fluorescence Resonance Energy Transfer (FRET)

FRET is an interaction of two fluorophore molecules with the emission band of one dye overlapping the absorption band of the other. In this case the energy from the first dye, the donor, can be transferred to the second one, the acceptor. The energy transfer itself does not involve any light emission and absorption [225, 226, 360]. Förster resonance energy transfer (FRET), or resonance energy transfer (RET), are synonyms of the same effect. The energy transfer rate from the donor to the acceptor decreases with the sixth power of the distance. Therefore it is noticeable only at distances shorter than 10 nm [360]. FRET results in an extremely efficient quenching of the donor fluorescence and, consequently, decrease of the donor lifetime.

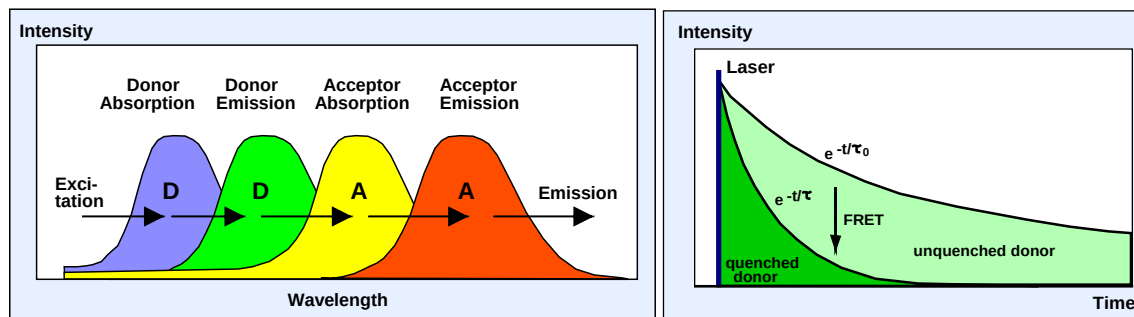


Fig. 387: Fluorescence resonance energy transfer

Because of its dependence on the distance FRET has become an important tool of cell biology [445, 447, 572]. Different proteins are labelled with the donor and the acceptor; FRET is then used to verify whether the proteins are physically linked and to determine distances on the nm scale.

The problem of steady-state FRET techniques is that the concentration of the donor and the acceptor varies throughout the sample. The techniques therefore depend on ratios of the donor and acceptor intensities. However, the FRET-excited acceptor intensity is not directly available. There is ‘donor bleedthrough’ due to the overlap of the donor fluorescence into the acceptor emission band. Moreover, some of the acceptor molecules are excited directly. Steady-state FRET techniques therefore require careful calibration, including measurements of samples containing only the donor and only the acceptor [256, 280].

The calibration problems can partially be solved by the acceptor photobleaching technique. An image of the donor is taken, then the acceptor is destroyed by photobleaching, and another donor image is taken. The increase of the donor intensity is an indicator of FRET. The drawback is that this technique is destructive, and that it is difficult to use in live cells. In fixed cells, however, the protein structure is changed already by the fixation so that the results are not necessarily correct.

All steady-state techniques have the problem that there is usually a mixture of interacting and non-interacting proteins. Both the fraction of interacting proteins and the distance between the proteins influence the FRET efficiency derived. It therefore cannot be told whether a variation in the FRET efficiency is due to a variation in the distance or a variation in the fraction of interacting proteins.

Single-Exponential FLIM FRET

The use of FLIM for FRET has the obvious benefit that the FRET intensity is obtained from a single lifetime image of the donor [17, 60, 64, 150, 202, 449, 485]. Donor bleedthrough and directly excited acceptor fluorescence therefore have no influence of FLIM-FRET measurements. The only reference value needed is the donor lifetime in absence of the acceptor. It

will be shown later that even this reference lifetime can be obtained from the FLIM-FRET data themselves.

Fig. 388 shows a single-exponential lifetime image of a cultured HEK (human embryonic kidney) cell expressing two interacting proteins labelled with CFP and YFP. The excitation wavelength was 405nm. The fluorescence was recorded through a 480 ± 20 nm filter. It can clearly be seen that the CFP lifetime is reduced in the membrane of the cell and in the Golgi apparatus.

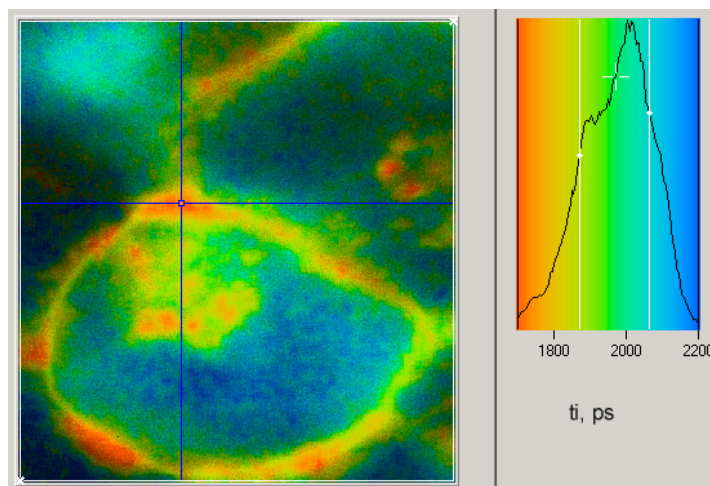


Fig. 388: HEK cell expressing two interacting proteins labelled with CFP (donor) and YFP (acceptor). Single-exponential lifetime image of the donor fluorescence, blue to red corresponds to a lifetime range of 1.7 to 2.2 ns. A lifetime distribution over the pixels is shown on the right. Cells courtesy of Burkhard Wiesner, Leibniz Institute of Molecular Pharmacology (FMP), Berlin, Germany

Single-exponential lifetime images as the one shown in Fig. 388 are very useful to locate the areas in a cell where the labelled proteins interact. It has been shown that for a given efficiency of the optical system and detector and a given excitation power FLIM-based FRET measurements give better accuracy than steady-state techniques [443]. Single-exponential decay measurements do, however, not solve the general problem of the FRET techniques that the total decrease of the donor fluorescence intensity or fluorescence lifetime depends both on the distance of donor and acceptor and the fraction of interacting donor molecules. In the simplest case, a fraction of the donor molecules may not be linked to their targets, or not all of the acceptor targets may be labelled with an acceptor. This can happen especially in specimens with conventional antibody labelling [357]. But even if the labelling is complete by far not all of the labelled proteins in a cell are interacting, and the fraction of interacting protein pairs varies throughout the cell.

Double-exponential FLIM FRET

TCSPC FLIM solves the problem of interacting and non-interacting donor by double-exponential lifetime analysis. The resulting donor decay functions can be approximated by a double exponential model, with a slow lifetime component from the non-interacting (unquenched) and a fast component from the interacting (quenched) donor molecules. If the labelling is complete, as it can be expected if the cell is expressing fusion proteins of the GFP variants, the decay components directly represent the fractions of interacting and non-interacting donor molecules. The composition of the donor decay function is illustrated in Fig. 389.

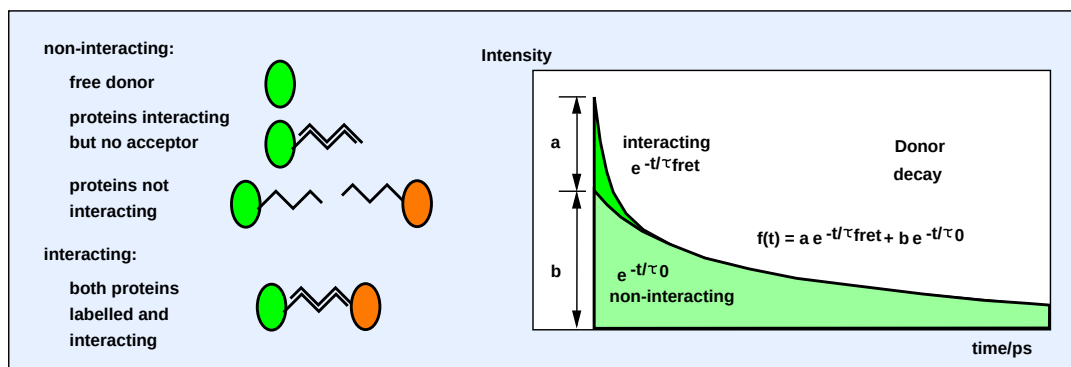


Fig. 389: Fluorescence decay components in FRET systems

Double exponential decay analysis delivers the lifetimes, τ_0 and τ_{fret} , and the intensity factors (amplitudes), a and b , of the two decay components. From these parameters can be derived the true FRET efficiency, E_{fret} , the ratio of the distance and the Förster radius, r/r_0 , and the ratio of the number of interacting and non-interacting donor molecules, N_{fret}/N_0 :

$$E_{\text{fret}} = 1 - \tau_{\text{fret}} / \tau_0$$

$$(r/r_0)^6 = \tau_{\text{fret}} / (\tau_0 - \tau_{\text{fret}}) \quad \text{or} \quad (r/r_0)^6 = \frac{1}{E_{\text{fret}}} - 1$$

$$N_{\text{fret}} / N_0 = a / b$$

Fig. 390 shows the fluorescence decay curves in a selected spot (array of 4 adjacent pixels, selected by the blue crosshair) of Fig. 388.

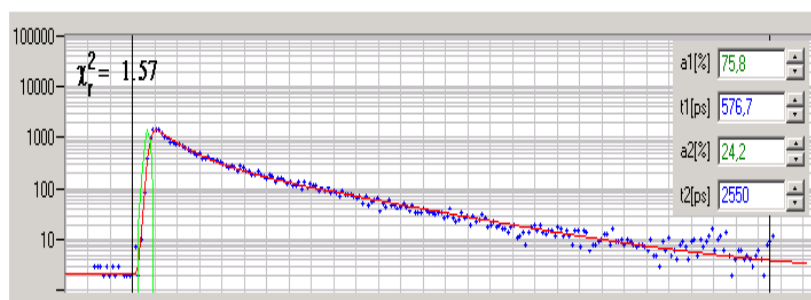


Fig. 390: Fluorescence decay curve in a selected spot of Fig. 388. The decay profile is clearly double-exponential.

The fluorescence decay is indeed double-exponential, with a fast lifetime component, τ_{fret} , of 590 ps, and a slow lifetime component, τ_0 , of 2.41 ns.

Fig. 391 shows the result of a double-exponential analysis of the data. The left image shows the ratio of the lifetimes of the non-interacting and interacting donor fractions, $\tau_0 / \tau_{\text{fret}}$. The distribution of $\tau_0 / \tau_{\text{fret}}$ in different regions is shown far left. The locations of the maxima differ by only 10%, corresponding to a distance variation of about 2%. However, the variation in the intensity coefficients, a/b , is about 10:1.

The results show clearly that the variation in the single-exponential lifetime (Fig. 388) is almost entirely caused by a variation in the fraction of interacting proteins, *not* by a change in distance. In other words, interpreting variations in the single-exponential lifetime (or classic FRET efficiencies from steady-state experiments!) as distance variations leads to wrong results.

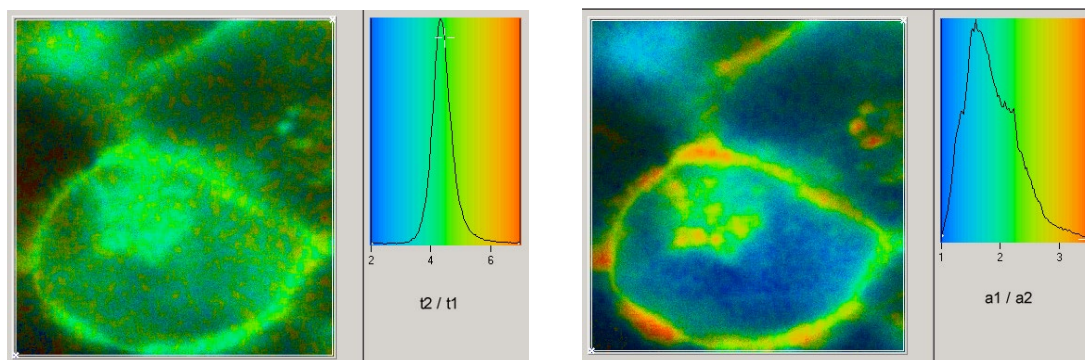


Fig. 391: FRET results obtained by double-exponential lifetime analysis. Left: Ratio of the lifetime components, $t_2/t_1 = \tau_0/\tau_{\text{fret}}$, Right: Ratio of the interacting and non-interacting donor fractions, $a_1/a_2 = N_{\text{fret}}/N_0$. Cells courtesy of Burkhard Wiesner, Leibniz Institute of Molecular Pharmacology (FMP), Berlin, Germany

Similar double exponential decay behaviour is commonly found in FRET experiments based on multi-dimensional TCSPC [17, 60, 62, 64, 116, 138, 202, 216, 449]. Double-exponential decay profiles have also been confirmed by streak-camera measurements [114, 115]. For many years, the double-exponential decay was discussed controversially. The discussion was enhanced by double-exponential decay behaviour found for some FRET donors themselves. Now there is no doubt any more that FRET induces a double-exponential donor decay, and that the components represent interacting and non-interacting donor fractions.

The fact that the slow decay component comes from the non-interacting donor can be exploited for FRET analysis: The lifetime of the slow component is the reference lifetime for the FRET calculation. The advantage is that this lifetime comes from the same cell and from the same pixels, and that it was measured simultaneously with the lifetime of the interacting donor. Possible variations by variable local environment have therefore no influence on the FRET result. Moreover, it can be expected that the slow lifetime does not vary noticeably throughout the cell. The slow lifetime can therefore often be used as a global parameter in the fit process [23].

Although the double-exponential approach yields good results it should be noted that FRET experiments can be more complicated than described above. There is the problem that a protein can be expressing (or be labelled with) several donor or acceptor molecules. The effect of multi-labelling is energy migration between the donors, and an increase in the energy transfer rate due to the presence of several acceptors. For physical background and possible pitfalls please see [337, 338, 605].

It should also be mentioned here that double-exponential FRET measurement faces the problem that the fluorescence decay of the unquenched donor itself is often multi-exponential. This happens especially for the CFPs and its variant, Cerulean. Strong multi-exponential behaviour can also be induced by fixation procedures (see Fig. 398) and by excessive photobleaching [281].

The situation can be complicated if the donor fluorescence is contaminated by autofluorescence [301]. The decay profiles of autofluorescence are multi-exponential, and can look very similar to the decay of the donor in a FRET system, see Fig. 399. Negative tests with unlabelled cells and with donor-only cells are therefore mandatory.

FRET results can also be biased by photobleaching or photoconversion of the fluorophores [281]. Even photo-induced changes in the protein conformation cannot entirely be excluded, see Fig. 400. In the last years, photo-induced artefacts have been reduced by better efficiency of the detectors, better efficiency of the microscope optics, and by new fluorescent proteins with higher photostability [174, 175].

FLIM-FRET experiments need not necessarily be performed in single cells. The same data analysis principles can, of course, be applied to fluorescence decay data obtained from protein or cell suspensions in cuvettes or micro-cuvettes [126, 264, 290]. The advantage of these measurements is that photobleaching is not a problem, the disadvantage is that only average FRET parameters over the whole volume of the cells and over a large number of cells are obtained. Usually, not all cells express the acceptor. Consequently, the result can be biased towards lower FRET efficiency, and a lower fraction of interacting proteins.

Using Information from the Acceptor Decay Function

There is continuous discussion about the shape of the acceptor decay function, whether it can be used to improve the accuracy of FRET measurements, and whether FRET results can be derived from an acceptor lifetime image. Fig. 392 shows how the acceptor decay function in an ideal FRET experiment should look like. Acceptor excitation occurs via the S1 state of the interacting donor molecules. The number of interacting donor molecules in the S1 state is represented by the fast donor fluorescence component. Consequently, this component acts as an excitation pulse to the acceptor. The acceptor fluorescence decay function should therefore have a slow rising edge with a time constant equal to the decay time of the interacting donor fluorescence.

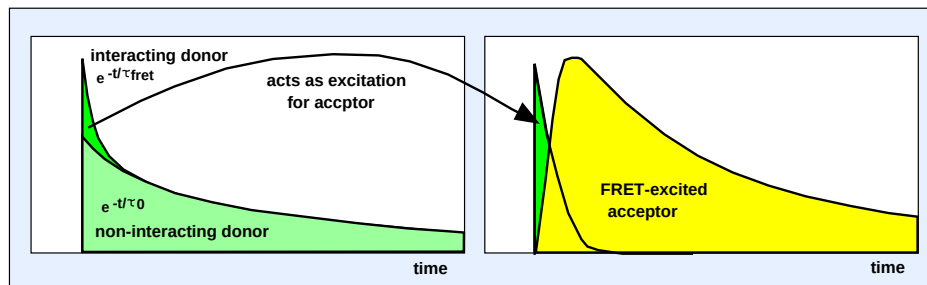


Fig. 392: Donor and acceptor decay functions expected in an ideal FRET experiment: The excitation of the acceptor comes from the interacting donor

However, nothing like this is observed in practical FLIM FRET experiments. Fig. 393 shows a lifetime image of the acceptor of the HEK cell shown in Fig. 388. The mean lifetime is between 2.3 and 2.6 ns. The fluorescence decay from the region of strong FRET has lifetime components of 2.2 ns and 3.2 ns. The shape is close to a single-exponential decay. There is neither a trace of a slow rise, nor is there a decay component with a negative amplitude.

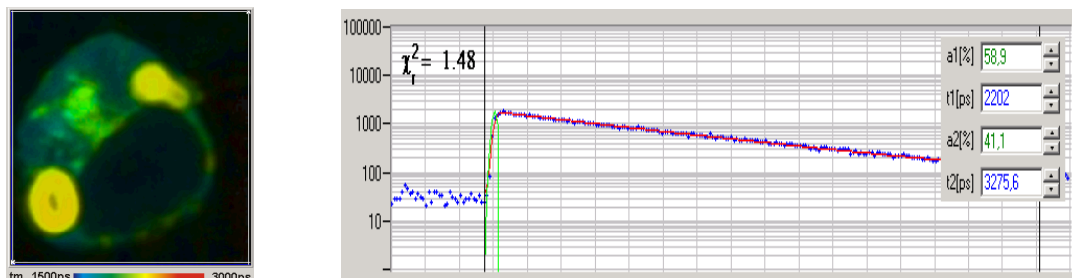


Fig. 393: Left: Acceptor lifetime image of the HEK cell shown in Fig. 388. Right: Acceptor decay function in region of strong FRET (right)

The explanation of the observed donor decay profile is in the fluorescence spectra. There is a strong overlap of the donor emission into the acceptor emission. The observed acceptor decay is therefore a sum of the donor emission overlapping into the acceptor spectrum, the decay of the FRET-excited acceptor, and a contribution from directly excited acceptor. This is in

agreement with the decay components shown in Fig. 393: The lifetimes of the decay components are close to the average lifetime of the donor, CFP, and the typical lifetime of YFP.

In CFP-YFP experiments the amount of donor fluorescence in the spectral range of the acceptor can be almost 50% of the observed acceptor fluorescence. Moreover, the decay time of the fast donor component is exactly the rise time of the FRET-excited acceptor. Both effects cancel, and the result is a near-single-exponential decay function, as shown in Fig. 394.

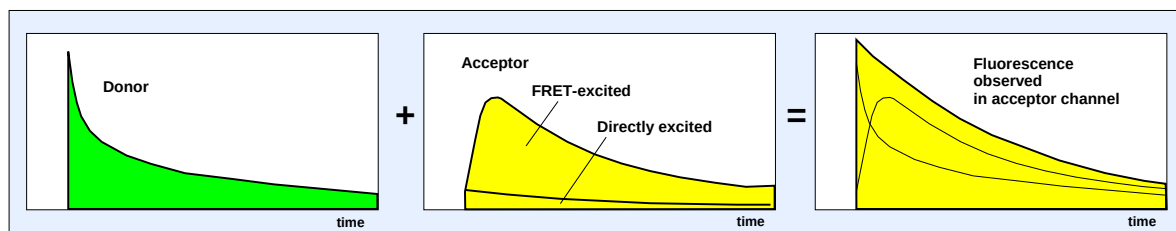


Fig. 394: The observed acceptor decay is a sum of FRET-excited acceptor fluorescence and donor fluorescence emitted in the spectral range of the acceptor.

The prospects of using the acceptor decay functions in FLIM FRET are therefore not good. It has been attempted to obtain additional FRET information from the acceptor emission measured simultaneously with the donor emission in a dual-detector TCSPC system [289]. An attempt was made in [65] to subtract the donor bleedthrough from the acceptor decay and to build up an acceptor lifetime image free of donor bleedthrough. The result showed indications of the slow-rising acceptor component but also a large amount of directly excited acceptor fluorescence. The slow rise of the acceptor fluorescence has been observed in [126] and [561]. These measurements were performed in protein solutions in cuvettes. The reason that the slow rise of the acceptor fluorescence stands out more prominently than in the FLIM data is possibly that the cuvette experiments used more restrictive filtering: [126] detected through a 557 nm filter, [561] in the 540 nm channel of a bh PML-16 multi-wavelength detector. Typical CFP / YFP beamsplitters used in microscopy split the signals at 510 to 520 nm and thus record more donor bleed-through.

Even if the FRET-excited component of the acceptor fluorescence is correctly extracted from the decay data as shown in [561], all these attempts are facing the same problems as intensity FRET: It is not possible to record the FRET-excited acceptor fluorescence without accurate correction of the donor bleedthrough and the contribution from directly excited acceptor fluorescence.

A more promising approach to the exploitation of the acceptor fluorescence is multi-wavelength detection [61, 69]. Reference spectra of the donor and the acceptor would be recorded, and the FRET fluorescence would be fitted by a model containing these spectra and the unknown intensity coefficients and lifetimes of the donor and the acceptor. Thus, multi-wavelength FLIM may lead to a combination of FLIM-based and sensitised-emission FRET techniques. A demonstration of the technique has been given in [117, 118, 119, 561].

Dark acceptors

An exciting possibility to improve FLIM FRET results is to use a dark acceptor. The advantage of this approach is that donor-acceptor pairs with almost ideal overlap of donor emission and acceptor absorption spectra can be selected. The FRET efficiency for a given donor-acceptor distance is therefore higher than for conventional FRET pairs.

The situation for the GFP-YFP pair is shown in Fig. 395. The figure shows that the pair would be almost impossible for use for conventional FRET: The emission of the GFP and the YFP cannot be spectrally separated, and excitation of the GFP anywhere close to its absorp-

tion maximum would also excite a large amount of YFP. However, there is an excellent overlap between the GFP emission and the YFP absorption. With a dark YFP, the GFP emission can be detected, and the FRET efficiency determined by FLIM FRET. A dark YFP mutant (called resonance energy accepting chromoprotein, REACh) has indeed been developed, and the GFP- (dark)YFP pair been used for FRET experiments [236, 237, 420, 447].

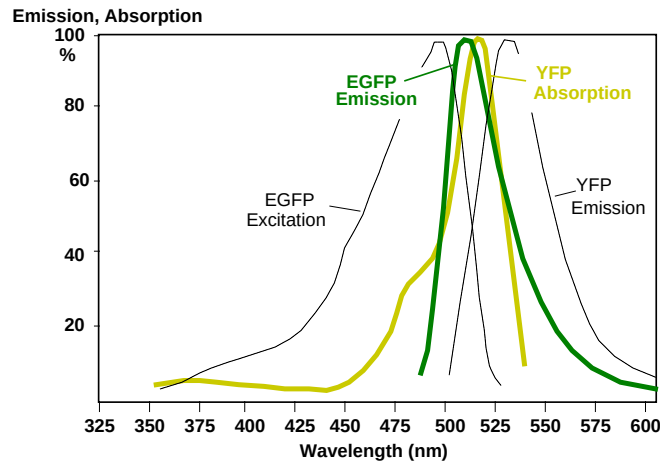


Fig. 395: Absorption and emission spectral of GFP and YFP, after [169]

One argument against FRET experiments with a dark acceptor is that the acceptor is not entirely free of fluorescence - the fluorescence is only strongly quenched. The donor emission would therefore be contaminated by an unknown amount of acceptor fluorescence. Such contamination may indeed exist. What happens in detail depends on whether the fluorescence of the acceptor itself is quenched dynamically or statically [360] is shown in Fig. 396, left and right. Dynamic quenching results in an extremely short fluorescence lifetime of the acceptor. The decay function of the FRET-excited acceptor is then identical with the decay component of the interacting donor. Contamination would result in a slight over-estimation of the amount of interacting donor. Static quenching (if incomplete) would result in a weak acceptor fluorescence with the normal lifetime. The result would be contamination by a FRET-excited or directly excited acceptor decay, and thus an over-estimation of the non-interacting donor fraction. The size of both effects can be determined by recording FLIM of acceptor-only samples.

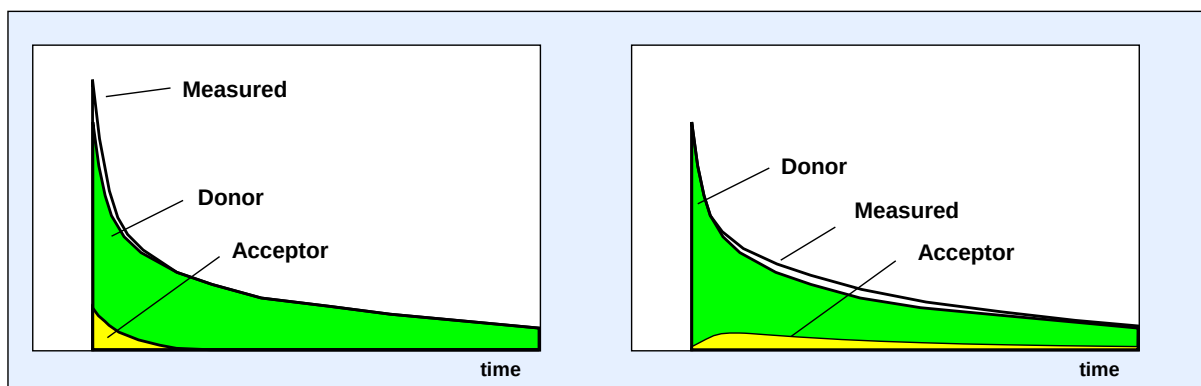


Fig. 396: Contamination by 'dark' acceptor fluorescence. Left: Acceptor quenched dynamically. Right: Acceptor quenched statically.

Another argument against dark-acceptor FRET is more serious: There is no way to prove that the cells are expressing the acceptor. If there is no acceptor FRET will not occur, even if the labelled proteins are perfectly interacting. Consequently, the *absence* of a FRET signature in the donor decay function is no proof of the absence of protein interaction.

FRET between Endogenous Fluorophores

There is a number of endogenous fluorophores with overlapping emission and absorption spectra. That means FRET can, in principle, occur between the fluorophores, and be exploitable to investigate interactions of the associated proteins. Serrano et al. used FRET from an amino acid (the donor) into tryptophane to detect structural heterogeneity [525]. Tryptophane can also be used as a FRET donor. The problem of these experiments is that amino acids or tryptophan must be excited deep in the UV, around 250 nm. The optics of a normal microscope and of the scan heads of normal laser scanning microscopes is transparent only down to about 360 nm. Li and Seeger therefore built a confocal UV microscope with 266 nm excitation wavelength, a PMH-100-6 detector and an SPC-630 TCSPC module. They were able to obtain decay curves from tryptophane, and show that the fluorescence lifetime decreases on interaction with other proteins [372]. There are currently attempts to reach tryptophane via three-photon excitation in multiphoton microscopes. The problem of this approach is that this wavelength excites NADH and FAD via two-photon excitation. At a given laser power, two-photon excitation is far more efficient than three-photon excitation. It can easily kill the cells as we have found in our own test experiments. Nevertheless, it has turned out that three-photon excitation of tryptophane - and the detection of its fluorescence - is possible. With UV-optimised optics in the detection beam path, hybrid detectors, and carefully adjusted laser power three-photon excited TCSPC FLIM images of tryptophane were obtained. Possible FRET would occur between the tryptophane and the bound component of the NADH. Indeed, FLIM experiments have shown a decrease in the tryptophane lifetime with the bound-unbound ratio of the NADH fluorescence [304, 447].

FRET in Diffraction-Limited Spots

Problems may occur when FRET occurs in image details too small to be resolved in the optical microscope. To show up in the image at all, a sub-resolution structure must emit fluorescence at an intensity much higher than the environment. That means the fluorophore concentration in the structure itself must be very high. High concentration bears the risk of interaction of the donor molecules with several acceptors [338]. The FRET efficiency for a given donor-acceptor distance is then higher than expected. The second problem is on the detection side. The fluorescence decay function from a sub-diffraction structure will necessarily be contaminated by fluorescence from the closer environment. If there is no FRET in the environment (which is the purpose of the experiment) the FRET efficiency and the fraction of interacting proteins come out too low.

Typical cases are cell membranes or synapses between neurons. A beautiful FLIM FRET image of a live cultured neuron is shown in Fig. 397. FRET is expected to occur in synapses. As can be seen in the image on the left there are a large number of small spots with decreased lifetime. Magnified images of two typical details are displayed on the right.

The interpretation of such data is far more difficult than in a cell like the one shown in Fig. 391, page 236. The spots of decreased lifetime are hard to distinguish from photon noise and from variation due to inhomogeneity of the local environment. Conclusions can only be made by correlating the spots of decreased lifetime with the morphology, by comparing a large number of measurements, and including negative tests with acceptor-free samples.

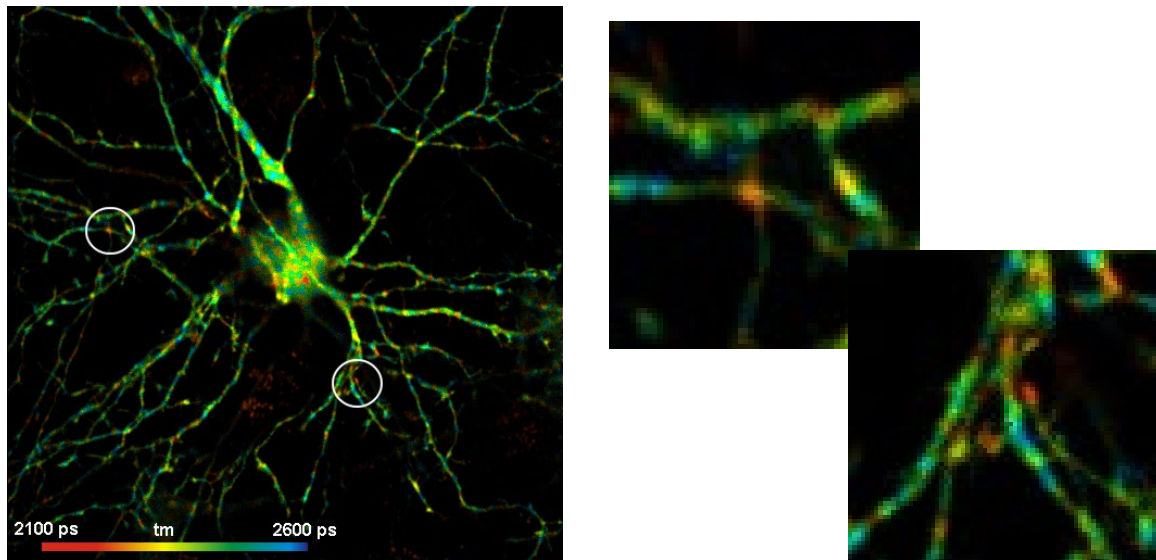


Fig. 397: Live cultured neurons. FRET expected in synapses. Left: Full image, interesting candidates marked. Right: Magnified images of details marked. Zeiss LSM 7 MP microscope with Simple-Tau 152 FLIM system and HPM-100-40 hybrid detectors. Analysis by SPCImage, incomplete decay model with two exponential components. Data courtesy of Inna Slutsky, University Tel Aviv.

FLIM FRET in synaptic structures has been published by Bosch et al. and by Fogel et al. Bosch et al. were not only able to detect protein interaction, they could even follow the reorganisation of post-synaptic substructures during long-term potentiation by time-series FLIM [127]. Fogel et al. [224] used a combination of intensity FRET measurement, FLIM FRET measurement, and single-molecule imaging to show that the amyloid precursor protein homodimer constitutes a resynaptic receptor that transduces a signal from amyloid-beta peptides to glutamate release. Dore et al. and Murokashi et al. performed FLIM-FRET experiments in single dendritic spines [195, 420].

Homo FRET

The FRET technique described above is based on resonance energy transfer between *different* fluorophores. It should be mentioned, that FRET is also possible between *similar* molecules. Resonance energy transfer between similar molecules is called homo-FRET. Homo-FRET leaves the fluorescence decay profiles unchanged: The energy transferred from the first molecules to a second one is emitted by the second molecule, and the sum of the decay rates does not change. What changes, however, is the fluorescence anisotropy. Homo FRET has thus to be detected by comparing the intensities and decay profiles in two detection channels of perpendicular polarisation [423, 606]. This is possible by using a polarising beamsplitter in the detection beam path. The DCS-120 scanner has one in its secondary-beamsplitter wheel. In most scanning microscopes polarising beamsplitter are not built in. Thaler et al. therefore detected through the condenser beam path of an LSM 510 NLO. Their paper on structural rearrangement of CaMKII α catalytic domains is based on homo FRET and can be recommended as an amazing piece of scientific detective work [583].

Ultra-Fast Energy Transfer

It is normally believed that the energy transfer rate between fluorescent proteins is limited by the geometry of the barrel structure of the molecules. However, Kim et al. found in homo-FRET experiments that the limiting anisotropy of Venus_{a206} dimers is reproducibly lower than expected. They concluded that the dimers must behave more or less like a single emitter. They investigated the effect by spectral measurements, anisotropy-decay measurements, FSC, molecular-brightness measurements, and anti-bunching measurements. After exclusion of all

other conceivable mechanisms, the results left coherent behaviour of the Venus molecules as the only possible explanation [316].

Practical Hints for FRET Measurements

Multi-Exponential Decay of the Free Donor

Double-exponential FRET measurements are faced with the problem that the fluorescence decay of the unquenched donor itself can be multi-exponential. This happens especially for the CFPs and, at a smaller extend, Cerulean. CFP has two lifetime component of about 1.3 ns and 2.9 ns with an amplitude of 20 to 40% and 60 to 80%, respectively [64]. Sarkar et al. [496] found an almost similar composition (60% of 3.7 ns and 40% of 1.5 ns) for Cerulean excited at 405 nm. Interestingly, there is no trace of a second component in our own data from live cells, see Fig. 398, left. It is therefore possible that the composition of the decay depends on the molecular environment, or on the excitation wavelength.

Fortunately, in FRET experiments the fast lifetime component in the donor decay is usually in the range of 400 to 800 ps. Under these conditions a double-exponential fit routine tends to combine the 1.3 ns and the 2.9 ns component into one component of about 2.5 ns. Thus, the interacting and non-interacting donor fractions can still be distinguished.

Almost ideal single-exponential decay profiles are obtained from GFP. GFP or EGFP are therefore increasingly being used as FRET donors.

Please note that strong multi-exponential behaviour can be induced by fixation procedures. The frequent complaints about strongly multi-exponential donor decay probably result from measurements of fixed samples, see below.

Live Samples versus Fixed Samples

FRET measurements aim at measuring changes in the conformation and binding of proteins. That means, that the molecular structure of a sample must be preserved during sample preparation. In other words, FLIM measurements normally have to be performed on live cells or live tissue. Fig. 398 shows what can happen if this simple fact is ignored.

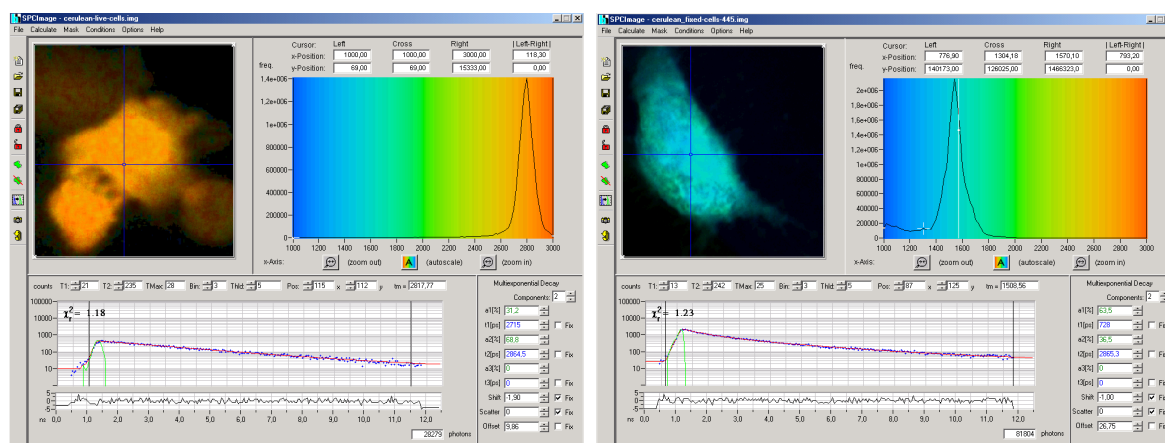


Fig. 398: Lifetime differences between a live cell (left) and a fixed cell (right). Colour-coded lifetime image, histogram of lifetimes over the pixels, decay curve in selected spot of the image, excitation 445 nm. Both cells are expressing Cerulean, without any FRET acceptor. Nevertheless, the fixed cell shows a decay profile typical for FRET.

Both images show cells of the same type, transfected with Cerulean. The excitation wavelength was 445 nm, the detection wavelength 480 ± 20 nm. The left image was obtained from a live cell, the right image from a fixed cell. Both cells were analysed by a double-exponential model.

The live cell delivers two almost identical lifetime components. This is a clear indication that the decay is almost perfectly single-exponential. The lifetime is about 2.8 ns. This is exactly what one would expect for a cell expressing Cerulean alone.

The fixed cell (Fig. 398, right) shows a strongly double-exponential decay profile, with 60% of 770 ps and 40% of 2.8 ns. The amplitude-weighted mean lifetime is around 1.56 ns. Moreover, the lifetime histogram shows a broad background of shorter lifetimes from the embedding medium. No matter what kind of experiment this result would be used for, it would lead to significantly wrong results. Consider a FRET experiment: The decay profile of the fixed Cerulean cell is similar to the decay profile of a cell showing FRET, compare Fig. 390. An inexperienced user may interpret the decay profile as 60% of interacting proteins, with a FRET efficiency of 0.725. The average FRET efficiency would be 0.44. However, the cell does not contain an acceptor, and consequently, cannot do any FRET.

Similarly wrong lifetimes and decay profiles are found also for other fluorescent proteins in fixed samples. It appears likely that the frequent complaints about strong multi-exponential decay of CFPs at some extent originate from measurements on fixed samples.

Autofluorescence

A frequent pitfall of FRET experiments is autofluorescence. If the cells are not expressing the fluorophores as expected autofluorescence dominates the FLIM signals. Autofluorescence has a fast and a slow decay component. The shape of autofluorescence decay functions can be very similar to those of FRET, see Fig. 399. FLIM can help to identify autofluorescence problems: If the slow decay component (circled) is not as expected for the free donor something must be wrong with the sample. However, even this is not sure. If autofluorescence wants to fool you it will, as can be seen in Fig. 399, right. Negative tests with un-transfected cells and with acceptor-free cells are therefore mandatory for proving the relevance of FRET results.

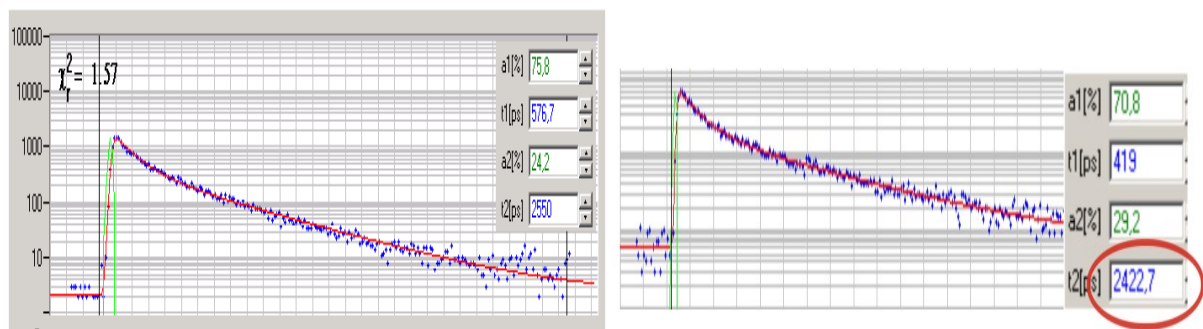


Fig. 399: Fluorescence decay from the donor of a CFP-YFP FRET system (left) and fluorescence decay of autofluorescence

Photobleaching

Photobleaching acts differently on the interacting and the non-interacting donor fraction. The non-interacting donor component photobleaches faster, so that the fraction of interacting donor (and thus the conventional FRET efficiency) is biased towards higher values. It is also possible that the acceptor photobleaches. In that case, photobleaching decreases the fraction of interacting donor. Moreover, photobleaching can create photoproducts that are fluorescent.

Emission from these compounds can change the composition of the decay profiles unpredictably [281].

As a rule of thumb, the count rates should not decrease by more than 20% during the measurement. If you notice excessive photobleaching, try with a lower laser power and a correspondingly longer acquisition time. In one-photon systems, you may use a larger pinhole and thus trade count rate against depth resolution. Even if you have to sacrifice a number of cells for these experiments the higher number of photons obtained at less photobleaching pays off at the end.

It should also be taken into regard that the exposure may induce real changes in the protein conformation in the cells. A possible example is shown in Fig. 400. The images shows a live-cell sample expressing interacting proteins fused with CFP and YFP.

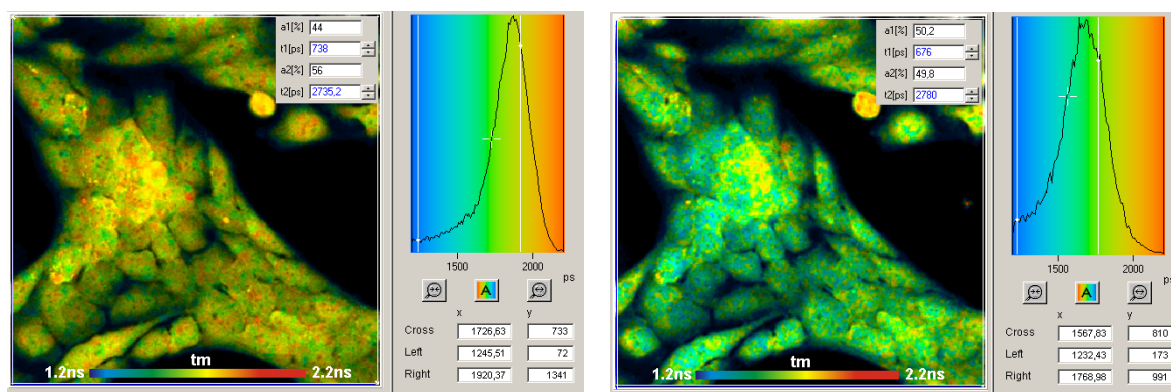


Fig. 400: Two FLIM FRET measurements of the same sample, lifetime images and lifetime histograms. Left: Initial recording, acquisition time 30 seconds. Right: Second recording, acquisition time 30 seconds, after 90 seconds of total exposure. Insert: Amplitudes and lifetimes of the decay components. Analysis by bh SPCImage, incomplete decay model with two exponential components

Two-photon excitation at 860 nm was used, the laser power regulator of the microscope was set to 4%. This is a power commonly used in two-photon live cell imaging. Both images were recorded at the donor emission wavelength. After getting the sample into focus, a 30 seconds FLIM recording was taken. Then the scanning was continued for another 60 seconds, and another 30 second image was taken. As can be seen from the images, the lifetime (amplitude weighted lifetime, t_m) in the second recording is significantly shorter. The shorter t_m comes from a larger amplitude and a shorter lifetime of the fast decay component. The slow decay component remained virtually unchanged. This is exactly what should be expected if the protein interaction had become stronger. This interpretation may be a bit speculative, but it shows that real changes in the sample should be considered at least as a possibility.

Photobleaching, and, possibly, photo-induced changes in the samples, has been a frequent problem of FRET measurements in early FLIM systems. These systems used PMTs with conventional photocathodes and, in many cases, inefficient ports of the microscopes. In the last years the photobleaching problem has been reduced by hybrid detectors [72] and highly efficient microscope optics. There is also a continuous development of new photostable fluorescent proteins which further reduce photobleaching artefacts [174, 175].

FRET Literature

An enormous amount of FRET papers based on the bh FLIM technique has been published in the last few years. An overview about FRET techniques, their applications and the related pitfalls is given in [605]. A detailed description of a TCSPC-FLIM-FRET system is given in

[202]. The system was used for FRET between ECPF-EYFP and FM1-43 - FM4-64 in cultured neurones. A number of papers give a general characterisation of TCSPC-FLIM FRET for monitoring protein interactions [116, 150, 151, 449, 553]. A combined FLIM-FRET microscope / endoscope system is described in [232]. The system simultaneously scans FLIM images through the beam path of an inverted microscope and records fluorescence from the back of the sample through a fibre bundle. Elder et al. reviewed different methods of FRET signal normalization and verified them by TCSPC FLIM [214].

A comparison of the accuracy of FRET measurements by different techniques is given by Pelet et al. [443]. The authors compared FLIM, spectral imaging, and two-channel intensity imaging with calibration of excitation and emission crosstalk. For the individual measurements they used identical optical paths, comparable data acquisition times and comparable fluorescence intensities. It turned out that the FRET results obtained from the FLIM data had a three-times smaller standard deviation than those obtained from the dual-channel and spectral measurements. Sun & Periasamy compared conventional FRET efficiencies obtained by filter-based FRET measurements with FRET efficiencies obtained by FLIM FRET [571] and found that additional correction is needed for filter-based measurements.

The construction of FRET standards and their verification by FLIM measurement are described in [143, 336]. Although FLIM FRET actually does not need calibration the standards are required to verify and compare the results obtained by different FRET techniques.

There is a number of FRET applications which are directly related to clinical research [394]. Because FRET yields information about protein interaction it can be used to investigate the formation - and possible dissolution - of amyloid plaques of Alzheimer's disease [17, 18, 107, 108, 109, 262, 279, 302, 375, 377, 596]. Mechanisms of Huntington disease (a progressive neuro-degenerative disorder) were investigated in [228] and [421].

FLIM FRET in synaptic structures has been published by Bosch et al., Fogel et al. and Ueda et al. [127, 224, 594, 595]. These experiments are difficult because FRET occurs in sub-resolution volumes of the sample. The effects are therefore difficult to detect, see above, 'FRET in Diffraction-Limited Spots'. Bosch et al. were not only able to detect protein interaction, they could even observe the re-organisation of post-synaptic substructures during long-term potentiation by time-series FLIM [127]. Fogel used a combination of intensity FRET measurement, FLIM FRET measurement, and single-molecule imaging to show that the amyloid precursor protein homo-dimer constitutes a resynaptic receptor that transduces a signal from amyloid-beta peptides to glutamate release.

Kelleher et al. demonstrated deep tissue FLIM-FRET in cancer cells and animal models. Two-photon excitation was used to obtain FRET images from layers as deep as 100 μm [313]. Ahmed et al. studied the interaction of growth factor receptor-bound protein (Grb2) and Ras/mitogen-activated protein (MAP) and found a relation to normal versus oncogenic function of the cells [5].

Another potential FRET application is the investigation of infection mechanisms. The technique was demonstrated by Ghukasyan et al. for the infection of HeLa cells with enterovirus 71 [248]. Jeshtadi et al. [299], Danquah et al. [171] and Cheung et al. [153] used FRET to investigate interaction of virus proteins with proteins of the host cell. The FRET pair was GFP-DsRed. The spreading of viruses in plant cells was studied by Amari et al. [8], fungus infection of plant cells by Shen et al. [536].

Almost all the FLIM-FRET applications are more or less related to interaction interactions of various proteins or to protein organisation [2, 3, 4, 9, 114, 116, 124, 128, 127, 195, 224, 230, 231, 234, 243, 257, 293, 300, 309, 315, 318, 341, 346, 378, 379, 383, 386, 420, 425, 444, 462, 472, 495, 509, 523, 524, 557, 589, 607, 643]. Monitoring HIV-1 Protein Oligomerization by

FLIM FRET was described in [465]. Protein interaction studies include detection of enzymatic activity in neuronal micro-compartments by FRET [639], Na^+ channels [401], or intracellular transport mechanisms and membrane trafficking [173, 409, 466]. Rochel et al. used FRET to study the role of DNA in the spatial organisation of nuclear hormone receptors (NHRs) [475]. Interactions between the PCK and $\text{NK}\kappa\text{B}$ signalling pathways have been investigated in [416]. [562] investigated the association of protein kinase C alpha ($\text{PKC}\alpha$) with caveolin. FRET between GFP and RFP and FRET cascades from GFP via Cy3 into Cy5 are demonstrated in [450] and [9]. The agglutination of red blood cells by monoclonal antibodies was studied using FRET between Alexa 488 and DiI [473]. Interaction of the neuronal PDZ protein PSD-95 with the potassium channels and SHP-1-target interaction were studied in [114, 116]. Interaction of αB -crystallin with focal adhesion kinase (FAK) was investigated in by Pereira et al. [448] and shown to confer protection to FAK against calpain mediated proteolysis in cardio-myocytes. It has also been shown that conformational changes of proteins in cells can be monitored by FLIM-FRET [138, 377]. Tynan et al. and Martin-Fernandez used FRET to gain insight into the structure of the human epidermal growth factor (EGFR) [389, 593]. Mergenthaler et al. investigated the function of mitochondrial HKs in different states of metabolic deprivation during hypoxia and hypoglycemia [399]. A new Ras sensor was developed and characterised by Oliveira and Yasuda [426].

A variety of papers are devoted to the conformation and interaction of SNARE proteins and mechanisms modulating their interactions [354, 394, 433, 466, 467, 468, 552, 598]. In [467] double-exponential FRET was used to distinguish interacting and non-interacting protein fractions. Studies of the interaction dynamics on the millisecond time scale are described in [468].

Ariola et al. used a Bodipy-cholesterol derivate (Bdp-Chol, donor) and Dil-C12 (acceptor) to probe fluidity and lipid order in cell membranes [12]. They found a reduction in lifetime of the Bdp-Chol in ordered domain which they attributed to FRET. The results were confirmed by anisotropy decay measurement.

The influence of shear forces on protein conformation was investigated in [140, 145, 646]. The authors used a micro-fluidic setup to induce dynamic forces on the cells. The setup was placed under a microscope and illuminated by a frequency-doubled Titanium-Sapphire laser. The donor fluorescence was detected by a bh PML-16 multi-wavelength detector with polychromator, and recorded by an SPC-630 TCSPC module. A similar setup was used to study the influence of oxygen concentration [647]. The Oxygen supply was turned on and off by starting and stopping the flow in the cell. The oxygen concentration was monitored via the emission intensity of a phosphorescence probe.

FRET between ECFP and EYFP in plant cells was demonstrated in [125]. FRET measurements in plant cells can be difficult because of the strong autofluorescence of the plant tissue. The authors showed that two-photon excitation can be used to keep the autofluorescence signal at a tolerable level. The autofluorescence problem has also been solved by using the GFP Mutant TSapphire and the DsRed Variant mOrange as FRET donor and acceptor, respectively [26]. Protein interactions upon intrusion of fungal pathogens into plant cells were investigated in [131, 121, 536]. Interactions between Golgi tethering factors and small GTPases was studied by Osterrieder et al. [432].

There is a continuing search for new FRET pairs with higher photostability, better overlap of donor emission with acceptor absorption bands, and (important for steady-state FRET techniques) less donor and acceptor bleedthrough. Please see [26, 174, 175, 570] for new fluorescent proteins for FLIM FRET and their characterisation. An interesting approach is the use of a GFP as a donor and a dark YFP as an acceptor [236, 237].

It is also attempted to use quantum dots or combinations of quantum dots (as donors) and fluorescent proteins [395] or quantum dots and metal-organic complexes [558] as FRET partners. Spectroscopically, quantum dots make excellent FRET donors and acceptors. The photostability is excellent, and almost any absorption and emission wavelength can be obtained. The drawback is that quantum dots cannot be easily be linked to target proteins by transfection techniques as fluorescent proteins can.

Autofluorescence FLIM of Cells and Tissues

Biological tissue contains a wide variety of endogenous fluorophores [163, 251, 328, 471, 356, 510, 545]. For many years, autofluorescence measurements of cells and tissue have mainly been performed by spectrally resolved imaging techniques. The problem of purely spectrally resolved techniques is that the emission and excitation spectra of the endogenous fluorophores are broad and poorly defined. Many of the fluorophores are mixtures of closely related compounds. The shape of the spectra can therefore vary. Moreover, absorbers present in the tissue may change the apparent fluorescence spectra. It is therefore difficult to disentangle the fluorescence components by their emission spectra alone. The most serious drawback of purely spectral techniques is, however, that different molecular environment and binding of fluorophores to proteins cannot be distinguished.

Fluorescence lifetime imaging improves the contrast of separation of the different fluorophores. This has been demonstrated by Krasieva et al. [339] who used TCSPC FLIM and phasor plot analysis to separate the fluorescence components of different melanins, and the melanin fluorescence from the NADH fluorescence. Autofluorescence imaging has also benefits when the reaction of tissue to optical radiation is to be investigated, such as skin ageing [490] or photodamage and tumor induction by UV [323] and IR irradiation [331]. Such experiments forbid the use of exogenous fluorophores because energy or electron transfer between the fluorophores and the proteins can induce additional photodamage. Moreover, TCSPC FLIM is more and more introduced into clinical applications. In these applications ‘label-free’ imaging is needed because staining the tissue with exogenous fluorophores is either not possible or not permitted. FLIM delivers a wealth of additional image information which often makes the use of exogenous fluorophores unnecessary [170, 328, 330, 332, 333, 476, 492, 490, 491, 510, 512, 513, 528, 529, 545] or favourably supplements the information from exogenous fluorophores [621].

The most important argument for using lifetime imaging is, however, that the fluorescence decay functions of many endogenous fluorophores bear information on molecular parameters, such as pH, oxygen saturation, ion concentrations, and, especially, on the binding to proteins. The most promising signals come from NAD(P)H (nicotinamide adenine (pyridine) dinucleotide) and FAD (flavin adenine dinucleotide). NAD(P)H and FAD are coenzymes involved in the cell metabolism. NAD(P)H and FAD are unique in the sense that their fluorescence intensities and fluorescence decay functions bears direct information on the metabolic state of the cells. There is an increasing number of papers about this subject [22, 113, 122, 123, 154, 156, 200, 199, 273, 358, 360, 376, 380, 381, 410, 440, 451, 463, 464, 484, 492, 511, 512, 528, 529, 548, 549, 573, 575, 603, 615, 616, 618, 627, 637, 638, 636]. The section below will discuss the use of the NAD(P)H and FAD fluorescence in detail.

Metabolic Imaging by NAD(P)H and FAD FLIM

Excitation and Emission Spectra

The signals from NAD(P)H and FAD are not easy to separate. Failure to record metabolic effects in most instances results from incorrect excitation and detection wavelengths. Also the numerous inconsistent ‘tumor lifetimes’ reported in the past may result in part from using wrong wavelengths.

Approximate excitation and emission spectra of NAD(P)H and FAD are shown in Fig. 401. The spectra were taken from [360] and [512]. Both the excitation spectra and the emission spectra are overlapping. To obtain clean signals NAD(P)H must be excited in the range from

330 to 370 nm, and detected from 425 nm to no more than 475 nm. FAD could, in principle be excited in the entire range from 330 to 470 nm. However, excitation below 400 nm would excite also NADH. The emission spectrum of NADH extends into that of FAD, and a clean FAD signal would not be detected. Therefore FAD should not be excited at wavelengths shorter than 400 nm. NAD(P)H excitation is then negligible, and a clean FAD signal can be obtained in the range from 500 to 600 nm. However, it is technically inconvenient to have the FAD excitation wavelength within the detection range of NAD(P)H. If the NADH and the FAD excitation are multiplexed (as in the bh metabolic FLIM systems) scattered light from the FAD excitation would shut down or damage the NADH detector. In practice this limits the FAD excitation wavelength range to the interval from 410 to 425 nm. For two-photon excitation a technical limitation for the FAD excitation does not exist. NAD(P)H can therefore be excited at 750 to 780 nm, and FAD at 860 to 900 nm.

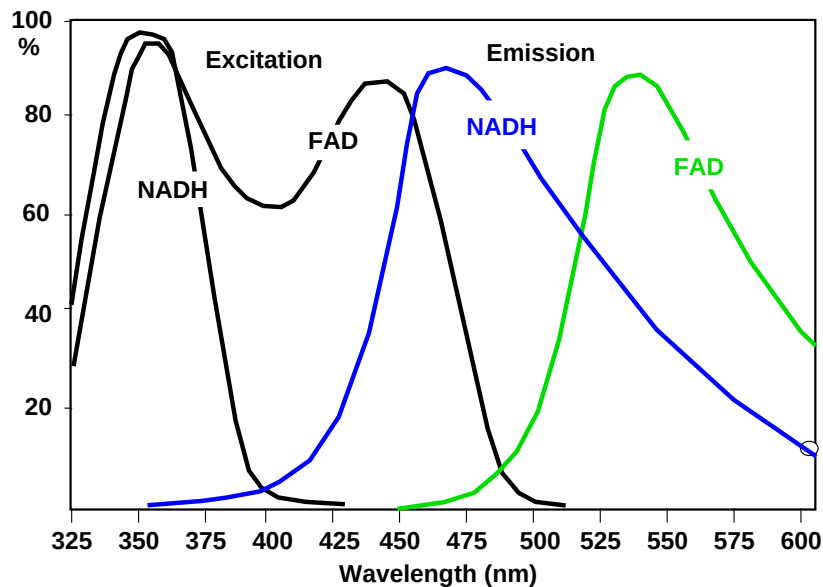


Fig. 401: Excitation and emission spectra of NADH and FAD. After [512] and [360].

NAD(P)H and FAD Intensities

Both NAD(P)H and FAD form redox pairs. NAD(P)H is fluorescent in its reduced form but not when oxidised to NAD^+ . FAD is fluorescent when oxidised to FAD^+ , and loses fluorescence when reduced. The fluorescence intensity ratio of FAD and NAD(P)H therefore changes with the redox state of the tissue. The intensity ratio shows whether the metabolism in a cell is more oxidative (oxidative phosphorylation) or more reductive (glycolysis) [551]. The redox ratio is closely related to the ‘Warburg Effect’: In normal cells oxidative phosphorylation dominates, in cancer cells reductive glycolysis [623, 624].

The influence of the redox state can be shown by recording FLIM from yeast cells. Fig. 402, left, shows yeast cells in a medium saturated with CO_2 and virtually free of oxygen. Except for a few (probably apoptotic) cells the fluorescence is extremely weak. When the sample has taken up oxygen from the air the fluorescence intensity increases, accompanied by a change in the lifetimes.

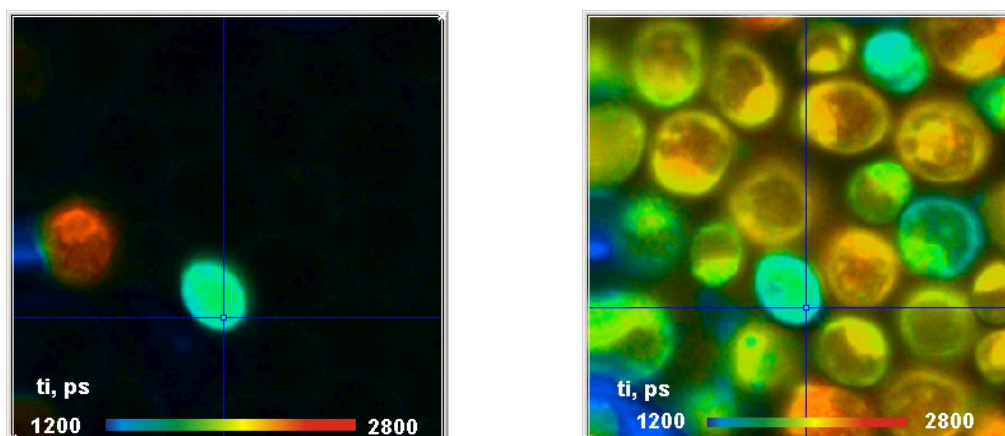


Fig. 402: FAD FLIM images of yeast cells in a cell dish saturated with CO₂ (left) and saturated with O₂ (right). Confocal FLIM, excitation 405 nm, detection by bh MW FLIM, 540 nm

Fluorescence Decay Functions

The fluorescence lifetimes of NAD(P)H and FAD depend on the binding to proteins [358, 360, 440]. Unbound NAD(P)H has a fluorescence lifetime of about 0.3 to 0.5 ns. When NAD(P)H binds to proteins the lifetime typically increases to a few ns [141, 360]. For FAD the effect of binding is opposite: Bound FAD has a lifetime of a few 100 ps, unbound FAD of a few ns. The resulting decay functions are thus (at least) double exponential. Typical decay functions of NAD(P)H and FAD in cells are shown in Fig. 403. The lifetimes and decay times of the decay components are shown in the inserts. The net lifetimes (amplitude-weighted, t_m) for NADH and FAD are 1.86 ns and 0.68 ns, respectively.

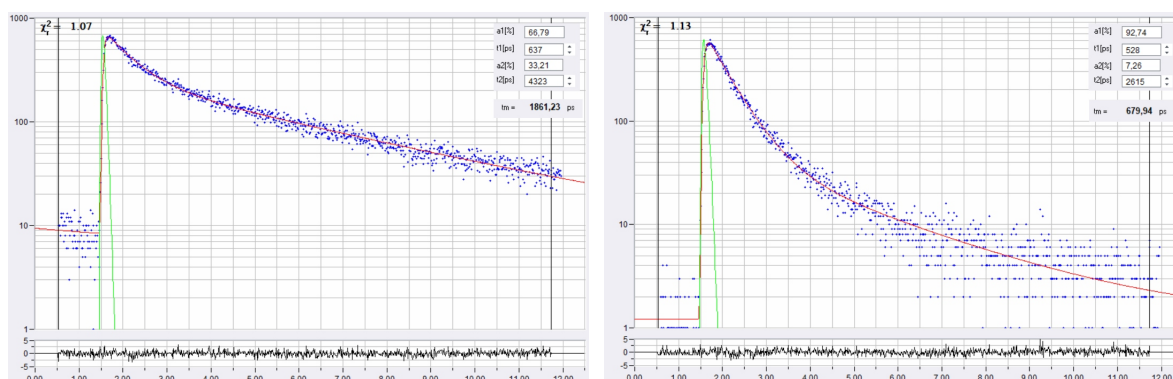


Fig. 403: Typical decay functions of NAD(P)H (left) and FAD (right). The blue dots are the data points, the red curve is a fit with a double-exponential decay model.

Change in the Lifetimes with the Metabolic State

What happens to the decay functions when cells pass from oxydative phosphorylation to glycolysis? Pioneering work in this direction has been done by the group of Melissa Skala at Vanderbilt University (now Morgridge Institute, Madison). A result from [549] is shown in Fig. 404. The figure shows NADH (left) and FAD (right) lifetime images for normal cells and different grades of tumors. It is clearly visible that the NADH lifetime gets shorter and the FAD lifetime longer as the tumor progresses. In other words, the lifetime changes for NADH and FAD go in opposite directions.

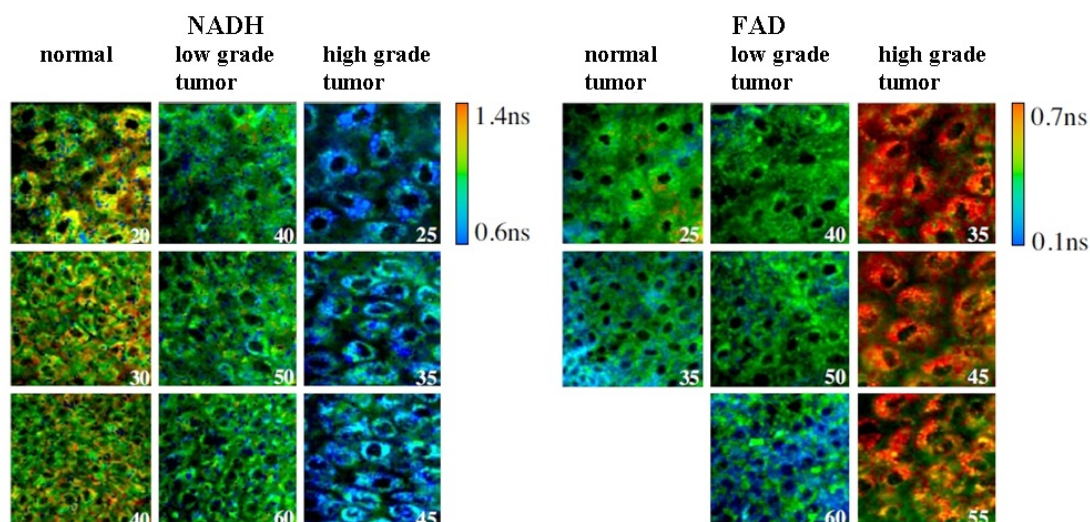


Fig. 404: Change in the mean (amplitude-weighted) lifetime of NADH and FAD with the state of the cells, from normal (oxidative phosphorylation) to high-grade tumor (glycolysis). With permission, from [549].

Amplitudes of the Decay Components

The fluorescence lifetime does not tell whether a change in the lifetime is caused by a change in the lifetimes of the bound and/or unbound NADH and FAD or by a change in the amount of bound and unbound NADH and FAD. However, there are strong indications from biochemistry that the primary cause of the lifetime change is in the bound/unbound ratios. The bound/unbound ratio is reflected by the amplitudes, a_1 and a_2 , of the decay components. a_1 and a_2 can be obtained by double-exponential decay analysis. Because the amplitudes do not depend on other cell parameters, such as mitochondrial-matrix pH [422, 498, 499] and extracellular pH [501] they are better - and more quantitative - indicators of the true metabolic state than the net lifetimes.

A comparison of NADH images of the amplitude-weighted lifetime, t_m and the amplitude of the fast component, a_1 , for different cells is shown in Fig. 405.

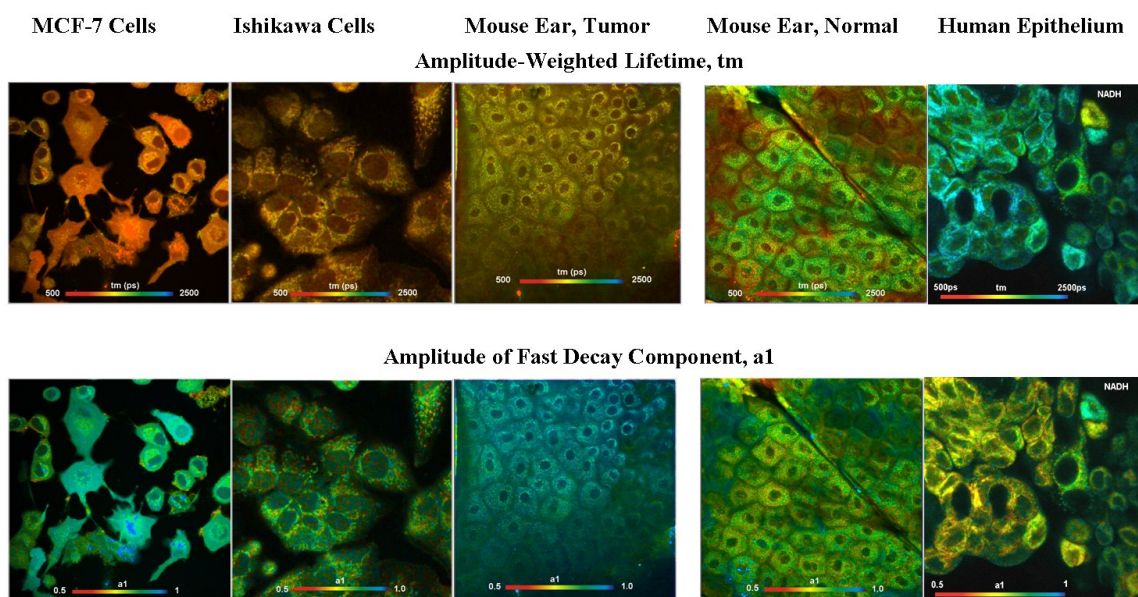


Fig. 405: Images of amplitude-weighted lifetimes, t_m , (upper row) and amplitudes, a_1 , of various cells. The left three cells are tumor cells, the right two are normal.

The figure shows two things: First, the change in a_1 goes in step with the change in t_m . Second, a_1 is obtained at almost the same signal-to-noise ratio as t_m . The good signal-to-noise

ratio of a_1 may be surprising. a_1 is obtained from a double-exponential fit, which is intrinsically less stable than a single-exponential one. The explanation is in the speed of the detector. The data in Fig. 405 were recorded with fast HPM-100-40 and -06 detectors. The IRF of these detectors is several times shorter than the IRF width of the previously used H7422 detectors, and several times shorter than the lifetime of the fast decay component. The fast IRF significantly reduces the ambiguity in the fit and thus results in a high accuracy of a_1 .

Summary of Effects Ruling NADH and FAD Fluorescence

A summary of the mechanisms affecting the NAD(P)H and FAD fluorescence is given in Fig. 406. NAD(P)H is shown on the left, FAD on the right. Please note that the change in intensity is caused by the change in oxidised/reduced forms, not by the change in the apparent lifetime. The first effect dominates, and the intensity does not (as might be expected) change in step with the lifetime.

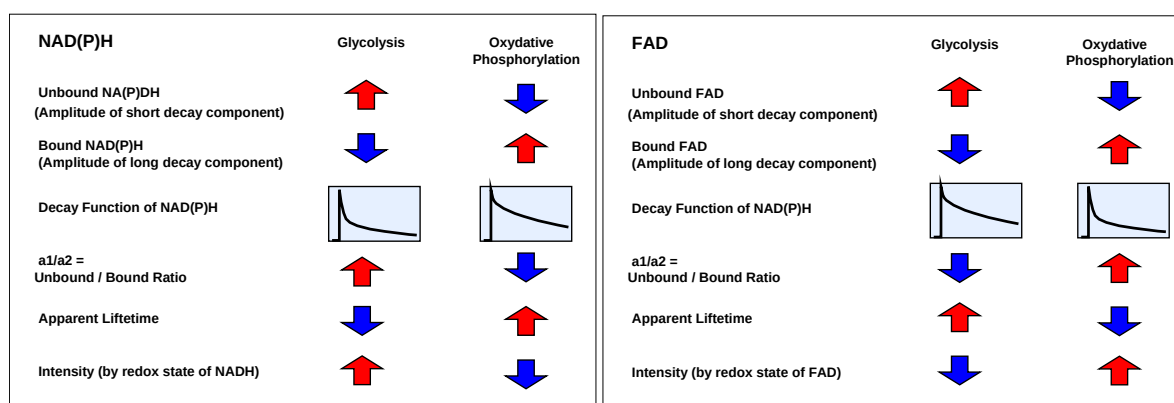


Fig. 406: Response of NAD(P)H and FAD fluorescence parameters to type of metabolism

Indicators Characterising the Cell Metabolism

As described above, there is a confusing complexity of molecular processes influencing the fluorescence of NADH and FAD. Different optical techniques have been developed that deliver insights into different aspects of the problem, but none of them is able to cover the problem in its full complexity. The technique that comes closest to the ideal is multi-dimensional TCSPC FLIM. But it is not only the technique that matters, it is also the researcher who has to understand the technical capabilities of a technique and exploit them to advantage. In this situation, it is not surprising that different approaches have been developed to quantify the metabolic state of cells. The most frequently used indicators are described below.

Redox Ratio

The Redox Ratio was introduced by Chance et al. in 1976 [146, 147]. The redox ratio is defined as

$$RR = I_{FAD} / I_{NAD(P)H} ,$$

with I_{FAD} and $I_{NAD(P)H}$ being the fluorescence intensities of FAD and NAD(P)H. There is some inconsistency in the definition of the redox ratio. Sometimes it is defined as the intensity ratio of FAD/ NADH [381], and sometimes as the intensity ratio of NADH /FAD [551]. Sometimes also the ratio of $FAD / (FAD + NADH)$ is used [217, 303]. Here we use the definition of Britton Chance (1979) [147], which is the intensity ratio of FAD / NADH.

The Redox Ratio makes use of the fact that both NADH and FAD exist in an oxidised and in a reduced form. NADH is fluorescent, NAD^+ is not. For FAD the effect is opposite: FAD^+ is fluorescent, FAD is not. The fluorescence intensity ratio of FAD and NADH therefore changes with the redox state of the cell. Often, the Redox Ratio is used in combination with the fluorescence lifetime. For practical applications please see [235, 376, 380, 413, 458, 526, 550, 551, 614, 617].

The redox ratio can, in principle, be derived from NADH / FAD FLIM data. The total number of photons in a given pixel is a measure of the intensity. Therefore the redox ratio can be determined by calculating the ratio of the photon numbers in the corresponding pixels of an FAD image and an NADH image of the same cell. This is especially attractive if NADH and FAD data are recorded simultaneously by laser-wavelength multiplexed FLIM. For calculation and display of Redox-Ratio images please see 'Classic Redox Ratio' page 386.

The problem of the redox ratio is that it depends on the ratio of the concentrations of NAD(P)H and FAD in the cells. The concentration ratio can hardly be considered to be the same for different cell types. Therefore, it is a good parameter to observe a given cell shifting its metabolism between glycolysis and oxidative phosphorylation, but it does not tell quantitatively whether a given cell is a tumor cell or a normal one.

Fluorescence Lifetimes

When the metabolism shifts from oxidative phosphorylation to glycolysis the apparent lifetime of NAD(P)H decreases, and the lifetime of FAD increases. Since the lifetimes (and the lifetime ratio of NAD(P)H and FAD) do not depend on the absolute concentrations they are more robust parameters than the redox ratio. They are therefore frequently used as indicators of the metabolic state [139, 235, 413, 460, 461, 458, 526, 579, 622, 627].

However, some pitfalls remain. The component lifetimes of NAD(P)H and FAD depend on the mitochondrial-matrix pH [422, 498, 499], extracellular pH [501], temperature, and a number of other environment parameters [141, 528]. They are clearly different in different compartments of a cell [464, 638], see Fig. 374, page 218.

There is increasing evidence that the primary effect of the metabolic state on the decay function is a change in the ratios of bound/unbound NADH and FAD, i.e. in the amplitudes of the decay components. However, the net lifetimes of NADH and FAD are influenced both by the amplitudes and the decay times of the components. The net lifetime is therefore not a quantitative indicator of the metabolic state.

Another source of confusion originates from the definition of the 'lifetimes'. The decay functions of NAD(P)H and FAD are double-exponential. Therefore the apparent 'lifetime' depends on the way the components are averaged. There are two definitions of this lifetime: The amplitude-weighted lifetime, t_m , and the intensity-weighted lifetime t_i . t_i and t_m are different and so are the metabolic states derived from one or the other.

Another simple mistake originates from incomplete (or missing) separation of the fluorescence signals of NADH and FAD, see Fig. 407. The many inconsistent 'tumor lifetimes' found in the literature probably originate from this mistake.

OMI Index

Skala and Walsh combined the normalised value of the redox ratio RR, the normalised amplitude-weighted fluorescence lifetime, $\tau_m \text{ NADH}$, and the normalised $\tau_m \text{ FAD}$, of NAD(P)H and FAD into a single 'Optical Molecular Imaging', or OMI index.

$$\text{OMI} = \text{RR} + \tau_m \text{ NADH} - \tau_m \text{ FAD} \quad (\text{all parameters normalised to reference values})$$

The OMI index turned out to be a sensitive and robust parameter to characterise the metabolism of a cell. The authors use it successfully to check the response of human cancer cells from clinical biopsies to various anti-cancer drugs [529, 614, 615, 616, 618, 617].

The OMI index is, however, not free of technical problems. The problem of variable NAD(P)H and FAD concentration has already been mentioned above. OMI values are therefore difficult to compare for different cell types. Moreover, the NAD(P)H and FAD intensities have to be measured at different excitation wavelengths (see Fig. 401). That means that changes in the relative laser power, focusing errors or aberrations in the microscope lens can influence the result. That means the OMI index is a robust and sensitive metabolic indicator for a given type of cell but it is not useful to compare different types of cells.

Statistics of Cell Clusters of Different Decay Signature

Shirshin et al. used non-parametric analysis based on identification of cells in the images, and discrimination of cells of different τ_m , phasor values, or decay shapes [546]. Distributions of the number of cells over the discrimination parameter were built up and used to identify different cell populations. The most successful discrimination technique was 'K-means', as it was demonstrated by simulated and measured data. Although the technique proved to be efficient in finding different cell populations it was not shown how it reacts to the pure metabolic state in presence of heterogeneity in the molecular environment, such as pH or oxygen concentration.

Mitochondrial-Cytoplasmic Ratio, MCR

Evers et al. introduced the ratio of the NAD(P)H fluorescence intensity in the mitochondria and in the cytoplasm, MCR, as an indicator of the metabolism [217]:

$$\text{MCR} = \frac{I_{\text{NADHmitochondrial}}}{I_{\text{NADHcytoplasmic}}}$$

The MCR reflects changes in the NAD(P)H concentration in the different cellular compartments.

Amplitude Ratios of NAD(P)H and FAD Decay Functions, a_1/a_2

After almost two decades of NADH FLIM there is more and more evidence that the primary effect of the metabolic state on the decay functions is a change in the amplitudes of the decay components. If this is correct, why not using the amplitude of the fast component or the ratio of the amplitudes as an indicator of the metabolic state? Changes in the component lifetimes, possibly induced by different molecular environment, pH, or different NADH / NAD(P)H ratio, then have no influence on the results. a_1/a_2 values are therefore comparable between different types of cells.

A problem of the amplitudes is that they can be difficult to extract from the decay data. Double-exponential decay analysis is required, so that the fit routine has several parameters to obtain a match of the model parameters to the data. The uncertainty in the amplitudes can therefore be higher than in a single-exponential fit of the fluorescence lifetime. This is especially the case if the IRF width is on the same order as the lifetime of the fast decay component, as it was the case for the H7422 detectors used in earlier FLIM systems. The poor signal-to-noise ratios of a_1 and a_2 obtained with these detectors have probably led to some reluctance in using the amplitudes as metabolic indicators. However, if the IRF of the FLIM system is significantly shorter than the lifetime of the fast decay component the amplitudes can be obtained with almost the same accuracy as the net lifetime of the decay. Compared with

the old H7422, the accuracy is much better for HPM-100-40 hybrid detectors, and the problem has entirely been solved with the ultra-fast HPM-100-06 (see 'NADH FLIM with Ultra-Fast Detectors', page 217) in combination with the MLE algorithm of SPCImage NG, see chapter 'SPCImage NG Data Analysis Software'.

In the past few years, the a_1/a_2 ratio (and a_1 , see below) have more and more replaced the other metabolic indicators, please, see, [7, 78, 164, 200, 235, 273, 241, 292, 303, 322, 376, 381, 492, 493, 527, 541, 569, 602, 635, 644, 645].

Amplitude of the Fast Decay Component, a_1

The a_1/a_2 ratio represents the unbound/bound and bound/unbound ratio of NAD(P)H or FAD. However, the a_1/a_2 ratio has a disadvantage. Because $a_1 + a_2 = 1$, the a_1/a_2 ratio can also be expressed as

$$a_1/a_2 = a_1 / (1-a_1)$$

That means, the a_1/a_2 ratio is a nonlinear function of a_1 . This is no problem as long as a_1 remains in the range around 0.5 to 0.6. However, if a_1 gets higher than 0.8 (which is often the case for FAD) the function becomes highly nonlinear. The images then start to look noisy, and it is hard to evaluate the pixel histograms. These get more and more stretched toward larger a_1/a_2 values - please note that a_1/a_2 goes to infinity for $a_1=1$. We therefore find it more useful to base the evaluation of metabolic FLIM data on the amplitude coefficient of the fast component, a_1 . Of course, the amplitude of the slow component can be used as well because $a_1 + a_2 = 1$ [142, 235].

FLIRR Ratio

To navigate around the problems of the classic redox ratio Alam, Wallrabe and Periasamy defined a fluorescence-lifetime redox ratio (FLIRR) [6, 142, 613]. It is the ratio of the fractional amplitudes of the bound decay components of NAD(P)H, $a_2/(a_1+a_2)_{\text{NADH}}$, and FAD, $a_1/(a_1+a_2)_{\text{FAD}}$.

$$\text{FLIRR} = \frac{a_2/(a_1+a_2)_{\text{NADH}}}{a_1/(a_1+a_2)_{\text{FAD}}}$$

The authors have shown that the FLIRR is a robust parameter for evaluation of the cell status. The advantage of the FLIRR is that it does not depend on the relative amounts of NAD(P)H and FAD in the cells, and not on the laser power, the focus accuracy or focus quality, or other instrument parameters. It should be noted that the FLIRR is not the same as the redox ratio. The redox ratio describes the relative amounts of oxidised and reduced NAD(P)H and FAD, the $a_{1\text{FAD}} / a_{2\text{NADH}}$ ratio the relative amounts of bound coenzymes in the cell.

Kalinina et al. [306] have extended the definition of the FLIRR ratio. They found that the FAD decay functions often contain a noticeable contribution from FMN, see 'FAD: The FMN Problem', page 268. FMN has a fluorescence lifetime of about 4.5 ns. This is distinctly longer than the lifetime of bound FAD. The authors therefore run triple-exponential decay on the data in the FAD channel. The FMN component then constitutes the slowest component, a_3 and τ_3 , and a_2 and τ_2 describe the true decay of unbound FAD. Based on the triple-exponential decay parameters in the FAD channel and using the ratios $a_{2\text{NADH}}/a_{1\text{FAD}}$, $a_{2\text{NADH}}/a_{2\text{FAD}}$, and $a_{2\text{NADH}}/a_{3\text{FAD}}$ the authors calculate three different FLIRR ratios.

Metabolic Imaging with the bh FLIM Systems

Due to their high time resolution and clean instrument response function bh FLIM systems are excellent metabolic FLIM systems. Provided the right excitation wavelengths are available, metabolic FLIM can be performed with all bh FLIM systems that have the HPM-100 hybrid detectors. The fast and clean instrument response function in combination with the MLE fit of SPCImage data analysis software then permits efficient separation of the decay components of bound and unbound NADH and FAD - a basic requirement for metabolic FLIM. However, there is another technical feature important for metabolic FLIM with NADH and FAD. This is laser multiplexing. As shown above, NADH and FAD need different excitation wavelength to separate the signals of the fluorophores: Laser multiplexing. Laser multiplexing allows the signals of NADH and FAD to be recorded quasi-simultaneously, making the results for NADH and FAD exactly comparable.

The sections below present typical results obtained with these systems and show details of the NADH and FAD dynamics which have not been seen by any other FLIM techniques yet.

Principle

Metabolic FLIM is based on recording the fractions of bound and unbound NADH and bound and unbound FAD. FLIM data of NADH and FAD are recorded simultaneously by laser multiplexing and parallel dual-channel detection, see 'Excitation Wavelength Multiplexing', page 137. The decay data are analysed by a double-exponential model, yielding the amplitudes, a_1 and a_2 , and the lifetimes, t_1 and t_2 , of the two decay components. The relationship to the metabolic state is illustrated in Fig. 407.

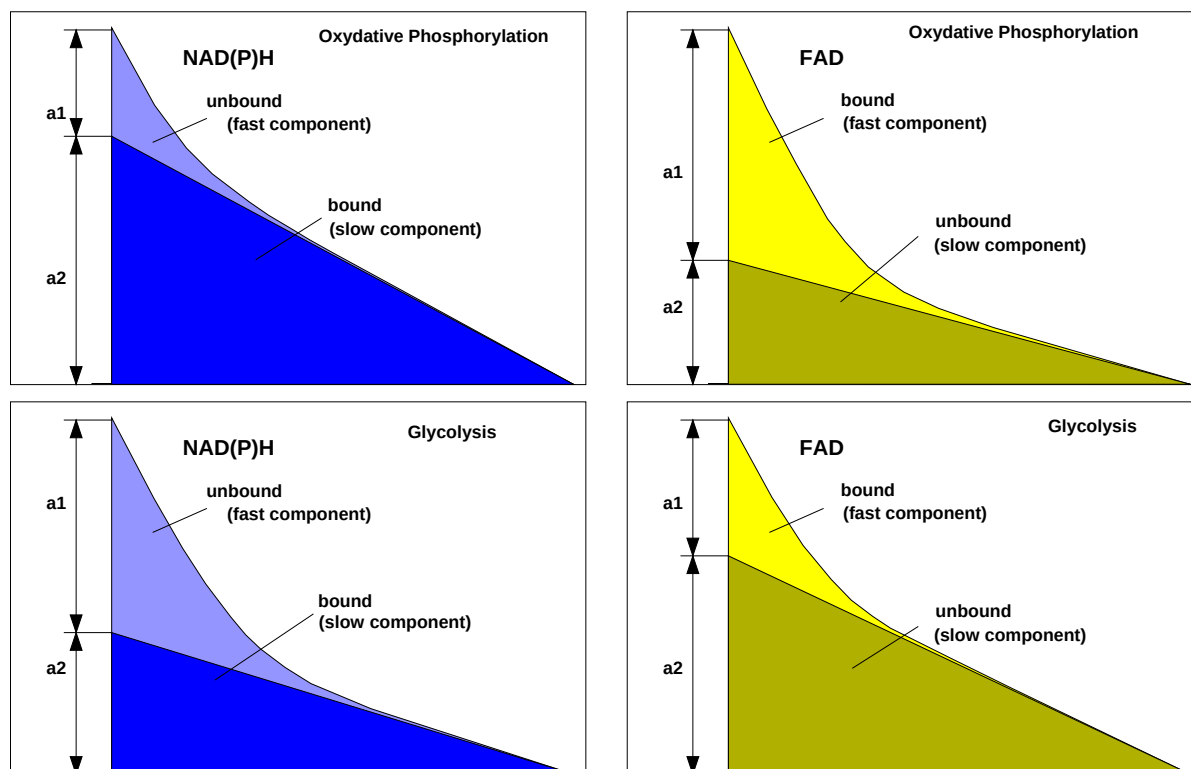


Fig. 407: The composition of the decay functions of NAD(P)H and FAD and changes with the metabolic state.

The decay components, a_1 and a_2 then directly represent the fractional amount of bound and unbound NADH or FAD. The ratio of the amplitudes a_1/a_2 , often called 'metabolic ratio', directly represents the concentration ratio of unbound/bound NADH or bound/unbound FAD.

The figure shows the general composition of the decay curves of NADH and FAD. In the NADH decay functions, there is a fast decay component from the unbound NADH and a slow component from the bound NADH. For the FAD it is the opposite. The fast component is from bound FAD, the slow component is from unbound FAD. The ratio of the amounts of bound and unbound NADH and FAD depends on the metabolic state. A cell can run both a oxidative metabolism (oxidative phosphorylation) and a reductive one (glycolysis). A shift from oxidative phosphorylation ('oxphos') to glycolysis is accompanied by a decrease of the bound fractions of NADH and FAD. Consequently, a shift from oxphos to glycolysis causes an increase in a_1 in the NADH decay and a decrease in a_1 in the FAD decay. A shift from glycolysis to oxphos results in the opposite: a_1 of NADH decreases, a_1 of FAD increases.

The changes in real decay data are shown in Fig. 408. The upper row shows decay functions from normal cells, NADH is shown left, FAD right. The lower row shows the corresponding decay curves for a tumor cell. The decay parameters are shown in the inserts. As expected, a_1 of NADH is higher in the tumor than in the normal cell. In the FAD curves, a_1 is lower in the tumor than in the normal cell. This is exactly the behaviour that has to be expected for the model illustrated in Fig. 407. The differences are not very large but, nevertheless, are clearly detected by TCSPC FLIM.

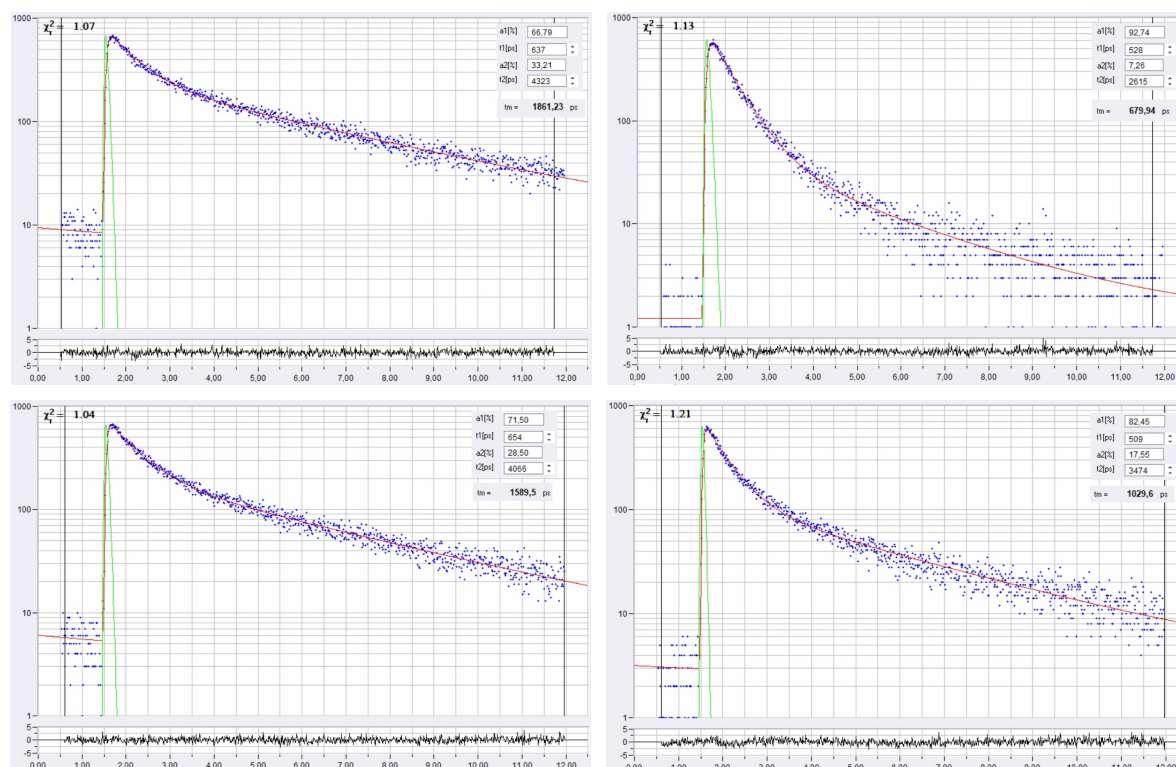


Fig. 408: Real decay data for NADH and FAD in a normal cell and in a tumor cell. Upper row: Decay curves of normal cell, NADH (left) and FAD (right). Lower row: Decay curves of tumor cell, NADH (left) and FAD (right).

The opposite changes in the decay parameters of NAD(P)H and FAD are a possible explanation of numerous discrepancies in fluorescence lifetimes measured in tumors. There have been as many reports for decreased lifetime as for increased ones. The source of the discrepancies is probably that the measurements did not cleanly separate the signals of NADH and FAD. Depending on which fluorophore dominates the net decay function the result can indeed be a decrease or an increase of the lifetime in the tumor compared to normal tissue. Another possible pitfall is in the detection of FMN, please see 'FAD: The FMN Problem', page 268.

Metabolic Imaging of Excised Human Bladder Tissue

The principles presented above were verified using a basic DCS-120 confocal FLIM system. The system was equipped with a 375 nm and a 405 nm BDS-SM ps diode laser. The instrument was placed in a hospital. FLIM data were obtained from human bladder cells excised during surgery. The patients had been diagnosed with bladder cancer or other suspicious lesions by classic endoscopy. Measurements on the excised material were performed immediately after surgery, before the material went to histology [565]. Representative results are shown in Fig. 410 through Fig. 412.

Fig. 409 shows NAD(P)H images of the amplitude-weighted lifetime, t_m . The left image shows normal cells, the right image tumor cells. It should be noted that the image of the 'normal' cells also contains some tumor cells, and the image of the 'tumor' cells some normal cells. This is unavoidable due to the procedure by which the cells were excised. Nevertheless, the behaviour is as expected - t_m is shorter in the tumor sample.

NAD(P)H images of the amplitude, a_1 , of the fast decay component are shown in Fig. 410. There is a clear difference in a_1 between the normal cells and the tumor cells. The normal cells have an average a_1 of about 0.65 (or 65% of the total amplitude). The cancer cells have an a_1 between 75% and 80%. We find similar values consistently in data of different cells.

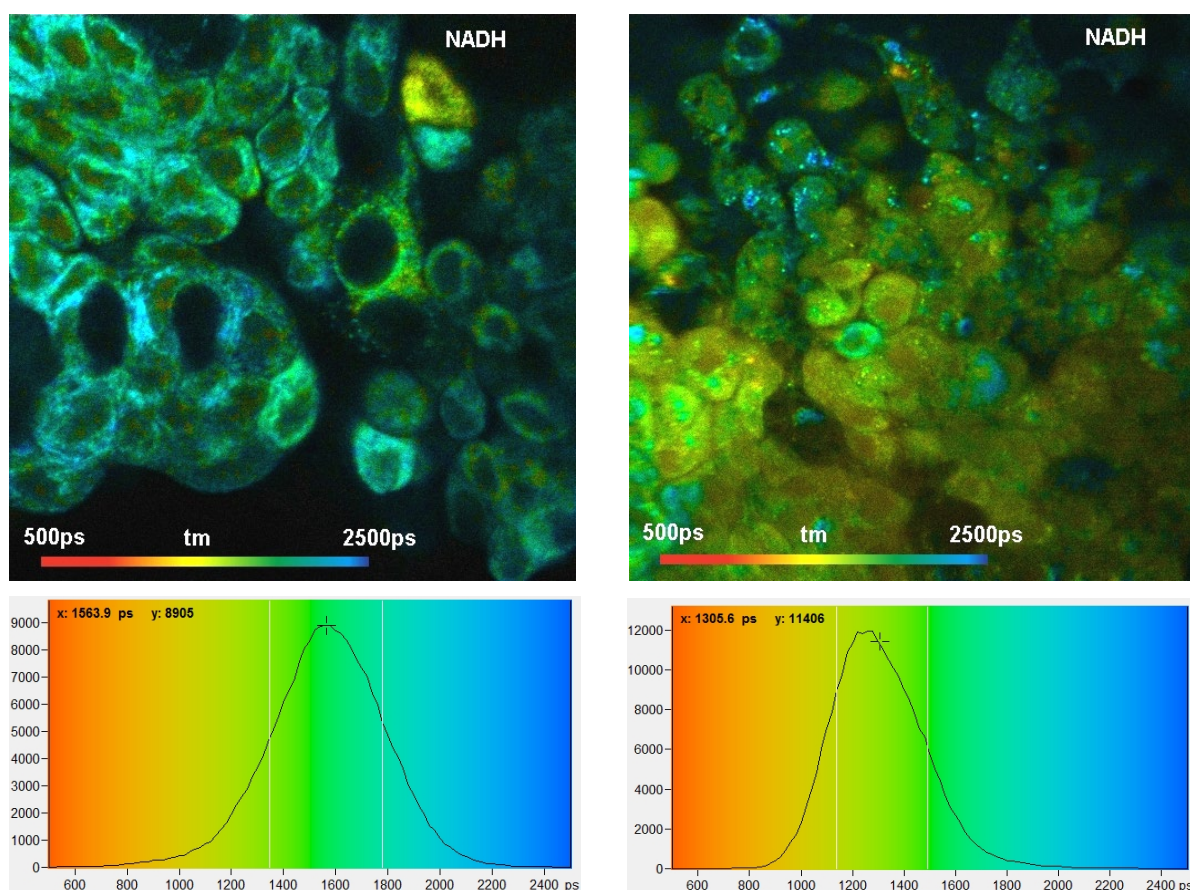


Fig. 409: NAD(P)H t_m images for normal cells (left) and tumor cells (right). Lower row: Histograms of t_m over the pixels of the images. As expected, t_m is shorter in the tumor sample.

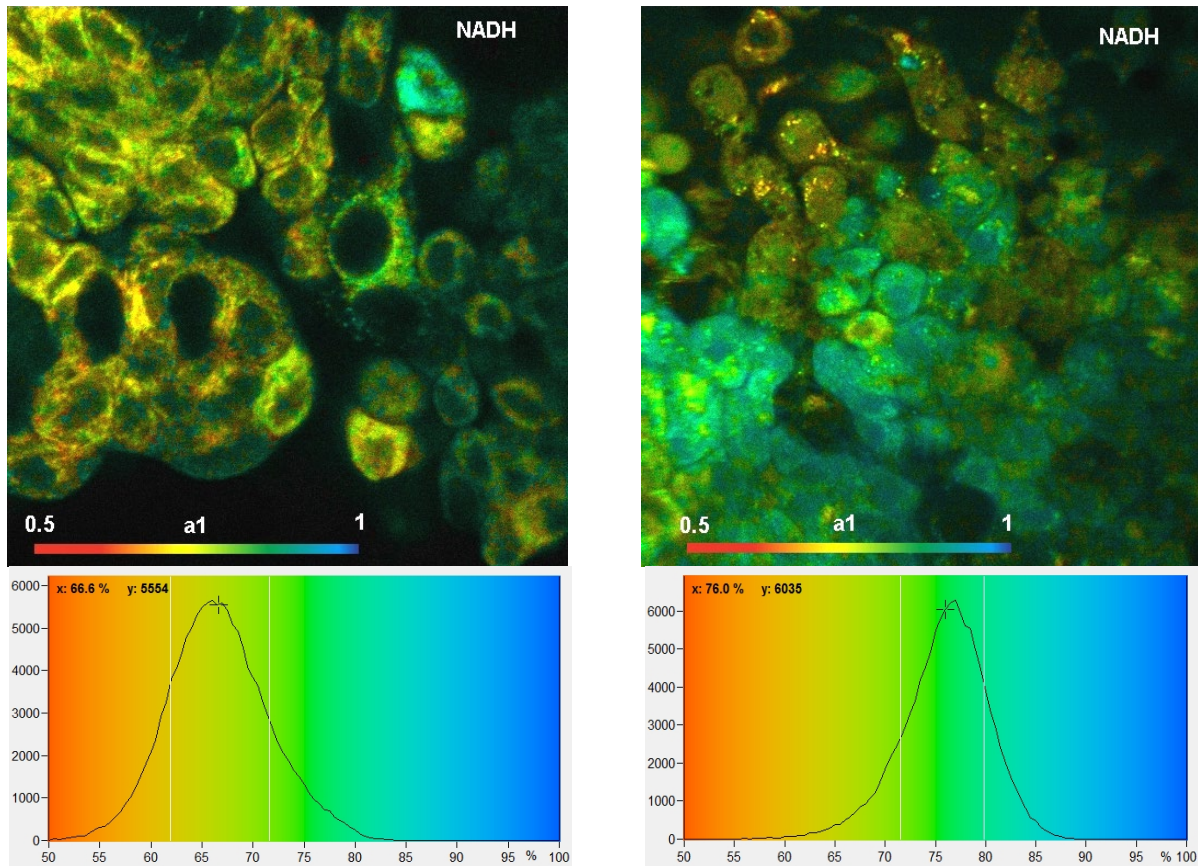


Fig. 410: NAD(P)H a_1 images for normal cells (left) and tumor cells (right). Lower row: Histograms of a_1 over the pixels of the images. The histogram of the tumor sample is from lower half of the image because the upper half contains also normal cells.

FAD data are shown in Fig. 411. In general, also the FAD data show the expected trend. a_1 decreases for the cancer cells, indicating that they contain less bound FAD (note a_1 is the *bound* component of FAD). However, some details are not entirely plausible. On the one hand, cells in the upper part of Fig. 411, right, have a significantly different a_1 than the cells in the normal-cell sample (Fig. 411, left). On the other hand, in the NAD(P)H images the same cells have *the same* a_1 as the cells in the 'normal' sample (compare Fig. 410, left and right). However, there is a possible explanation. The redox ratio in the tumor sample may be so low that the FAD intensity gets extremely weak. It is then possible that residual NAD(P)H fluorescence excited by the 410-nm laser is detected in the FAD channel. The fluorescence in the yellow cells in Fig. 411 may then be from NAD(P)H, not FAD. A inspection of the decay curves in the yellow areas shows indeed that the decay functions are more compatible with NAD(P)H than with FAD. An improvement can probably be obtained by using a slightly longer excitation wavelength for FAD excitation.

Fig. 412 shows the FLIRR calculated from the same data sets. As expected, the FLIRR shifts to lower values for the cancer cells.

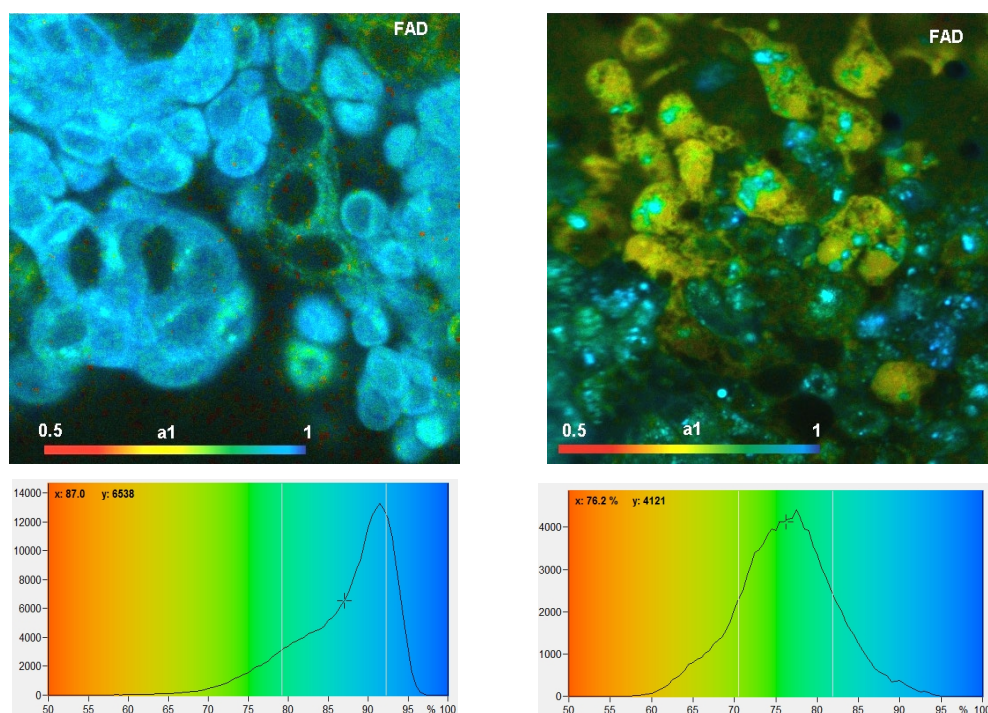


Fig. 411: FAD a1 images for normal cells (left) and tumor cells (right). Lower row: Histograms of a1 over the pixels of the images. The histogram of the tumor sample is from lower half of the image because the upper half contains also normal cells.

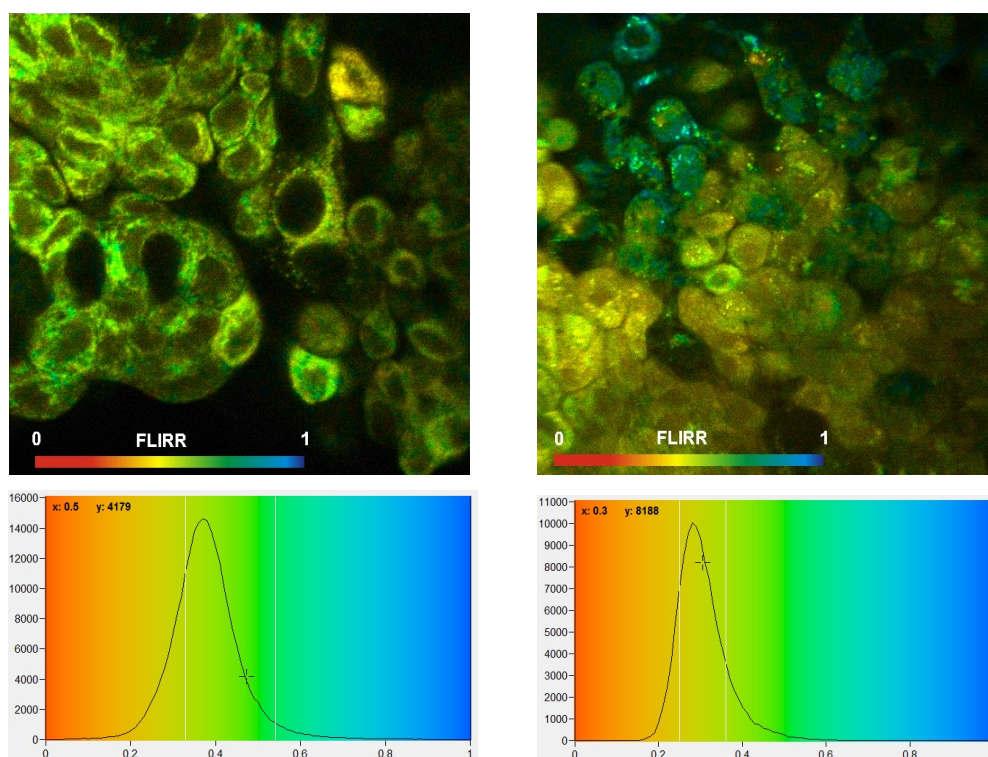


Fig. 412: FLIRR images of normal cells (left) and tumor cells (right). Lower row: Histograms of the FLIRR over the pixels of the images. The histogram of the tumor sample is from lower half of the image.

Comparison of NAD(P)H FLIM and Histology Results for Clinical Diagnosis

A comparison of the results of classic endoscopy, NAD(P)H FLIM, and histology is given in Fig. 413. FLIM data were obtained from the material from 7 patients [78, 565]. Initial endoscopy had diagnosed two of them with non-cancer lesions, and 5 of them with cancer.

The a_1 FLIM images showed normal a_1 for patients 1 and 2, a suspiciously increased a_1 for patients 3, an increased a_1 for some of the cells from patient 6, and a massively increased a_1 in the entire sample from patient 7. By using a selection criterion of $a_1 = 0.71$ as the tumor threshold, the FLIM-based diagnosis is as indicated in the figure. A threshold of $a_1 = 0.71$ may appear somewhat artificial but is supported by other NAD(P)H FLIM data [199, 381, 532].

Histology diagnosed the patients 1 and 2 as healthy, patients 2, 3, and 4 as inflammation, patient 6 as low-grade tumor, and patient 7 as high-grade tumor. The FLIM results were thus in perfect agreement with histology, and much better than the initial endoscopy results [565]. We therefore believe that FLIM can favourably complement histology. In this respect, a substantial benefit of FLIM is that, in contrast to histology, the results are available within minutes after surgery.

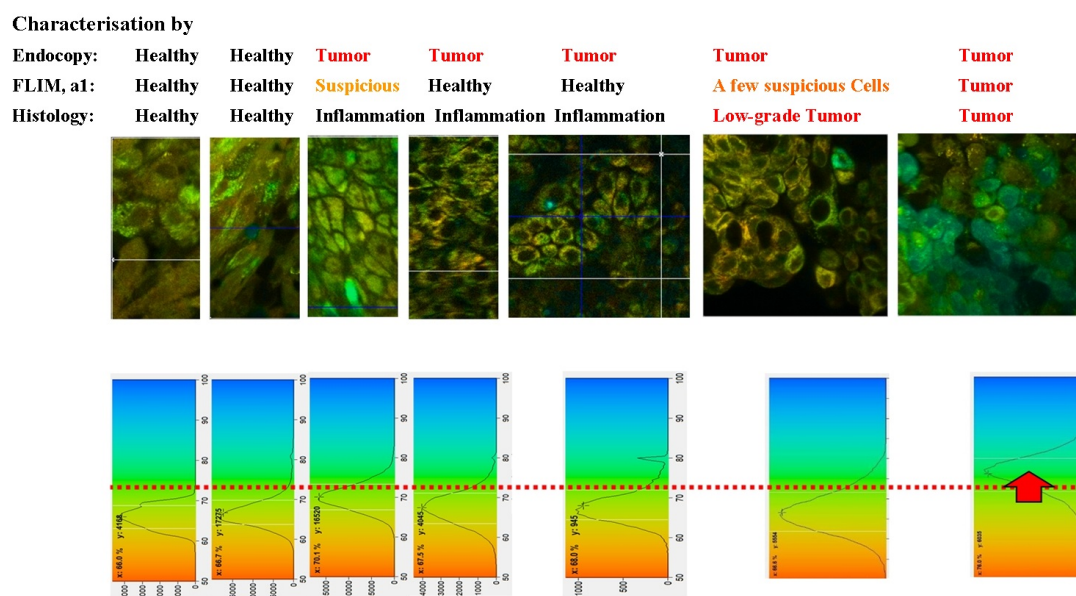


Fig. 413: Comparison of endoscopy, NAD(P)H FLIM, and histology results

Using the FAD FLIM Data for Clinical Diagnosis

Using the FAD data in combination with the NADH data turns out to be more difficult than expected. One problem is that there is a possible contribution of a decay component from of FMN, see page 268. The second problem is that the FAD signal can be extremely weak. Although the FLIM system is sensitive enough to detect images in these cases the decay data contain fluorescence of contaminations and residual fluorescence of NAD(P)H. Low FAD intensity was found especially in the data from cancer cells. Whether this is due to intrinsically low FAD concentration or extremely low redox ratio is not known. Moreover, there is still disagreement about the mechanism of the lifetime changes of FAD. Even the direction of the change with the metabolic state is discussed controversially. Be it as it may, additional research effort is required to make the FAD decay parameters a reliable indicator for clinical diagnosis.

To avoid spoiling metabolic data from NADH with possibly misinterpreted FAD data, FAD data can be used in a way that they supplement the NADH data but do not get entirely mingled with them. A possible way to do so is two-dimensional histogramming of the amplitudes. A histogram of a_1 of FAD (vertical axis) versus a_1 of NADH (horizontal axis) is calculated and displayed as shown in Fig. 414. Normal cells are characterised by low a_1 of NADH and high a_1 of FAD. They are expected to cluster in the upper left part on the histogram. Tumor cells have high a_1 of NADH and low a_1 of FAD. They are expected in the lower right part. If

the FAD data are of poor quality the histogram just washes out vertically, but horizontally the distribution remains conclusive.

An example of 2D histogramming is shown in Fig. 414. The data were taken from the images of the 'low-grade tumor' sample shown Fig. 413, second right. The histogram of the entire sample (Fig. 414, top) shows indeed indications of clustering.

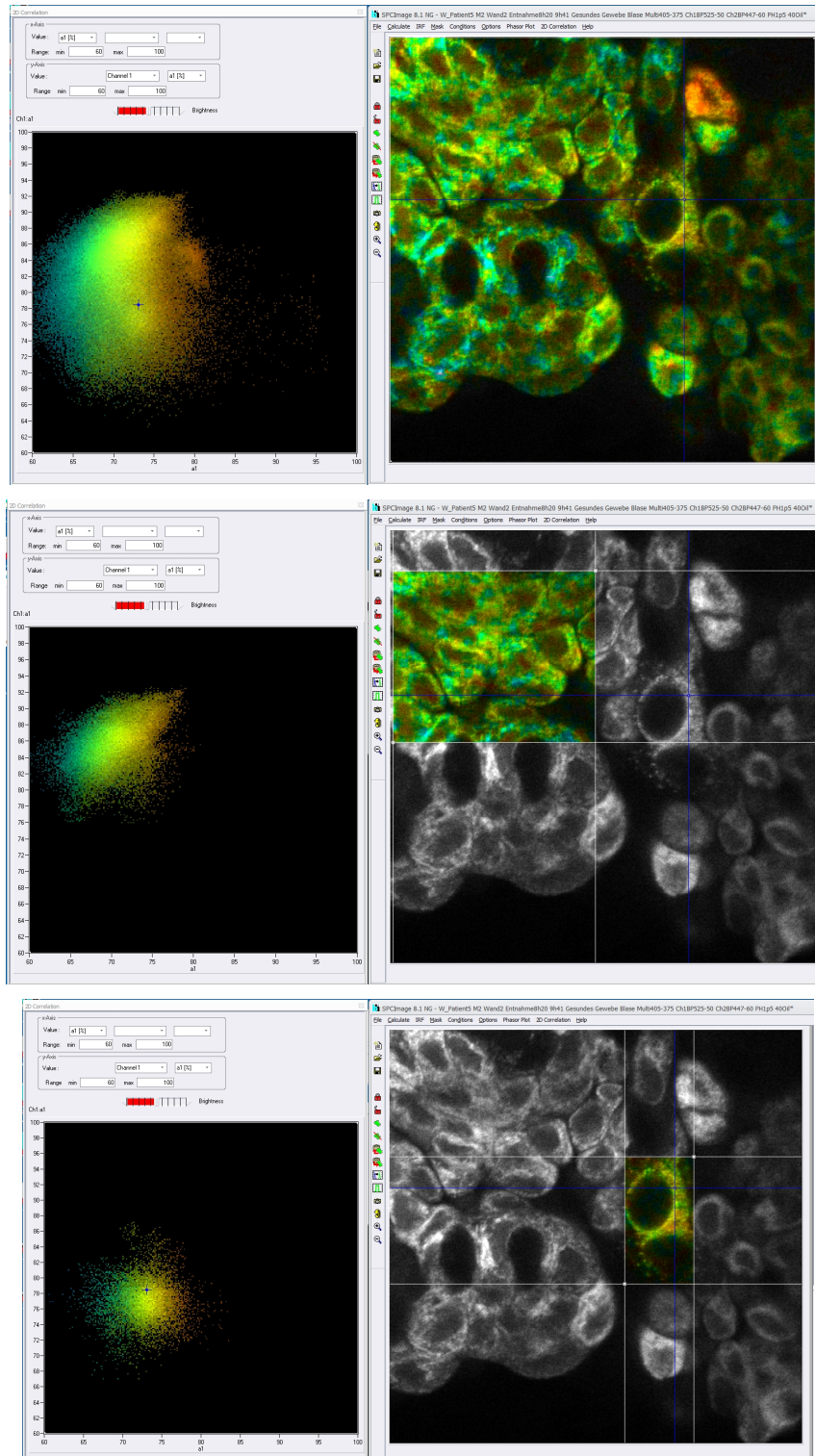


Fig. 414: Histograms of a_1 of FAD (vertical) versus a_1 of NADH (horizontal). t_m images from the NADH channel shown on the right. Top: Histogram from entire image. Middle: Histogram from mostly healthy cells. Bottom: Histogram from suspected tumor cell.

Fig. 414, middle, shows the histogram of cells in the upper left of the image. These cells are presumed normal or close to normal. Fig. 414, bottom shows the histogram from a suspected tumor cell. Interestingly, the difference in a_1 of NADH (horizontal axis) between the normal cells and the tumor cells is not significant but the difference in a_1 of FAD clearly is. This shows that 2D histogramming of the two a_1 against one another is a possible way to combine the information from NADH and FAD data.

Another possibility would be to display the FAD/NAD(P)H intensity ratio on the vertical axis. If low FAD intensity, either by low concentration or low redox ratio, is a cancer indicator this would add important information to the NAD(P)H a_1 data. In any case, further work appears necessary to make the FAD signal a reliable source of clinical information.

Metabolic Imaging by Macro FLIM

Metabolic FLIM can be performed with the bh DCS MACRO system, see page 8. In the simplest case, the sample is excited by a 375-nm BDS or BDL laser, and the fluorescence of NADH is detected between 440 and 470 nm. To determine the metabolic state double-exponential decay analysis is required, which requires to record about 1000 photons within the binning area in SPCImage [91]. It has been shown that this is well within the reach of the DCS MACRO system [99, 381, 352, 353, 532, 544, 541, 644]. Metabolic FLIM with the DCS-120 MACRO system is therefore a real option for the investigation of tissue samples from tumor surgery. The FLIM data would not show whether the excised tissue is cancerous or not, but also deliver valuable information on the metabolism of different tumor tissues.

Two examples are shown in Fig. 415 and Fig. 335. Fig. 415 shows an open tumor in a mouse and a freshly excised rat brain with a glioblastoma. The image is false-colour images of the amplitude of the fast (unbound) decay component, a_1 , of NADH. Decay curves from the tumor region and the healthy tissue are shown on the right.

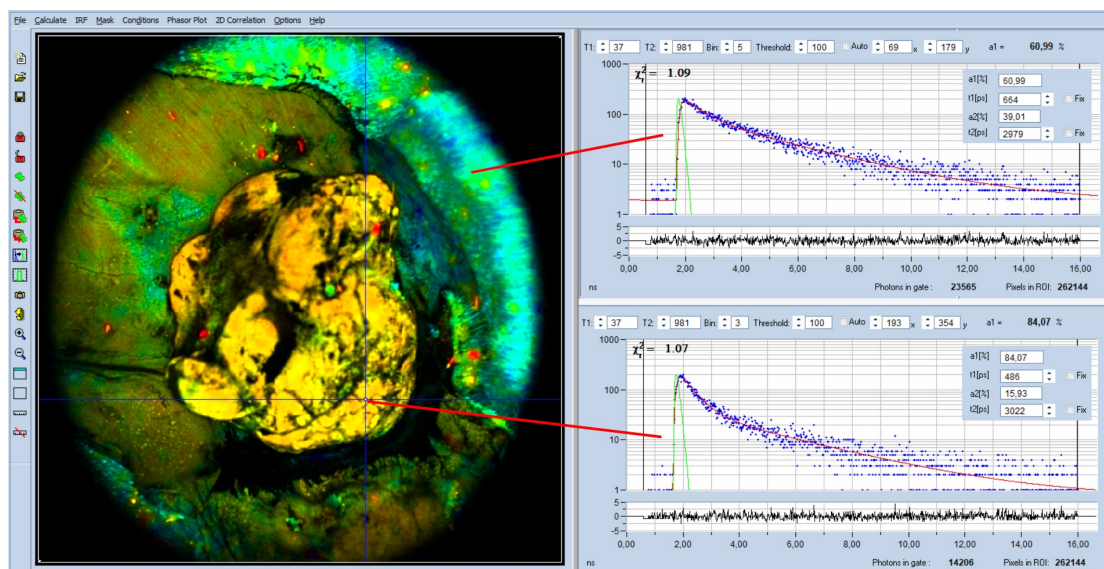


Fig. 415: Open tumor in a mouse. NADH image, excitation 375 nm, detection 430 to 475 nm. Image size 15 x 15 mm. False-colour image of the amplitude, a_1 , of the unbound NADH component.

Fig. 335 shows a similar image of a freshly excised rat brain with a glioblastoma. The acquisition time for both images was about 100 seconds, the data were analysed by the maximum-likelihood fit of SPCImage NG. The decay parameters of the mouse tumor are exactly as expected. The component lifetimes are the typical ones for NADH, and the amplitude, a_1 , is 61 % in the normal tissue and 84 % in the tumor.

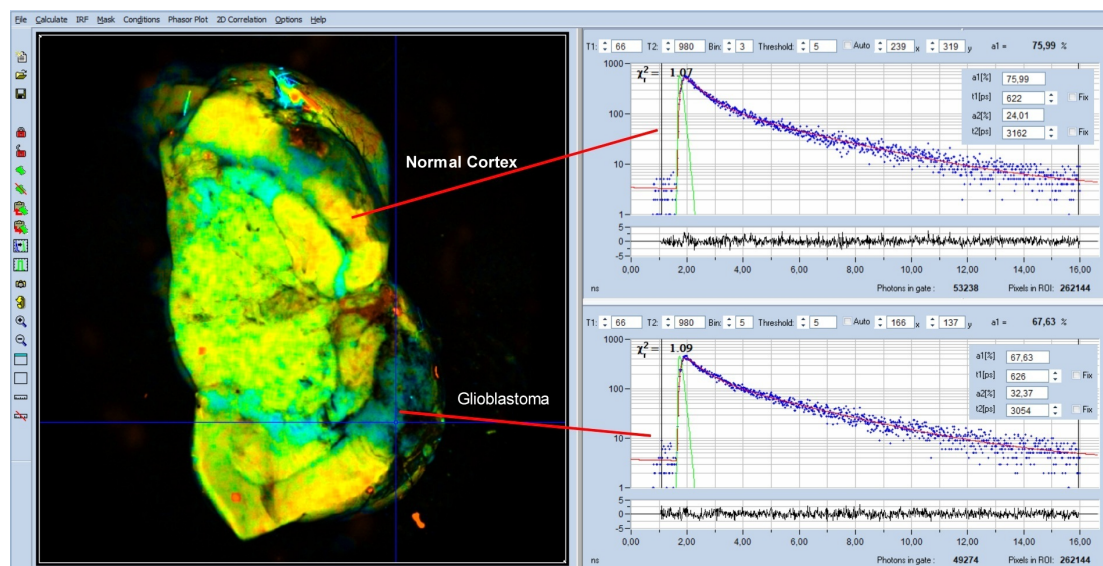


Fig. 416: Rat brain with glioblastoma (right). Autofluorescence, excitation 375 nm, detection 430 to 475 nm

The rat brain shows entirely different spectroscopic behaviour. Also here, the lifetimes are in the range expected for NADH. However, the amplitude, a_1 , does not follow the expected trend. It is 75% in the normal brain tissue and 67% in the tumor. This is the opposite of what has to be expected. It is not clear what causes this discrepancy. It is possible that glioblastoma just do not follow the usual trend. However, it is also possible that the unusual a_1 is a reaction of the tissues to the environment conditions. There is no perfusion, the temperature is not in the physiological range, and the partial pressure of O_2 and CO_2 is not in the correct range. Brain tissue has a high metabolic rate. From this point of view, deviations from the normal behaviour appear plausible. Especially the fact that a_1 of the normal brain tissue is in the tumor range supports the assumption that the tissue became hypoxic. The possibility of hypoxic conditions is supported also by [644] where a_1 is in the glycolysis range (above 70%) both for the glioblastoma and the normal brain tissue. Further investigation, such as measurement of a brain by a fibre-based system, are necessary to make final conclusions. Additional information could also be derived from the recording of FAD FLIM images.

The MACRO FLIM system can be extended to simultaneous measurement of NADH and FAD. Two lasers, 375 nm and 420 nm, would be multiplexed in time, and FLIM data of the two fluorophores recorded in two spectral emission channels. Please see 'Excitation Wavelength Multiplexing', page 137.

An inconvenience of MACRO FLIM is that the detection efficiency is lower than when the scanner is attached to a microscope. The acquisition times are therefore relatively long. The reason is the low NA of the detection light bundle in the image plane of the scanner. It could be increased by larger galvanometer mirrors in the scanner, but the options for that are limited by mechanical restrictions. The effect on the acquisition time can, however, be compensated by higher laser power. Due to the large scan area, the excitation light is distributed over a large area. An increase of the laser power by a factor of 10 or more should therefore not induce invasive effects on the sample. Suitable lasers, such as frequency-multiplied fibre lasers, OPOs or Ti:Sa Lasers are available. The only obstacle to their widespread application is the high price of these laser system.

Metabolic Imaging Combined with pH Imaging

The NAD(P)H lifetimes in the mitochondria of a cell are influenced by local pH. It has therefore been suggested to supplement metabolic FLIM measurements by pH measurement. In measurements using the fluorescence lifetime of NAD(P)H the pH can be used to correct the

results for the pH dependence, in amplitude-based measurements it delivers additional information on the status of the cell [498]. In such experiments, it is, of course, desirable to run the pH measurement simultaneously with the measurement of the metabolic state. This requires two parallel TCSPC FLIM channels. One is used for the NAD(P)H, the other for a pH-sensitive fluorescence probe.

A good candidate for a pH probe is BCECF (2',7'-bis-(2-carboxyethyl)-5(6) carboxyfluorescein). It has a relatively large pH dependence of its fluorescence lifetime [270], it can be excited at the same wavelength as NAD(P)H, its emission spectrum does not overlap much with the emission of NAD(P)H, and it has no toxic effects on cells and tissues.

For the experiments described here we used a DCS-120 MP two-photon FLIM system with a femtosecond fibre laser [87]. The laser has an emission wavelength of 785 nm. Two-photon excitation at this wavelength excites both the NAD(P)H and the BCECF at reasonable efficiency. The fluorescence of the two compounds was collected via a non-descanned beam path and split spectrally by a dichroic beamsplitter and filters. The signals were recorded in two parallel TCSPC FLIM channels of the DCS-120 system. With the HPM-100-40 detectors and the SPC-180NX FLIM modules used in this system the IRF width is about 85 ps, full width at half maximum.

A typical result is shown in Fig. 38. The NAD(P)H image (on the left) shows the amplitude-weighted lifetime, τ_m , as it is normally used in metabolic FLIM. For the BCECF image we used the intensity-weighted lifetime, τ_i , because it is the closest match to the phase-and-amplitude lifetime used in [270]. The τ -pH curve published in [270] therefore applies also to the BCECF lifetimes in Fig. 38, right.

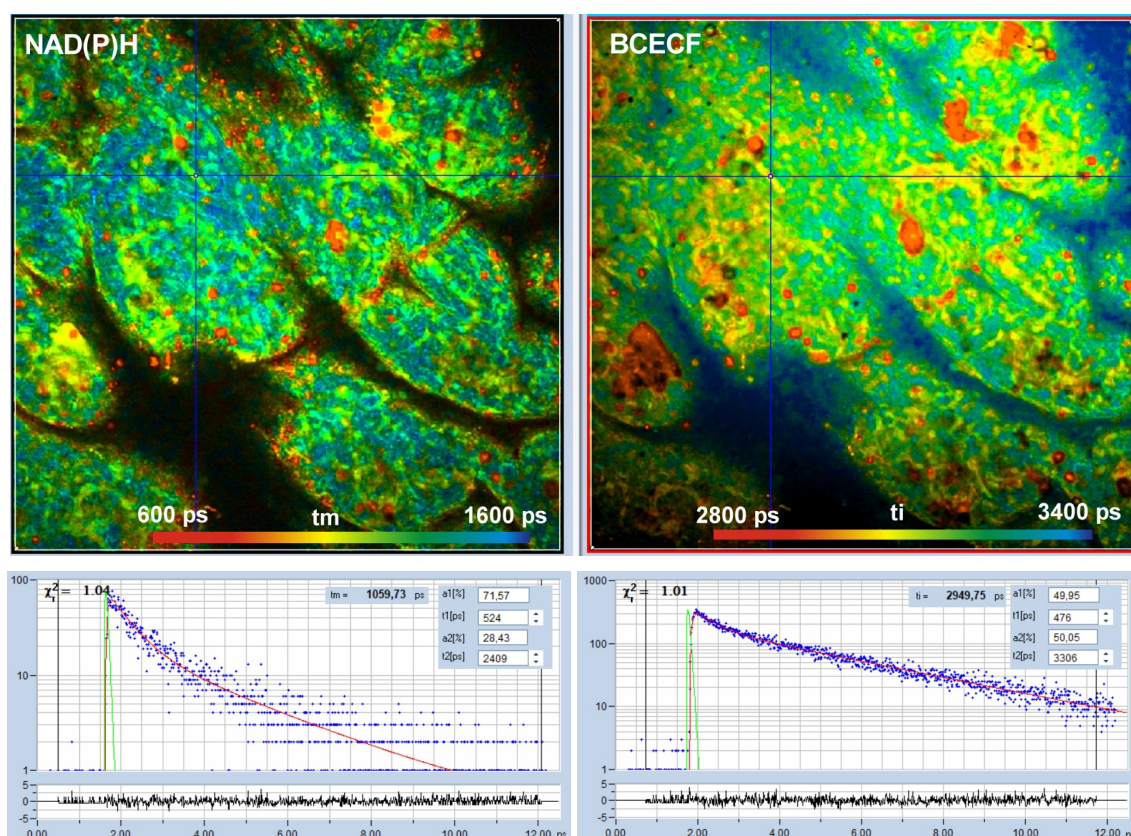


Fig. 417: Upper row: NAD(P)H image of the amplitude-weighted lifetime, t_m , and intensity-weighted lifetime image of BCECF. Lower row: Decay function of NAD(P)H and decay function of BCECF at cursor position. Both images 512 x 512 pixels, 1024 time channels. Data analysis with bh SPCImage NG, MLE fit.

Fig. 38 uses weighted averages of the component lifetimes, τ_1 and τ_2 , to characterise the metabolic state and the pH of the tissue. As mentioned above, fluorescence lifetimes are not the most reliable parameters to characterise the metabolic state. A more reliable way to characterise the metabolic state by the relative amount of free NAD(P)H, a_1 , or the ratio of bound/free NAD(P)H, a_1/a_2 .

A similar consideration applies also for the BCECF. Different pH induces differences in the relative amounts of protonated and de-protonated BCECF. These have different fluorescence lifetimes. As a result, a_1 shows the relative amount of protonated BCECF, a_1/a_2 the ration of protonated and de-protonated BCECF.

Images of the amplitude, a_1 , of the fast decay component for NAD(P)H and BCECF are shown in Fig. 418. For technical details and possible pitfalls please see [100].

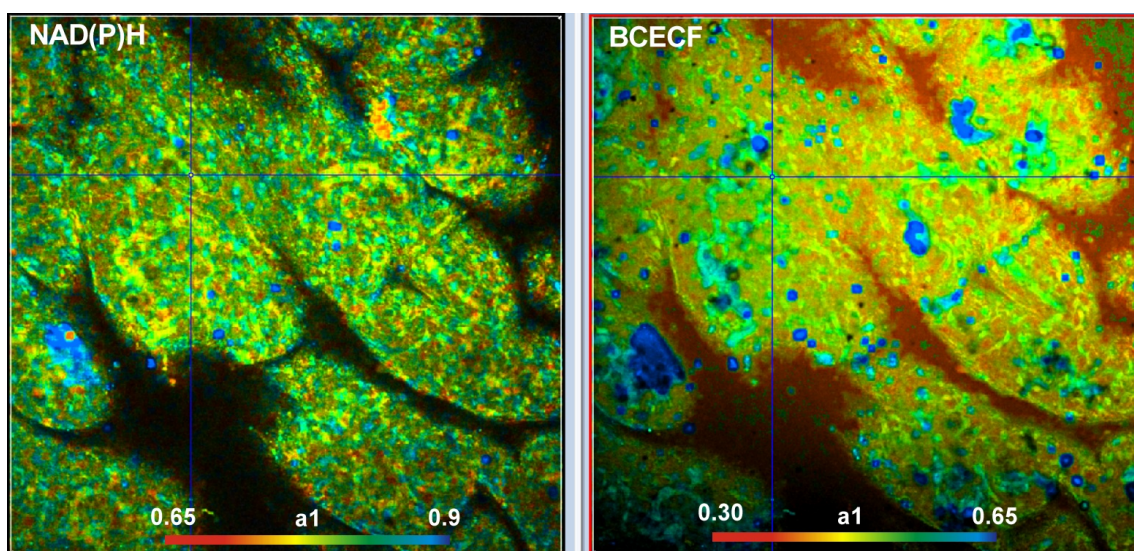


Fig. 418: NAD(P)H image (left) and BCECF image (right) of the amplitude, a_1 , of the fast decay component. In the NAD(P)H image a_1 represents the fraction of unbound NAD(P)H, in the BCECF image it represents the amount of protonated BCECF.

Complex Decay Behaviour of NADH and FAD

NADH: Additional Decay Components

The fact that a double-exponential model fits the decay curves of NADH well does not mean that there are only two decay components. In fact, there is direct evidence that the ‘fast’ and the ‘slow’ decay components consist of several sub-components. There are reports that the fluorescence of NAD(P)H in cells has at least three, if not four components [122, 217, 637, 638]. This is confirmed by data obtained with the bh ultra-fast hybrid detectors. Fig. 420 shows a 2-photon NAD(P)H image of live 'Ishakawa' cells. A double-exponential fit in an area of 5x5 pixels delivers a good fit with component lifetimes of 356 ps and 2546 ps. A triple-exponential fit splits the slow component into two, even though it does not yield a visibly better fit quality. The component lifetimes are 290 ps, 1393 ps, and 5868 ps, see Fig. 420.

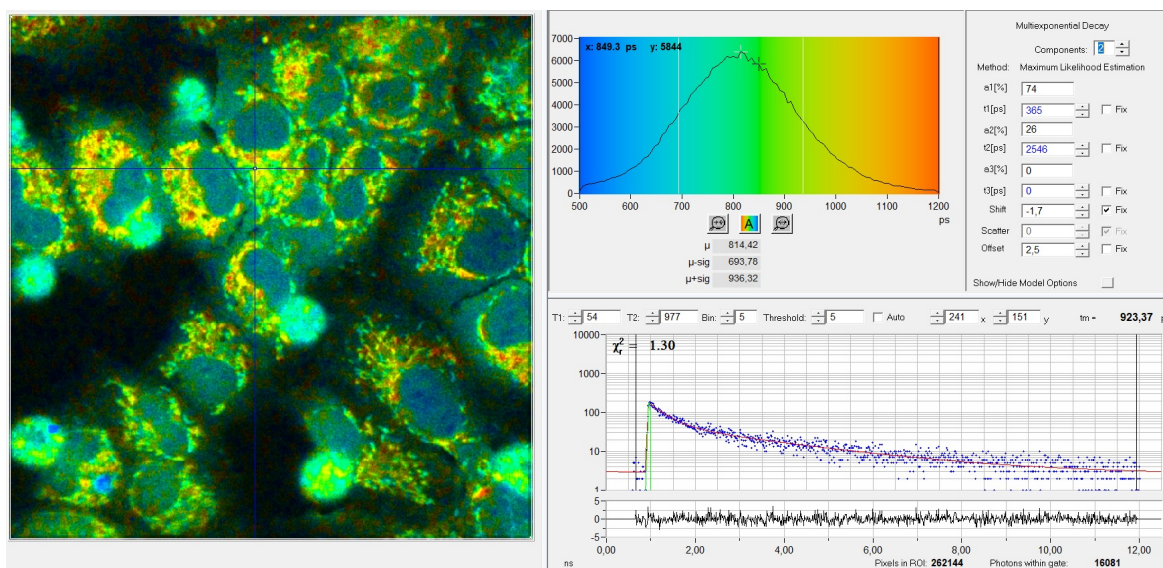


Fig. 419: NAD(P)H FLIM with ultra-fast detector, IRF width <20 ps. A fit with a double-exponential model delivers component lifetimes of 356 ps and 2546 ps.

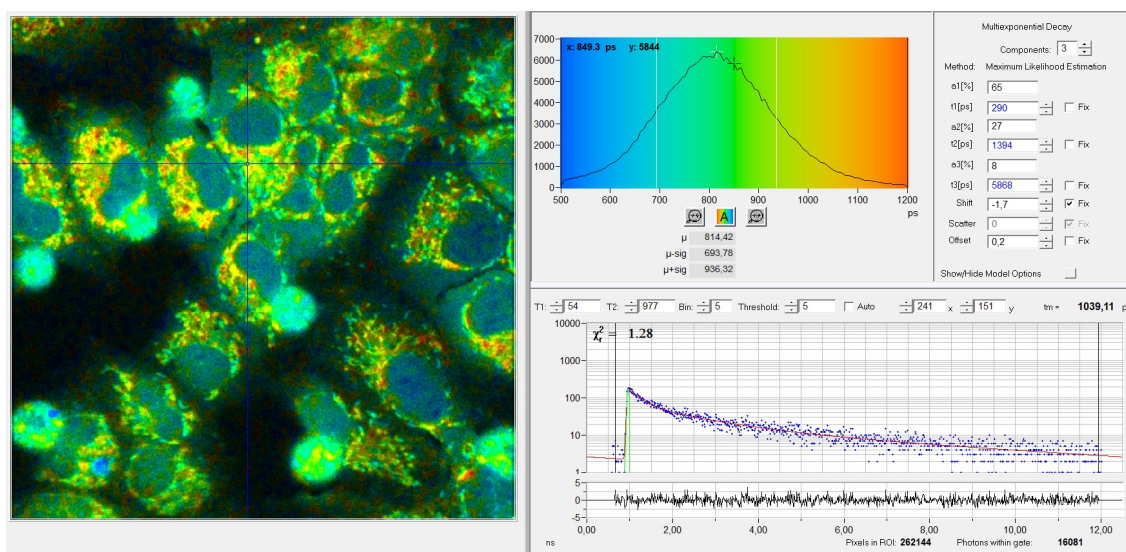


Fig. 420: NAD(P)H FLIM with ultra-fast detector, IRF width <20 ps. Triple-exponential analysis shows three decay components, with lifetimes of 290 ps, 1393 ps, and 5868 ps.

Often additional components can only be identified in data from larger areas, either by binning pixels, or by defining regions of interest and combining the decay data of the pixels. However, the component lifetimes can also be different in different compartments of the cell, and even different in different mitochondria. An example is shown in Fig. 421. It shows an image of the slow (bound) component of NAD(P)H obtained from the usual double-exponential fit. Similar differences can be found in the fast component, see 'NADH FLIM with Ultra-Fast Detectors', page 217.

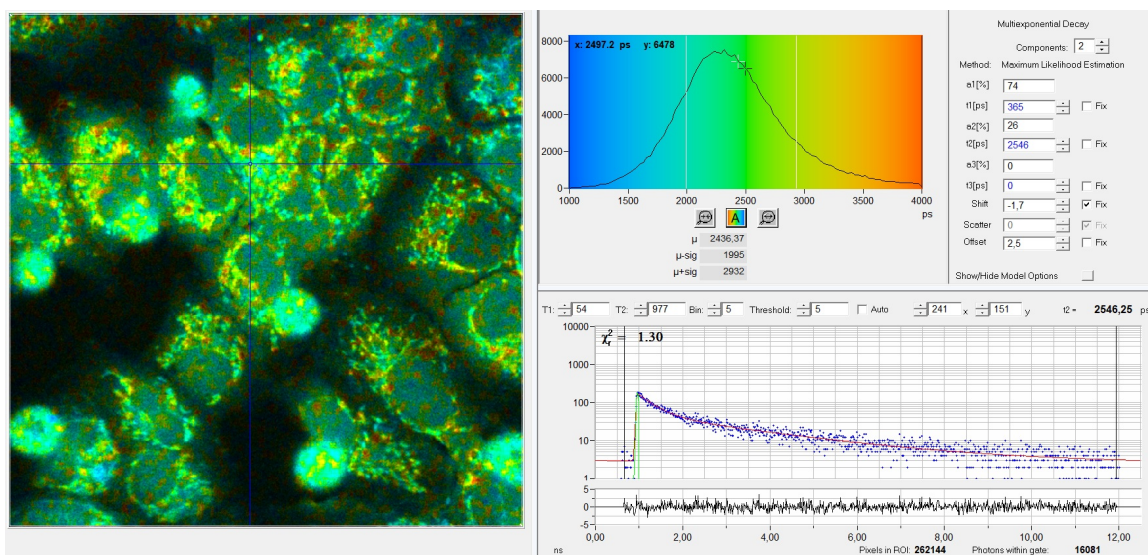


Fig. 421: Image of the lifetime of the slow component, t_2 , from the double-exponential model. Different mitochondria have different t_2 .

Caution is recommended when NADH FLIM is performed in tissue. Especially in skin tissue there can be other fluorophores which interfere with NADH. Especially Keratin has excitation and emission spectra which overlap those of NADH and FAD [384]. In how far contamination of the metabolic-FLIM signature can be avoided by image segmentation or by including the known lifetime of Keratin in the decay analysis as a fixed component is not known.

FAD: The FMN Problem

Also for the FAD, there are indications that the decay functions contain more than two components. More importantly, it seems that the signal recorded in the FAD channel does not always come from FAD (flavin adenine dinucleotide) alone but also from FMN (flavin mononucleotide). FMN has the same fluorescence spectrum as FAD but a longer lifetime.

Fig. 422 shows an FAD image of freshly excised human epithelial bladder cells, please see page 258 for details. The sample was imaged by laser-multiplexing and detection in an NADH channel and an FAD channel. According to the NADH images, the sample contains mostly normal cells, but also a few (possibly tumorous) cells with an enhanced fraction of free NADH. In Fig. 422, an ROI was chosen which contains only cells that qualify 'normal' in the NADH channel. The decay curve of the combined pixels within the ROI is shown on the right. Triple-exponential analysis clearly delivers a decay component of 4.8 ns, present with an amplitude of 3.9%. A lifetime of 4.8 ns cannot be attributed to FAD, but very well to FMN.

Fig. 423 shows the same data, but the ROI was set on cells which are classified as 'low grade cancer' in the NADH image. The lifetime of the third component is 3.38 ns, not much different than in Fig. 422. However, the amplitude has increased to 8.84%.

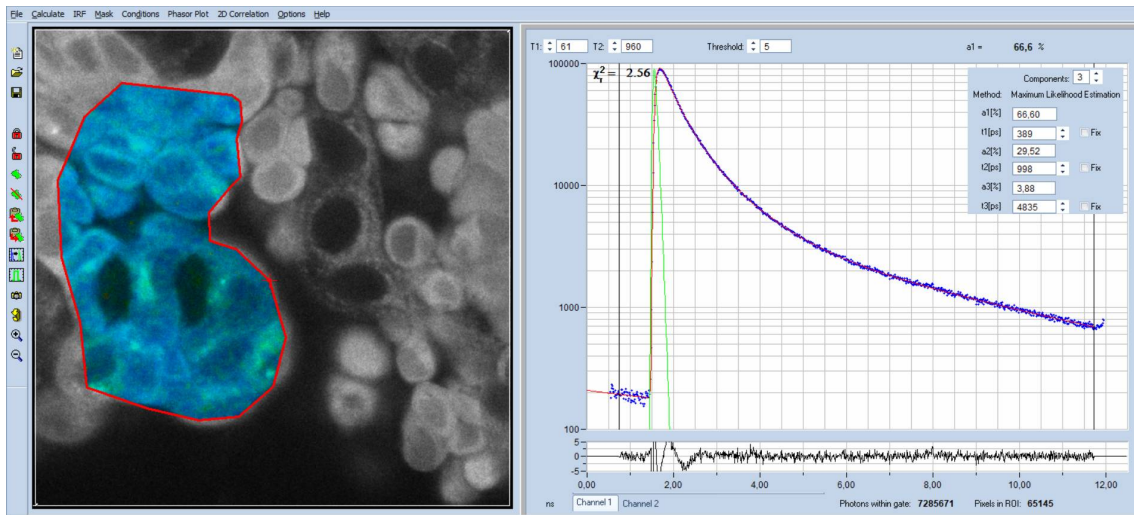


Fig. 422: Left: FAD Image of human epithelial bladder cells. The region of interest includes healthy cells only. Right: Decay curve of combined pixels in ROI, and triple-exponential fit. The third component, with $t_3 = 4.83$ ns and $a_3 = 3.88\%$, is compatible with FMN.

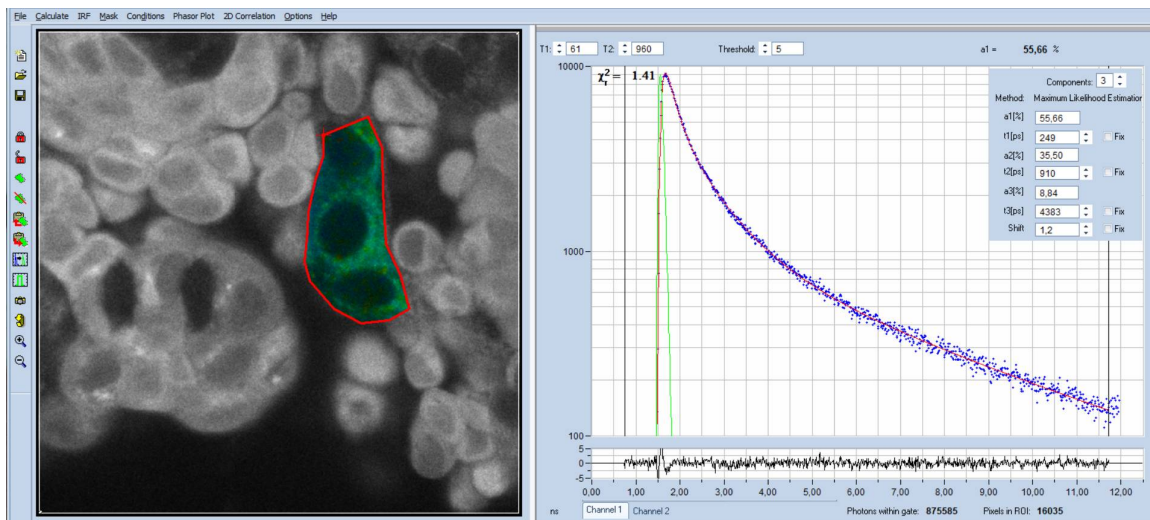


Fig. 423: Same data as in Fig. 422, but ROI on cells which are probably tumor cells. The third component is $t_3 = 4.38$ ns and $a_3 = 8.84\%$.

The presence of a decay component from FMN can have implications to metabolic imaging, especially if FAD data are used for cancer diagnosis. Certainly, an amplitude contribution of 3.8% or even 8.84% from FMN does not render the FAD data useless. It can, however, introduce unpredictable shifts in the metabolic parameters. The results can then no longer be considered quantitative. The shifts have not only consequences to the amplitudes, a_1 and a_2 which are determined for FAD, but also on FLIRR values derived from them. Kalinina et al. therefore suggested new definitions of the FLIRR based on triple-exponential analysis of FAD [306].

In Fig. 424 an attempt was made to extract the amplitudes of bound FAD (t_1), unbound FAD (t_3), and FMN (t_3) from FLIM data on a pixel-by-pixel basis. With all three lifetimes and amplitudes freely floating the result is not satisfactory [100]. However, when the lifetimes are fixed to the most frequent values (see SPCImage, Pseudo-Global Analysis) clean images of the amplitudes are obtained.

Of course, a pseudo-global fit with fixed component lifetimes is prone to user errors. If only one component lifetime is fixed to a wrong value large errors in the other lifetimes and, even more, in the amplitudes are induced. SPCImage NG, version 9.0 therefore has a global-fit

procedure implemented. The procedure fits the decay data under the assumption that the component lifetimes are unknown, but otherwise the same in all pixels of the image. For principles and pitfalls please see [100] and section 'Global Analysis' in this book. A result is shown in Fig. 424.

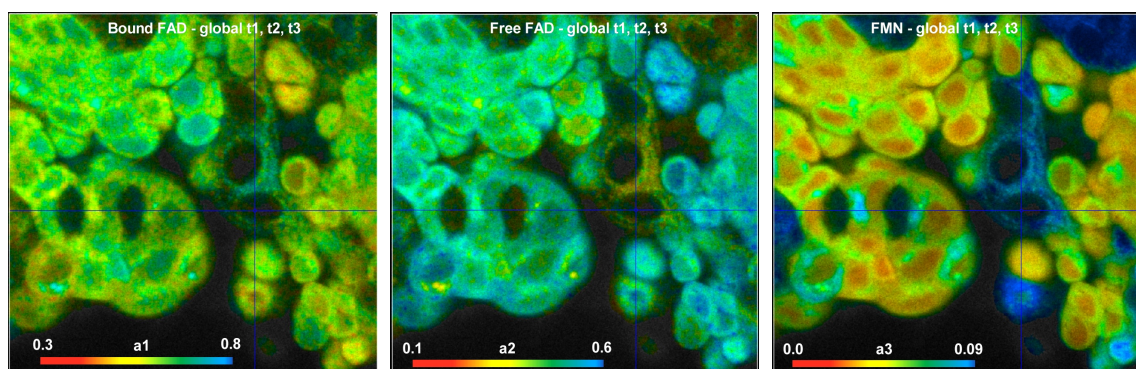


Fig. 424: Left to right: Amplitudes, a_1 , of bound-FAD decay component, a_2 , of unbound FAD component, and FMN, a_3 . Global Fit by SPCImage MLE.

As can be seen from Fig. 424, right, there is a large difference in the amplitude, a_3 , of FMN between the tumor cells and the normal cells. There may be different reasons for that. The first one is that there is simply less FAD in the tumor cells. Another plausible reason may be different redox ratio. The amount of oxydised FAD compared to reduced FAD is then lower, and so are the fractional contributions of the FAD component, a_1 and a_2 . Since the sum of the amplitudes is $a_1 + a_2 + a_3 = 1$ the amplitude of FMN, a_3 , increases. A third possibility is that there is more FMN in the tumor cells. However there is no theoretical explanation for that. More research on the FAD and FMN fluorescence dynamics and its dependence on the metabolic state is required to settle these questions.

Review of NAD(P)H / FAD FLIM Literature

General Evaluation of NAD(P)H decay Parameters

An overview on NADH / FAD fluorescence dynamics has been given by Chorvat and Chorvatova [159] and by Heikal [277]. Ghukasyan and Kao [249] have shown that the NADH decay parameters, in particular amplitudes of the fast and slow lifetime components, are indicators of physiological and pathological states of the cells. Changes in the unbound-bound ratio of NADH with the shift from oxidative phosphorylation to glycolysis were confirmed by using FLIM and phasor plot analysis [361]. Differentiation between apoptosis and necrosis by the NADH lifetime components was described by Wang et al. [620].

Early experiments of Skala et al. [548, 549] performed with two-photon excited autofluorescence FLIM delivered lifetime images of NADH in normal and pre-cancerous epithelia. They found a significantly decreased lifetime of the slow decay component, of the bound NADH, in precancerous tissue. A significant decrease of the lifetime of the protein-bound FAD was detected in high-grade pre-cancers [549]. Bird et al. used a similar approach for metabolic mapping of human breast cells [113]. Yu & Heikal found statistically relevant differences in the free/bound NADH ratio in normal cells and breast cancer cells [642].

Heikal et al, Vishwasrao et al, and Yu et al. showed that independent information on the unbound/bound ration of NADH can be obtained by recording fluorescence anisotropy decay data of intracellular NADH [165, 277, 603, 642].

Tumor Identification, Tumor Metabolism

Kantelhardt et al. [308] and Leppert et al. [367] recorded two-photon excited lifetime images of glioma and the surrounding brain tissue. Surprisingly, they found a significantly increased mean (amplitude-weighted) lifetime for the glioma cells compared to the surrounding brain cells.

Metabolic imaging of melanoma progression was performed by Pastore et al. [437]. They confirm a significant increase in the unbound / bound (a_1/a_2) ratio of NADH with the growth of a melanoma tumor, while the lifetimes of the decay components remained constant.

Effects of a nanosecond-pulsed electric field in normal and cancer cells was investigated by Awasthi et al [15]. They found changes both in the intensity and the lifetime of NADH. The NADH FLIM signature shows that apoptosis was induced in the cancer cells. Based on these results, the authors suggest the application of pulsed electric fields for drug-free cancer therapy.

Response to Chemotherapy

NADH FLIM is closely related to effect of cancer drugs in chemotherapy. Skala and Walsh used NADH FLIM to evaluate the effect of different cancer drugs on cells from biopsies from patients, and to develop the best treatment strategy [253, 530, 614, 615, 616, 618, 617]. The cells are cultured, the cell cultures are treated with the drugs, the cells are repeatedly imaged by FLIM. The OMI index is used as an indicator of the response to the drugs [529, 614, 615, 616, 618, 617]. Within a few days, the most efficient drug can be determined and a treatment strategy for the patient be developed. If the technique can be transferred into clinical use it has the potential to revolutionise cancer treatment.

Shah et al. [526] investigated heterogeneity in the response to ctuximab and cisplatin. The aim of this work was to avoid that subpopulations of cells escape the anti-cancer treatment and later cause relapse in cancer patients.

Stunz et al. [564] studied the response of cultured rat astrocytes and neurons to manganese treatment.

Shirmanova et. al. [542] demonstrated the relationships between the intracellular pH and the metabolic state derived from NAD(P)H / FAD FLIM in response to treatment with cisplatin. They found a metabolic shift from glycolysis towards oxidative metabolism during cisplatin-induced inhibition of cancer cell growth in vitro and in vivo. Lukina et al. report on experiments with paxlitaxel [381]. Yuzhakova et al. used NAD(P)H FLIM to explore the response of patient-derived glioblastoma to temozolomide (TMZ) [645].

Sergeeva et al. [522] performed metabolic imaging of NAD(P)H and FAD, and simultaneously used the genetically encoded pH-indicator SypHer1 and the FRET-based mKate2-DEVD-iRFP sensor for pH imaging and caspase-3 activity imaging. They found a relationship between the metabolic state derived from NAD(P)H FLIM, intracellular pH and caspase-2 activation during apoptosis. Induction of apoptosis was accompanied by a switch to oxidative phosphorylation, cytosol acidification and caspase-3 activation.

Response to Drugs

The effect of Ouabain, a pharmaceutical drug, and of 4-hydroxynonenal on live cardiomyocytes was studied by Chorvatova et al., see [161] and [162].

Infection and Infection Defence Mechanisms

NADH FLIM can be used to show metabolic changes during infections. Szaszak et al. [575] used the NADH fluorescence to track metabolic changes in the host and in the pathogen during intracellular infectious by *chlamydia trachomatis*. Buryakina et al. showed changes during infection of HeLa cells with enterohemorrhagic *escherichia coli* [136]. Szulczewski et al. used NADH FLIM for metabolic imaging of macrophages in a tumor environment [576], Gallego-Lopez measured metabolic changes caused by *Toxoplasma gondi* infection [235].

T Cells and Macrophages

Walsh et al. performed autofluorescence of T cells [619]. They were able to classify the cells based on the τ_m of NAD(P)H, the τ_m of FAD, and the redox ratio. Heaster et al. [275, 276] used τ_m of NADH and FAD in combination with the redox ratio to assess macrophage heterogeneity on the cell level.

Wound Healing

Gehlsen et al. [241] recorded NADH lifetimes and bound/unbound NADH ratios during wound healing of corneal epithelial cells. They compared the results to lifetimes obtained at different temperature, and during suppression of mitochondrial electron transport by Rotenone. Other wound healing studies were performed by Deka et al. [177]. They showed that the metabolic rate (revealed in the amplitude-weighted lifetime of NADH) increased after injury, and then gradually decreased. Simultaneously, collagen built up (revealed by SHG signal). A FLIM study on the uptake and excretion of fluorescein in hepatic reperfusion injury was presented in [586]. Alterations in NADH FLIM data of rat aorta induced by diabetes mellitus are described by Uherek et al. [597]. Batista et al. report on combined NADH FLIM and SHG measurements of porcine cornea [25]. Jones et al. used the redox ration and the $a1/a2$ ratio to compare wound healing diabetic and normal mice [303].

Metabolic FLIM of Oocytes

Sanchez et al. have shown that 2-photon excited FLIM of NAD(P)H and FAD can be used to differentiate normal oocytes from those with metabolic dysfunctions [492]. The authors used the fluorescence intensities, the component lifetimes, τ_1 and τ_2 , and the amplitudes of the bound component to quantify the metabolic state of mouse embryos. The authors also conducted studies of the impact of the illumination on the blastocyst development rates. They did not find a measureable differences between embryos that has been exposed to the FLIM measurement and reference embryos which had not been illuminated.

Liver Tissue

Rodimova et al. studied the metabolic state of liver tissue during the regeneration process [478]. They found a significant increase of bound NAD(P)H during liver regeneration. This indicated a metabolic shift towards oxydative phosphorylation. In parallel, they used mass spectrometry to study the lipid and amino acid composition. During regeneration, mass spectrometry showed an increase in the triglyceride signals and cholesterol signals, and a decrease in amino acids and sphingomyelin. Another study from the same group compared double-exponential and triple-exponential analysis approaches, and evaluated the influence of contributions of FAD, collagen and elastin to the detected signals [477]. Moreover, the authors extensively studied the influence of the preservation conditions on the FLIM results. Only with preservation of liver tissue in 10% BSA [352] and storage on ice the metabolic state remained stable within an observation time of 3 hours.

Barkanauskas et al. used nonlinear regression to estimate the lifetimes of unbound and bound forms of NAD(P)H and FAD, the amplitude ratio, a_1/a_2 , and the fluorescence-lifetime redox ratio (FLIRR) [24]. They also examined the impact of image segmentation. Both the amplitude ratio and the FLIRR were found useful for discrimination of diseased liver from healthy liver. These parameters provided a far better insight into the tissue state than the global mean fluorescence lifetime, t_m .

Brain Tissue

Metabolic-FLIM for Brain tissue has produced inconsistent and often contradicting results. Kantelhardt et al. [308] and Leppert et al. [367] found a significantly increased mean (amplitude-weighted) lifetime for the glioma cells compared to the surrounding brain cells. This is in contraction to the model that tumor cells have more free NAD(P)H and, consequently, a larger amplitude, a_1 , of the fast lifetime component. It could be suspected that the data were biased by inappropriate storing conditions or inappropriate filtering. However, more recent results show similar inconsistencies. Lukina et al. performed FLIM measurement of macroscopic brain samples by a bh DCS-120 MACRO system [353]. The excitation wavelength was 375 nm, the detection wavelength 435 nm to 475 nm. Both samples from rat brains and from human brains were investigated. The decay data were analysed by fitting with a double-exponential model. In both cases, the lifetimes of the decay components were significantly higher than normally found for NAD(P)H in biological tissue. In the rat brains $\tau_1 = 0.78$ to 0.94 ns and $\tau_2 = 3.32$ to 5.03 ns was found. The values for human brain samples were $\tau_1 = 1.6$ ns and $\tau_2 = 7.8$ ns. These values suggest that the fluorescence does not come from NADH or NADPH alone.

In rat glioblastoma t_m in the tumor was found shorter than in the tumor-distant cortex and the tumor-distant white matter, and shorter than in the healthy brain. In glioma t_m was only insignificantly shorter than t_m in the tumor-distant cortex and the tumor-distant white matter. It was, however, substantially shorter than in the healthy brain. t_m in anaplastic astrocytoma followed the same trend. However, the a_1/a_2 ratios were inconclusive or even went in the unexpected direction, i.e. a decrease of a_1/a_2 was found in the tumor.

In human glioblastoma the t_m in the tumor, in infiltrated white matter, and in non-infiltrated white matter did not differ significantly. However, the component lifetimes in non-infiltrated white matter were shorter. The amplitude of the fast component, a_1 , and the amplitude ratio, a_1/a_2 were lower in the tumor, i.e. went in the expected direction. Please see [353] for details.

As can be seen from these results, the fluorescence decay behaviour of brain tumors still resists explanation. Storing conditions are unlikely a source of the problem, as was carefully checked [352] by the authors. The clue may be in the unusual lifetimes of the decay components. It appears likely that the components contain contributions from fluorophores other than NAD(P)H. The fast and slow decay component then cannot be attributed to free and bound NAD(P)H, and a_1/a_2 cannot be considered a free/bound ratio. Possible sources of additional decay components are discussed in [353]. NAD(P)H is certainly present but cannot account for the extremely long lifetimes found in the data. FMN can be excluded because it is not detected through the emission filter used. Other possible sources are collagens, myelin, cholesterol, and various phospholipids. There are several approaches to solve the problem. One of them is precision triple- and quadruple exponential decay analysis on combined pixel data. It could show whether 'normal' NAD(P)H decay functions are hidden behind the decay functions of unknown fluorophores. Also measurements at different excitation and emission wavelength with careful multi-exponential decay analysis and multi-wavelength FLIM could help solve the mystery.

Neurons

Niederschweiberer et al. performed NADH FLIM measurements of neurons [422]. The authors had found earlier that the NADH decay times depend on the pH in the mitochondrial matrix [498]. Therefore, they used two detection channels, one from 430 to 490 nm for the NADH and another for SypHer, a pH sensor. The NADH lifetimes were then corrected for the particular pH. It was found that the NADH lifetimes correlated with the overexpression strength of Alzheimer's disease-related proteins.

Stem Cells

Gou et al. used FLIM of the decay components of NADH to distinguish human mesenchymal stem cells from differentiated progenies [263]. Uchugonowa & König applied two-photon autofluorescence lifetime imaging to human and animal stem cells [333, 334]. They found significant changes in the fluorescence lifetimes on differentiation and maturation of the cells. An example is shown in Fig. 425. The differentiated cells have smaller a_1/a_2 ratios and longer mean lifetime, t_m .

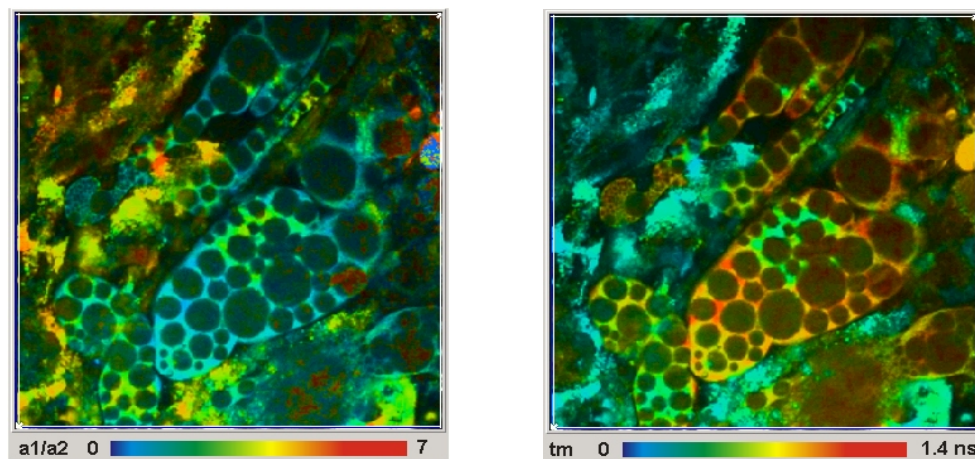


Fig. 425: FLIM of human salivary gland stem cells. Differentiated cells have significantly lower a_1/a_2 ratio and longer mean lifetime. Data courtesy of Aisada Uchugonova and Karsten König, Saarland University, Saarbrücken.

Meleshina et al. simultaneously tracked the redox ratio and the a_1/a_2 (unbound/bound) ratio during maturation of stem cells. For different stages of differentiation, the observed metabolic shifts both in the direction to glycolysis and to oxidative phosphorylation [397, 398]. Rodimova et al. investigated differentiation of stem cells on tissue-engineered constructs [479].

NADH / FAD FLIM with Tracking of the Cell Cycle

Shirmanova et al. developed a new variant of the cell cycle indicator FUCCI (fluorescent ubiquitination-based cell cycle indicator) [543]. The variant, FUCCI-Red, utilizes two fluorescent proteins, mCherry and mKate. Depending on the phase in the cell cycle, emission from one or the other fluorophore dominates the net fluorescence. Because the fluorescence lifetimes are different, the apparent lifetime is an indicator of the phase in the cell cycle. Different from the original FUCCI, the fluorescence spectra of the two emission components of FUCCI-Red are closely the same. The signal can therefore be detected in a single spectral channel. Importantly, the emission is in the red region of the optical spectrum, and thus does not interfere with the emission from NADH and FAD. It is therefore possible to detect NADH FLIM, FAD FLIM, and FUCCI-Red FLIM simultaneously. As a result, the metabolic state of cells can be related to the phase in the cell cycle. The authors demonstrated the method on CT26 cancer cells.

Unmixing NAD(P)H / FAD Fluorescence Components

Additional information on the NAD(P)H / FAD fluorescence can be obtained by multi-wavelength FLIM with the bh PML-16 C and PML-16 GaAsP detectors [27], see page 143. The separation of the signals from NAD(P)H and FAD can then be improved by spectral unmixing of the components, and distinct spectral components within the emission spectra of NAD(P)H and FAD can be determined [152, 156, 157, 159, 482, 481, 483]. Typical multi-wavelength autofluorescence data are shown in Fig. 426. Two-photon excitation at a wavelength of 750 nm was used. This wavelength excites both NAD(P)H and FAD. Each of the images covers a wavelength range of about 12.5 nm. The colour of the images represents the lifetime obtained from a single-exponential fit.

Due to the different fluorophores present in the tissue, the lifetimes in the individual wavelength intervals are different. The third left image contains a strong SHG component. SHG reveals itself as an infinitely short lifetime component, see the blue features in the third to left image.

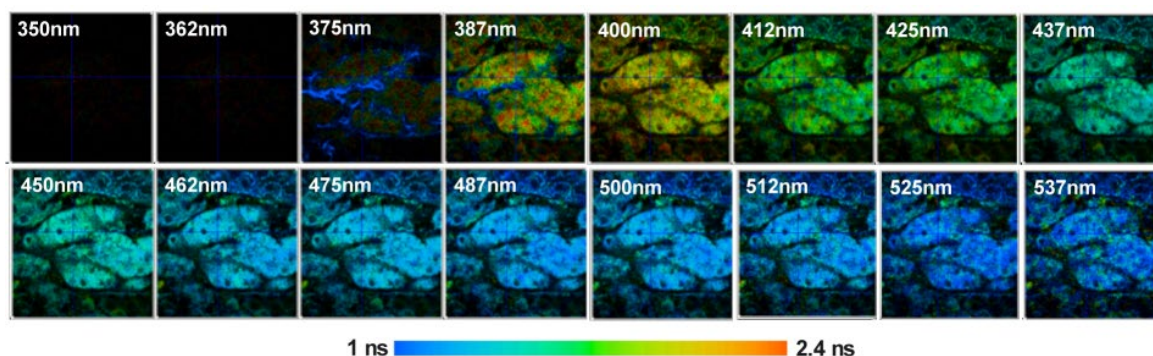


Fig. 426: Multi-wavelength fluorescence lifetime images of a mouse kidney section. Two-photon excitation at 750 nm. Images in subsequent wavelength intervals from 350 nm to 550 nm. (Christoph Biskup, University Jena, Germany. LSM 510 NLO with bh MW FLIM)

Fig. 427 shows the fast lifetime component, the slow lifetime component, and the amplitude ratio of both for a wavelength interval from 430 to 480 nm. The fluorescence in this interval is dominated by NADH. The lifetime of NADH is known to depend on the binding to proteins [358, 360, 440]. The images can therefore be expected to represent different binding states of NADH.

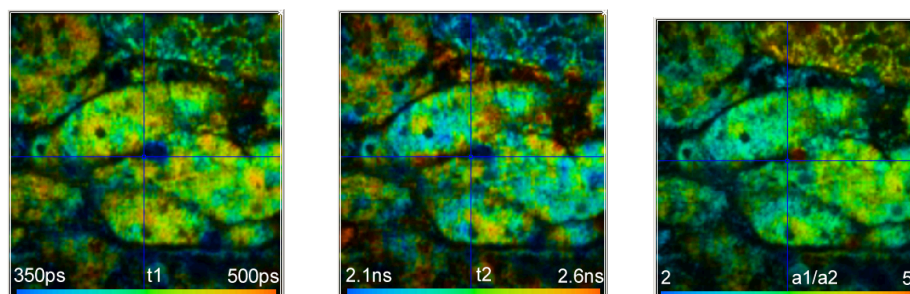


Fig. 427: Images of the lifetime components and amplitude ratio of a double exponential fit, 430 to 480 nm interval

An interesting application of multi-wavelength lifetime detection of NADH has been published by Chorvat et al. [160]: The authors used the data to detect early stages in the metabolism of isolated live cardio-myocytes in cases of rejection of transplanted hearts.

An overview about autofluorescence multi-wavelength FLIM is given in [159]. Full exploitation of multi-wavelength autofluorescence FLIM data requires a global fit with a suitable model that includes the effective spectra of the expected fluorophores. The task is extremely difficult because some of the endogenous fluorophores are in fact mixtures of slightly different compounds, with different absorption and emission spectra [512]. Moreover, there may be absorbers of unknown absorption spectra, inhomogeneous distribution, and unknown concentration in the tissue. Nevertheless, there is currently a number of approaches that are likely to solve at least a part of the problem [156, 157, 159, 456].

Two-Photon FLIM of Mammalian Skin

A typical application of two-photon excited FLIM measurements of tissue autofluorescence is multiphoton optical tomography of skin [328, 469]. The technique has been developed to the stage of clinical application [170, 330, 332, 333, 438, 476, 490, 491]. Typical in-vivo images obtained at human skin are shown in Fig. 428.

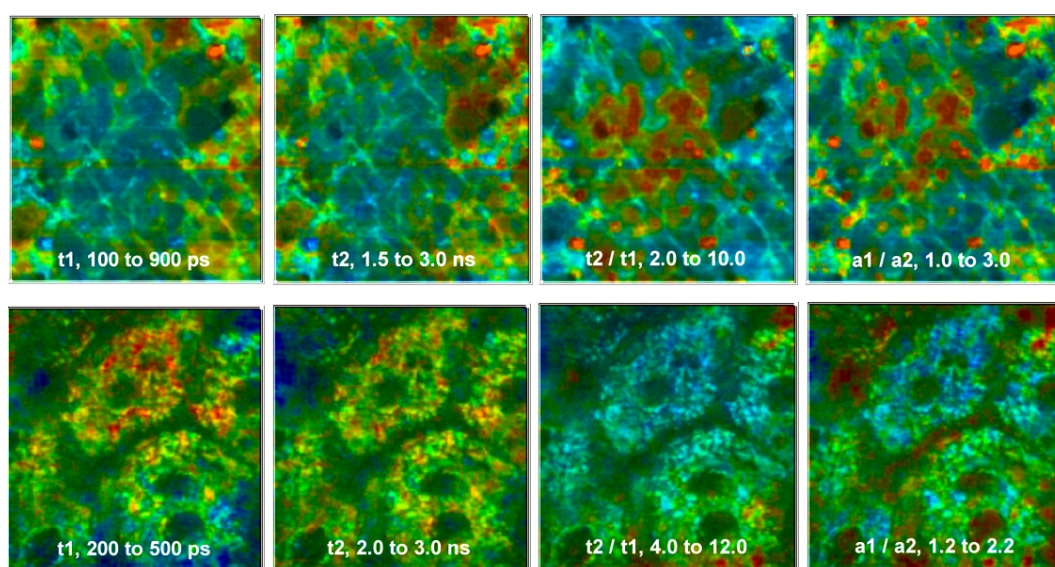


Fig. 428: Time-resolved in-vivo autofluorescence images of human stratum corneum and stratum spinosum. Left to right: Fast lifetime component, τ_1 , slow lifetime component, τ_2 , ratio of the lifetime components, τ_1/τ_2 , and ratio of amplitudes, a_1/a_2 . The indicated parameter range corresponds to a colour range from blue to red. Karsten König and Iris Riemann, Fraunhofer Institute of Biomedical Techniques, St. Ingbert, Germany.

The upper row shows the stratum corneum (upper row, 5 μm deep), the lower row the stratum spinosum (lower row, 50 μm deep). Double-exponential decay analysis was applied to the data. The colour represents the fast lifetime component, τ_1 , the slow lifetime component, τ_2 , the ratio of lifetime components, τ_1/τ_2 , and the ratio of the amplitudes of the components, a_1/a_2 .

Lifetimes, lifetime components, and intensity ratios in several spectral channels have been used by Patalay et al. to derive signatures of skin lesions [438]. They recorded skin FLIM data in a Dermainspect system with a bh SPC-830 module [77]. From the data of individual cells in images of several patients they built up scatter plots of component lifetimes vs. the fractional intensities of the components (note this is q_1 and q_2 in SPCImage), and of the mean (amplitude-weighted) lifetimes versus intensity ratios in different channels. They found differences in the distribution of the amplitude-weighted lifetimes for dysplastic nevi and nodular basal cell carcinoma. Differences also showed up in the scatter plots. The authors discuss whether the observed changes come from melanin or from NADH without coming to a final decision.

NAD(P)H imaging of melanoma progression was performed by Pastore et al. [437]. They confirm a significant increase in the unbound / bound (a_1/a_2) ratio of NADH with the growth of a melanoma tumor, while the lifetimes of the decay components remained constant.

FLIM measurements at human skin and the results obtained are supported by measurements with single-point tissue lifetime spectrometers [176, 585]. An interesting result has been published by Pires et al. [451]. The authors excited human skin with two BDL-SMC ps diode lasers with wavelengths of 378 nm and 445 nm. The 378 nm predominately excites NADH, the 445 nm laser FAD. The fluorescence was detected at wavelengths of 440 nm (NADH) and 514 nm (FAD) and recorded by two SPC-150 channels. The decay curves were analysed with a double-exponential model. This delivered the fast and slow decay times, τ_1 , and τ_2 , of the NADH and the FAD decay curves. With these values they plotted the frequency of the values of τ_{1FAD} versus τ_{2NADH} obtained from a large number of samples. The results delivered a clear discrimination between normal skin and melanoma, see Fig. 429.

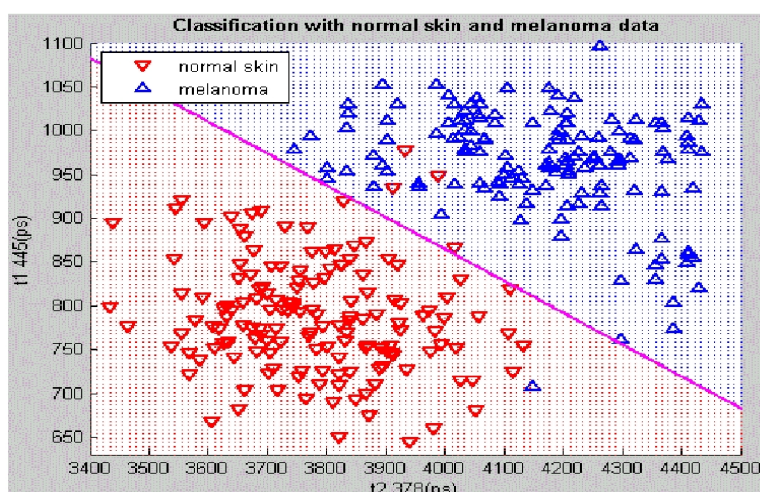


Fig. 429: Discrimination of normal skin and melanoma by a plot of τ_{1FAD} versus τ_{2NADH} . Adapted from [451]

If these results can be confirmed by FLIM experiments (they are in agreement with [6]) a similar discrimination for the individual pixels of the image, and thus of the of the tumor tissue could be achieved.

Earlier experiments of Teuchner et. al. [580] and Eichhorn et al. [213] have shown that the fluorescence spectrum of melanin in malignant melanoma is red-shifted compared to normal skin. The result has been confirmed by Dimitrow et al. who used multiphoton multi-spectral FLIM [186]. Consequently, there is an increasing interest in lifetime imaging of melanin. FLIM of melanin fibrils by an LSM 510 with a bh Simple-Tau 150 FLIM system has been described by [392], lifetime imaging of melanin in human hair in [212]. Two-photon excitation spectra of melanin are given in [132]. For an overview please see also [330, 333].

Combination with SHG

Additional information about the constitution of tissue can be obtained from second-harmonic generation (SHG) signals. Second-Harmonic generation has nothing to do with fluorescence but often shows up as an ultrafast component in two-photon autofluorescence data. SHG emission requires some global organisation of the molecules [390]. In biological system it comes mainly from connective tissue, especially from collagen in animal tissue and from cellulose and starch in plant tissue. The ratio of SHG and autofluorescence intensity in mammalian skin is an indicator of skin ageing [321].

An example is shown in Fig. 430. The images show a pig skin sample excited by two-photon excitation at 800 nm. The left image shows the wavelength channel below 480 nm. This

channel contains both fluorescence and SHG signals. The SHG fraction of the signal has been extracted from the FLIM data and displayed by colour. The right image is from the channel >480 nm. It contains only fluorescence, the colour corresponds amplitude-weighted mean lifetime.

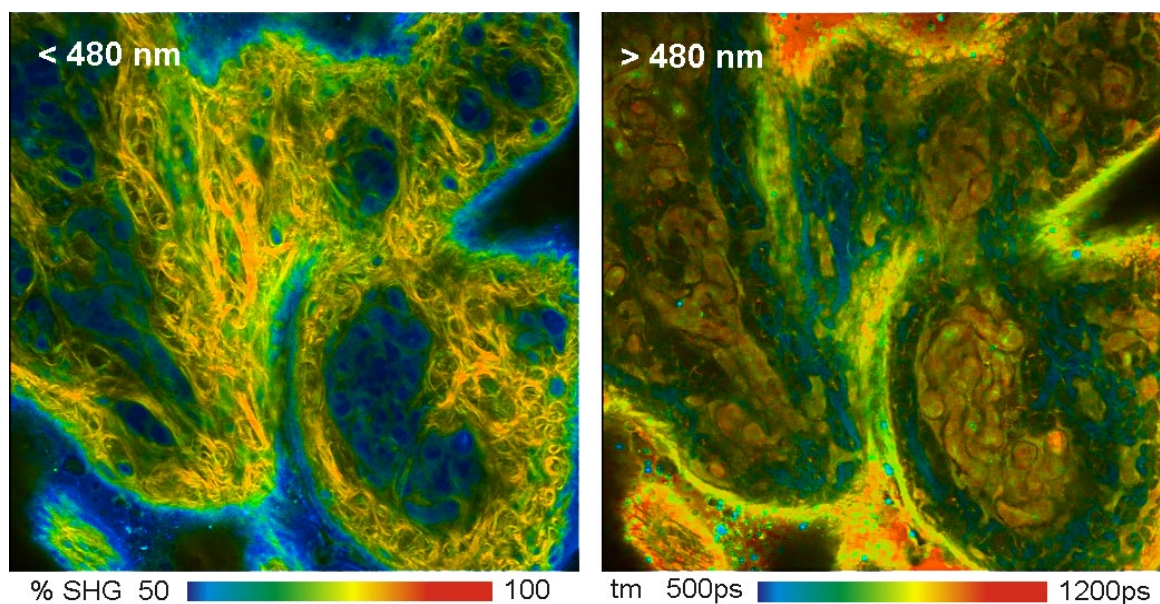


Fig. 430: Two-photon FLIM of pig skin. LSM 710 NLO, excitation 800nm, HPM-100-40, NDD. Left: Wavelength channel <480 nm, colour shows percentage of SHG in the recorded signal. Right: Wavelength channel >480 nm, colour shows amplitude-weighted mean lifetime.

Z Stacks of Autofluorescence FLIM

Measurements as the ones described above can favourably be performed by Z stack FLIM, see page 154 and page 158. Fig. 431 shows Z-stack FLIM data of a pig skin sample in the wavelength channel above 480 nm. The data were analysed by the batch-processing function of SPCImage. The colour of images shows the average (intensity-weighted) lifetime of a double-exponential fit to the decay data. The intensity of the images was normalised to the intensity of the brightest pixel.

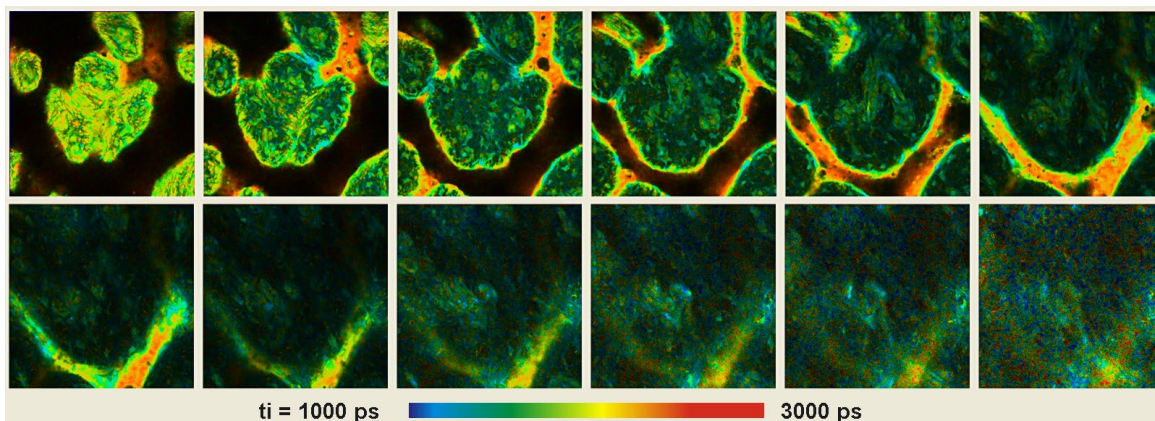


Fig. 431: FLIM Z stack recorded at a pig skin sample, excitation at 800 nm, emission above 480 nm. Z step width 5.09 μ m, scan area 212 x 212 μ m. Images from 5 μ m to about 60 μ m below the surface. Colour represents average (intensity-weighted) lifetime of a double-exponential fit. FLIM data format 256 x 256 pixels, 256 time channels. Normalised intensity.

Fig. 432 shows the SHG intensity extracted from the FLIM data in the channel below 480 nm. Also here, intensity of the images was normalised to the intensity of the brightest pixel.

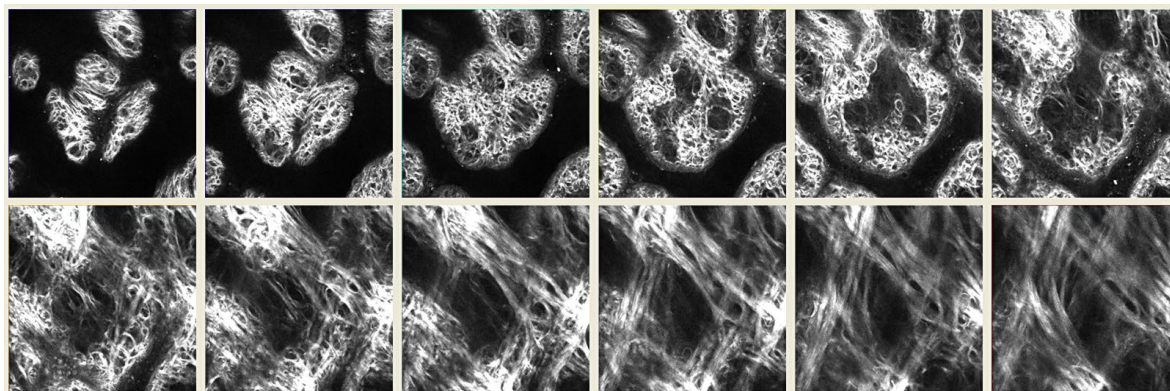


Fig. 432: Z stack recorded at a pig skin sample, excitation at 800 nm, emission below 480 nm, SHG signal, extracted by selecting photons in an early time window. Z step width is 5.09 μm , scan area 212 x 212 μm . Images from 5 μm to about 60 μm depth. Normalised intensity.

In all autofluorescence lifetime experiments it is important that the metabolism of the tissue be not changed before or during the measurement. This is critical especially for ex-vivo samples. Sanchez et al. have therefore studied the degradation of skin samples over the storage time and under different storage conditions [489].

FLIM of Other Endogenous Fluorophores

There are other endogenous fluorophores which can possibly be used for the identification of the tissue state and of cancerous and pre-cancerous situations. Melanin at its relationship to melanoma has been described above. The use of the tryptophane fluorescence, including FRET from tryptophane into NAD(P)H has been described in [6, 304, 447]. The problem of tryptophane is that it cannot easily be excited by wavelength available in one-photon and two-photon laser microscopes. The authors therefore used three-photon excitation. FLIM with endogenous bilirubins has been described by Shen et al. [537]. Bilirubin is present at high concentration in liver tissue. The authors excited it by two-photon excitation at 1230 nm. In differentiated hepatocellular carcinoma they found a fluorescence lifetime of 500 ps compared to 300 ps in non-tumor tissue. Endogenous fluorescence is also recorded from porphyrines. Porphyrines exhibit a strongly multi-exponential fluorescence decay which indicates that they are a mixture of slightly different compounds, different conformations, or, possibly, aggregates of different size. The composition of these mixtures may depend on the tissue state. The composition of the decay function may therefore contain biological information. Other endogenous fluorophores, such as lipofuscin [521] or advanced glycation end products (AGEs) [512] are important as indicators in ophthalmic FLIM, please see [77].

Diffusion of Nanoparticles in Skin

An important issue in dermatology is diffusion of drugs and nano-particles through human skin. On the one hand, diffusion through the skin may be desirable as a pathway of drug delivery. On the other hand, nanoparticles contained in sunscreens or cosmetic products should not permeate through the stratum corneum of human skin. Moreover, it is important whether the nanoparticles cause changes in their molecular environment. The use of two-photon imaging and FLIM to study these effects has been described in [247, 282, 365, 366, 373, 412, 455, 476, 491, 587, 654].

Autofluorescence of the Ocular Fundus

The bh FLIM technique is used in ophthalmic scanners, see [77]. The instruments have produced FLIM images of amazing quality. Clinical trials have shown that FLIM is able to detect metabolic changes that are early indications of a number of eye diseases [203, 204, 205, 206, 209, 513, 514, 515, 517, 518, 519]. The resolution of an ophthalmic scanner is limited by the low NA and the poor optical quality of the lens of the human eye. Data delivered by ophthalmic scanners have therefore to be supported by FLIM microscopy of ex-vivo samples.

Lifetime imaging of lipofuscin and melanin in retinal pigment epithelium cells has been described by Sun et al. [573]. An autofluorescence FLIM study of retinal pigment epithelium cells under oxidative stress has been published by Miura et al. [410].

A FLIM study of extra-macular drusen has been published by Schweitzer et al. [516]. Based on the FLIM data, the authors were able to clearly discriminate the RPE from Bruch's membrane, drusen, and choroidal connective tissue. An example of a FLIM image of the RPE with hard drusen is shown in Fig. 433. The data were acquired with the MW FLIM multi-wavelength detector. The figure shows the lifetime data in all combined wavelength channels. Triple-exponential decay analysis was used; the images show the fast decay component, the medium component, and the slow decay component. Interestingly, in the drusen and in the Bruch's membrane an extremely slow component with about 8 ns decay time occurs. The lifetimes in the RPE are much shorter.

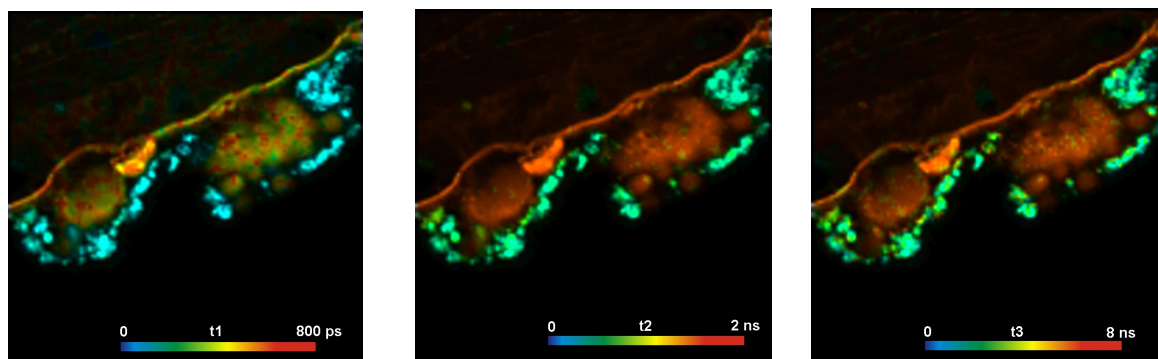


Fig. 433: Autofluorescence FLIM image of a human fundus sample. Triple-exponential analysis, left to right: Lifetimes of the fast, medium, and slow decay component. Courtesy of Dietrich Schweitzer, Martin Hammer, Sven Peters, Christoph Biskup, University Jena, Germany.

Spectrally resolved data are shown in Fig. 434. It shows single wavelength channels of the MW FLIM detector from 450 nm to 575 nm. The lifetime shown is the amplitude weighted average of a triple-exponential decay model. The intensities were normalised to the brightest pixel.

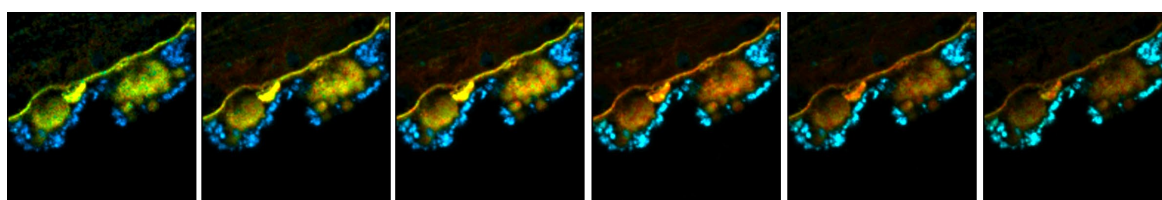


Fig. 434: Lifetime images of the amplitude-weighted lifetime, t_m , for single wavelength channels from 450 nm to 575 nm. The lifetime scale is blue to red, 0 to 2500 ps.

Oxygen Sensing by Phosphorescence Lifetime Measurement

Phosphorescence

Phosphorescence occurs when an excited molecule transits from the first excited singlet state, S1, into the first triplet state, T1, and returns from there to the ground state by emitting a photon [360]. Both the S1-T1 transition and the T1-S0 transition are ‘forbidden’ processes. The transition rates are therefore much smaller than for the S1-S0 transition. That means that phosphorescence is usually a weak and slow process, with low intensity and lifetimes on the order of microseconds or even milliseconds. Phosphorescence of organic dyes or endogenous fluorophores is extremely weak or even not detectable at room temperature. However, strong phosphorescence with lifetimes from the microsecond up to the millisecond range is obtained for lanthanide complexes [148] and organic complexes of ruthenium [360, 285], platinum [221, 360, 364, 486], terbium, palladium [364], and iridium [350, 411, 649]. Early TCSPC PLIM applications used the long lifetime of phosphorescence to suppress endogenous fluorescence of the sample [19, 20].

Of special interest for live-cell imaging is, however, that the phosphorescence of these complexes is quenched by oxygen, see Fig. 435. Phosphorescent dyes are therefore excellent oxygen sensors [245, 360, 364, 434, 435, 486, 539]. Most PLIM applications are therefore aiming at the measurement of oxygen partial pressure (pO_2) in biological objects. Of special interest is the correlation of changes in the oxygen concentration with the metabolism of the cells [305]. To reach this target it is necessary that PLIM and FLIM measurements are performed simultaneously [50, 73, 89, 80, 83, 531], see ‘Simultaneous FLIM / PLIM’, page 172. The oxygen concentration is derived from the PLIM data, the metabolic information from the FLIM data, preferably from the NAD(P)H fluorescence.

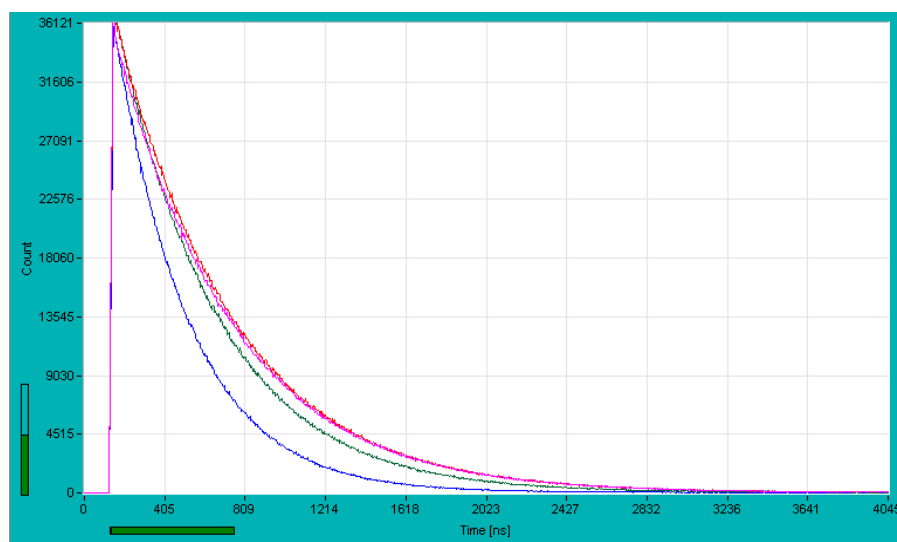


Fig. 435: Phosphorescence decay of a ruthenium dye for different oxygen concentration. Recorded with bh DPC-230

Oxygen Sensing by PLIM

Examples are shown in the figures below. Fig. 436 and Fig. 437 show cultured human embryonic kidney cells incubated with a palladium-based phosphorescence dye. Fig. 436 was recorded under atmospheric oxygen partial pressure. The maximum of the lifetime distribution over the pixels (upper right) is at 75 s. Fig. 437 was recorded under decreased oxygen partial pressure. As can be seen, the maximum of the lifetime distribution has shifted to 144 μ s.

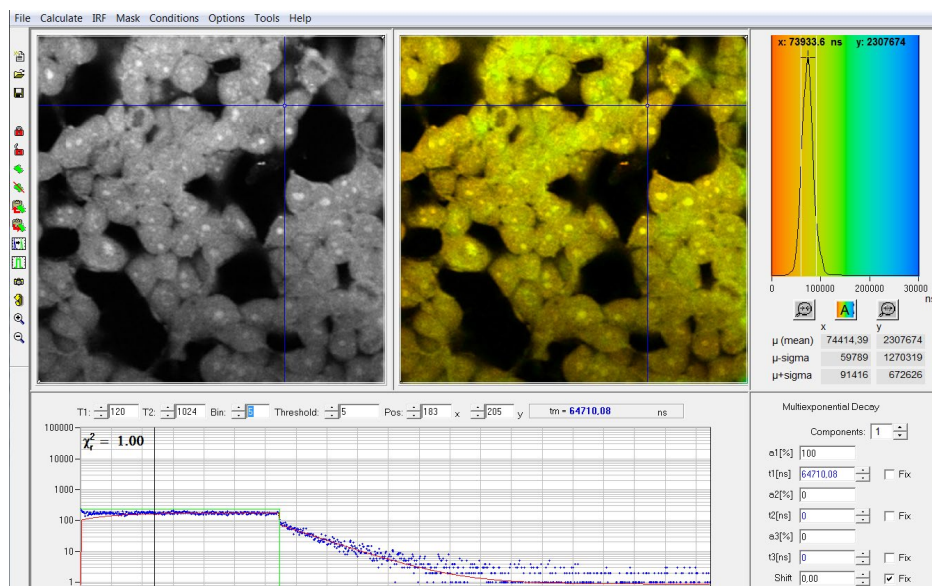


Fig. 436: HEK cells incubated with a palladium dye under atmospheric oxygen partial pressure. Lifetime scale 0 (red) to 300 μs (blue). Phosphorescence lifetime at the Cursor-Position 65 μs . The maximum of the lifetime distribution over the pixels is at 75 μs .

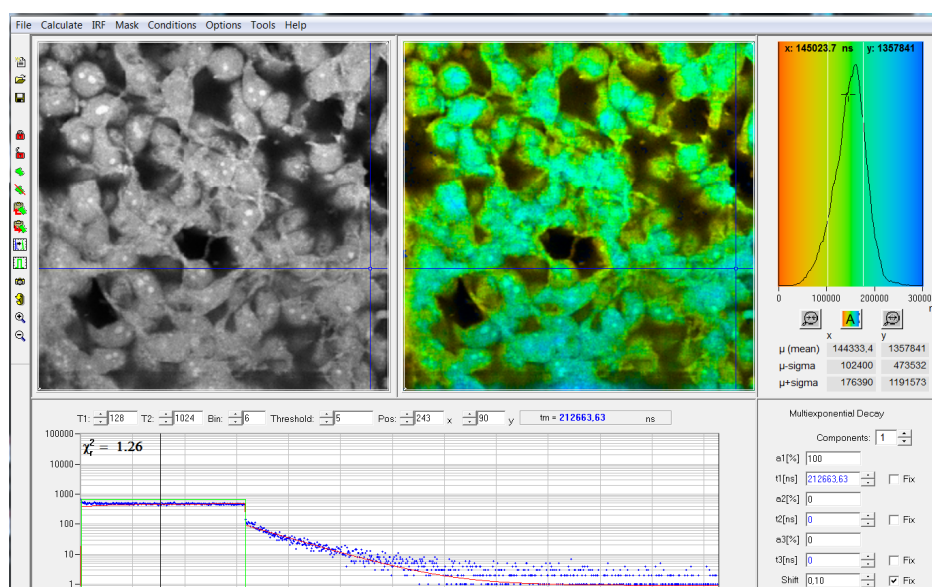


Fig. 437: HEK cells incubated with a palladium dye under reduced oxygen partial pressure. Lifetime scale 0 (red) to 300 μs (blue). Phosphorescence lifetime at the Cursor-Position 212 μs . The maximum of the lifetime distribution over the pixels is at 144 μs .

Simultaneous Recording of Oxygen and NAD(P)H Images

Simultaneously recorded fluorescence and phosphorescence lifetime images of live cultured human embryonic kidney cells stained with 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin-Pd(II) are shown in Fig. 438. The FLIM and PLIM images on the left were recorded under atmospheric oxygen concentration. The images on the right were recorded after adding sodium sulphite (Na_2SO_3). Na_2SO_3 binds oxygen. The oxygen concentration is therefore low. It can clearly be seen that the phosphorescence lifetime became longer, indicating there is indeed less oxygen (please note different colour scales). In the FLIM image, it can be seen that the amplitude-weighted lifetime, τ_m , of the NADH has decreased.

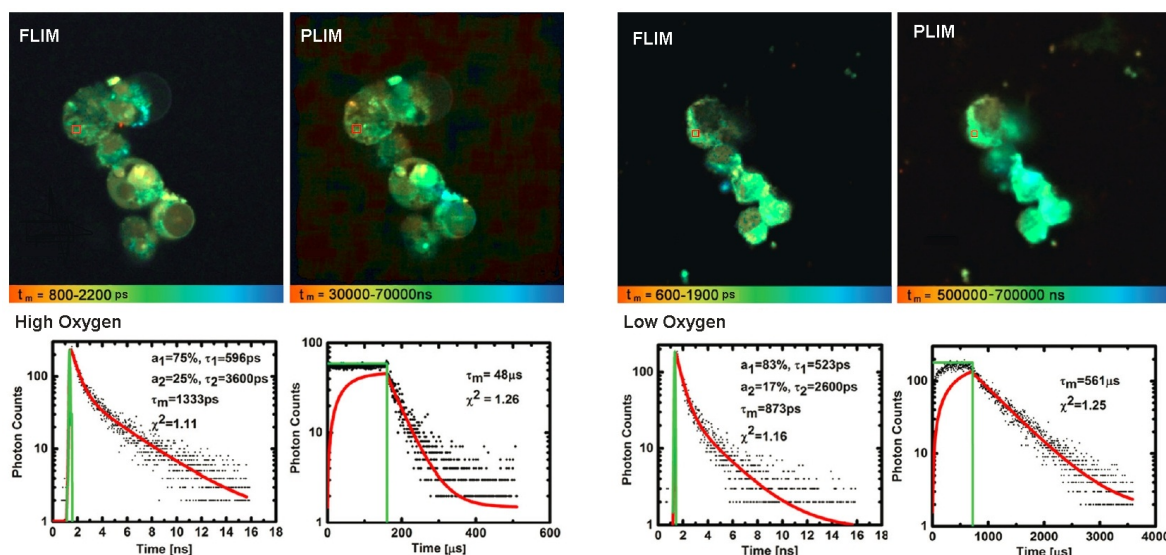


Fig. 438: Simultaneous FLIM/PLIM of human embryonic kidney cells. Left: High (atmospheric oxygen concentration). Right: Low oxygen concentration. Upper row FLIM and PLIM images, lower row decay curves in indicated position. Lifetime-colour scale given underneath the images. bh DCS-120 confocal scanning FLIM system, excitation wavelength 375 nm. Adapted from Shcheslavskiy et al. [531].

Delayed Fluorescence of PpIX

Protoporphyrin IX (PpIX) is an endogenous porphyrin. PpIX has been reported to emit delayed fluorescence in measurable amounts [402, 403]. Delayed fluorescence occurs when an excited molecule transits from the S1 state to the T1 state, and back to the S1 state. From there it emits through the normal S1-S0 relaxation process. Since the process goes through the triplet state the lifetime is long as in the case of phosphorescence. The triplet state interacts with oxygen, therefore the lifetime of delayed fluorescence has the same pO₂ dependence as phosphorescence. PpIX is there a potential (and probably the only) endogenous oxygen sensor.

Since the emission of delayed fluorescence occurs from S1 the emission spectrum is identical with that of prompt fluorescence. However, the intensity of delayed fluorescence is orders magnitude lower than that of prompt fluorescence. Delayed fluorescence is therefore difficult to record. The bh simultaneous FLIM/PLIM technique separates fluorescence and phosphorescence temporally, without the need of spectral separation. In principle, it is therefore able to record also delayed fluorescence.

To confirm the existence of delayed fluorescence a solution of PpIX was put in a cell dish and scanned with the 405 nm laser. The data were recorded by a DCS-120 system by bh's simultaneous FLIM/PLIM technique. A laser-on-off period of 412 μ s was used, with a laser-on time of 95 μ s. Fluorescence and phosphorescence (in this case delayed fluorescence) decay data of all pixels were combined in a single fluorescence and delayed-fluorescence curve. The result is shown in Fig. 439. The decay function of the prompt fluorescence is shown left. The decay is strongly multi-exponential, with a mean lifetime of about 9 ns. The luminescence in the 0 to 300 μ s range is shown on the right. A long-lifetime signal was indeed detected in the laser-off phases. It has a lifetime of about 25 μ s, which is in agreement with [402]. The lifetime increased when we decreased the oxygen concentration by adding Na₂SO₃ to the solution. We therefore believe that the signal is indeed delayed fluorescence from PpIX. However, the signal is extremely weak - almost 3 orders of magnitude weaker than the prompt fluorescence in the laser-on phases.

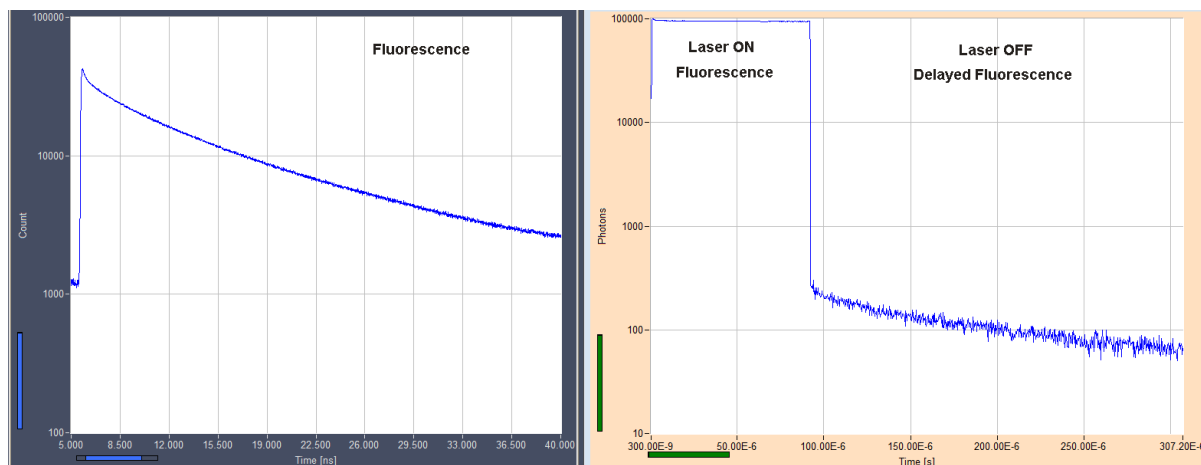


Fig. 439: Fluorescence (left) and delayed fluorescence of PpIX (right). DCS-120 system in FLIM/PLIM mode, excitation 405 nm, detection 600-700 nm.

Imaging data of the same solution are shown in Fig. 440. The figure shows the SPCImage data analysis panel, with the intensity image, the lifetime image, the decay data in a selected area of the sample, and a lifetime histogram over the pixels. Delayed fluorescence is also seen in the scan data. However, the intensity is uncomfortably low. For reasonable decay analysis we had to bin the data into 20 x 20 pixel areas. Within this binning area decay data of the delayed fluorescence are obtained as shown in the figure. (The prompt fluorescence is 3 orders of magnitude stronger, and out of the scale of Fig. 440).

These results show that the delayed fluorescence of PpIX can be recorded. Most likely, it can be used for sensing the average pO₂ in an entire sample or in reasonably large areas of a sample. However, it is probably too weak to image pO₂ in individual compartments of a cell or in individual structures within a tissue sample.

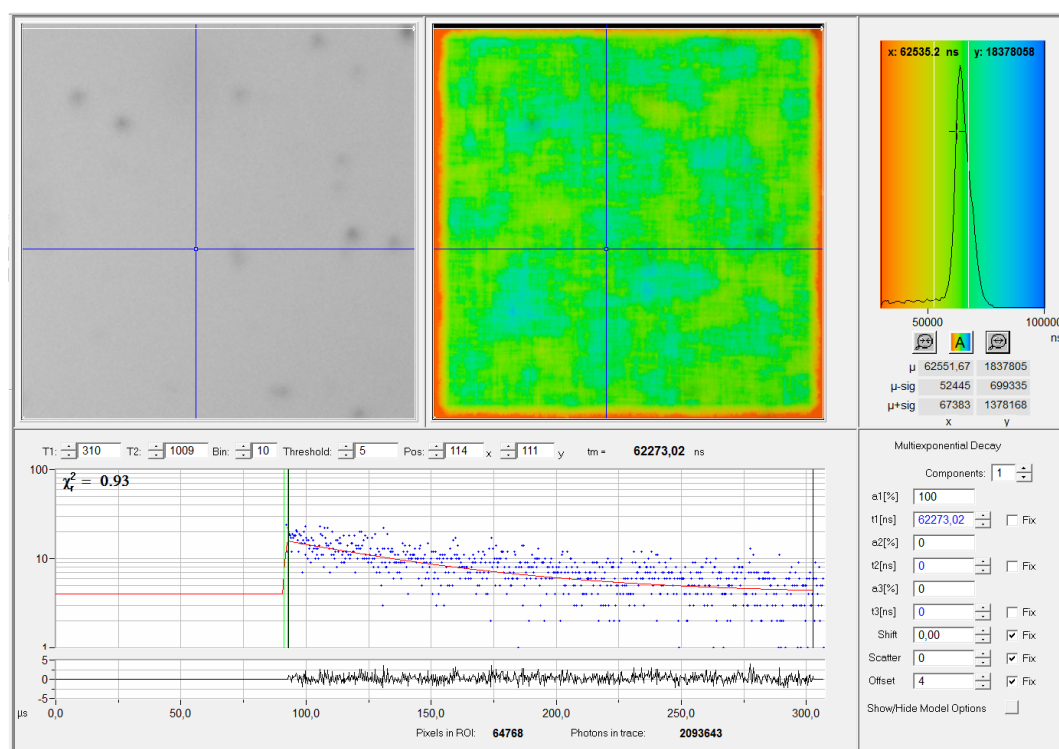


Fig. 440: Delayed fluorescence of PpIX, shown in scanning data. Total scan area 256 x 256 pixels, decay data from 20 x 20 pixel binning area. Lifetime histogram over the pixels shown upper right.

Singlet-Oxygen Phosphorescence

Singlet-oxygen detection with modified a DCS-120 system and a superconducting single-photon detectors (SSPD) has been described in [415]. For technical details please see [77].

The system was tested with solutions of Ce6 and methylene blue, and with Ce6 in Hela cells. In all cases, a weak signal of about 100 photon per second at 1300 nm was detected. The low intensity was not a problem, however, because the SSPD detector has a dark count rate of only a few counts per second. The acquisition time therefore could easily be extended to 10 minutes and more to collect enough photons. A result for a solution of methylene-blue is shown in Fig. 441. For a detailed discussion of the results please see [415].

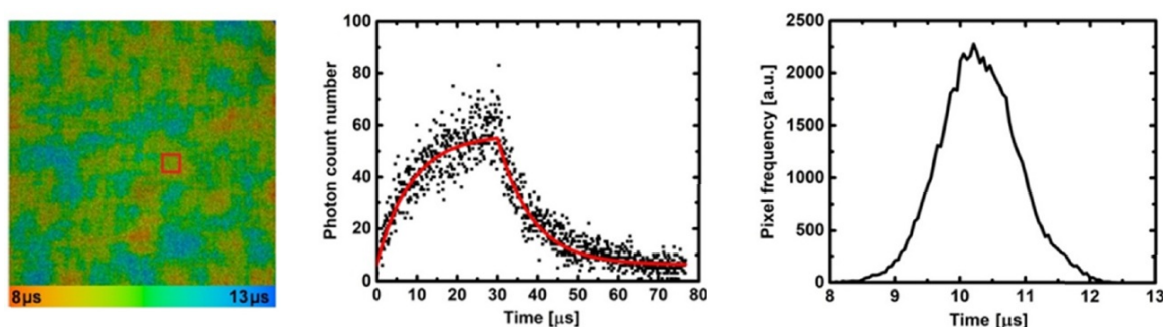


Fig. 441: Phosphorescence lifetime image of a methylene-blue solution. Colour coded image (left), waveform of the detected signal (middle) and distribution of the lifetime over the pixels of the image.

Applications

There is an impressive amount of publications based on the bh FLIM/PLIM technique. The first publications concentrated on the characterisation of phosphorescence probes. In [191], [193] and [436] Dmitriev and Papkovski give a summary on available phosphorescent probes, their O_2 sensitivity, brightness, phosphorescence lifetimes, cell-staining capability, and toxicity.

The problem of sensitivity of phosphorent dyes to other cell parameters, such as pH, viscosity, and oxygen diffusion time constant, is addressed by embedding the dyes in nanoparticles and nanostructured cell substrates. Phosphorescent nanoparticles, nanostructured films, scaffolds and fibres as pO_2 -sensors are explored in [189, 192, 294, 295, 298, 430, 590, 640]. Phosphorescent gold clusters as probes for lipid droplets were described in [335]. Dos Santos et al. describe a Bromine indirubin FLIM/PLIM sensor to measure pO_2 during PDT [197].

Oxygen imaging of neurospheres is described in [188]. Applications to Giant Umbrella Cells and to healthy and inflamed colon tissue are described in [651] and [653]. Changes in intracellular oxygen concentration with the metabolic activity were observed by Zhdanov et al. [652]. Jenkins et al. studied the function of SPCA ATPases in HCT116 cells. They found correlations of the expression of SPCA1 and SPCA2 with the O_2 concentration [296].

Kurokawa et al. [348] provided a detailed evaluation of TCSPC PLIM with Platinum(II) 5,10,15,20-tetrakis-[4-carboxyphenyl]porphyrin. They tested the sensor with MKN45 cells, colon26 spheroids under different O_2 concentration. They were able to correlate low pO_2 regions with mitochondria, to track the change in O_2 concentration in Colon26 cells after addition of antimycin A, and to visualise the O_2 change in MIN6 cells after glucose stimulation.

Simultaneous measurements of NADH FLIM and PLIM and the correlation of metabolic parameters and O_2 concentration were published first by Kalinina et al. [305]. The authors ob-

served changes in the NADH lifetime with the O_2 concentration and after addition of antimycin A. Changes of the NADH decay functions with the intracellular O_2 concentration were also demonstrated by Shcheslavskiy et al. [531].

Lukina et al. [380] used a fibre-based TCSPC system with implantable fibre tips [77] to record NAD(P)H decay curves simultaneously with phosphorescence decay curves of an oxygen sensor in mouse tumors. The authors found characteristic changes in the NADH decay parameters and the O_2 concentration between healthy tissue and tumor tissue.

Plant Physiology

The fluorescence of plant tissue is dominated by the emission of chlorophyll. Chlorophyll is excited at any wavelength throughout the visible spectrum. It emits strong fluorescence in the spectral range from 700 to 800 nm. The chlorophyll fluorescence is closely related to the efficiency of photosynthesis. Energy used in the photosynthesis process is not emitted as fluorescence, and energy emitted by fluorescence does not contribute to photosynthesis. The quantum efficiency of the chlorophyll fluorescence - and thus the fluorescence lifetime - is therefore an indicator of photosynthesis rate, and an indicator of plant health.

Early chlorophyll lifetime measurements have been performed by Schneckenburger et al. [502-508] and applied to the detection of plant diseases, especially forest decline by environment contamination.

Falkowski, Lin, and Gorbunov [218, 374] have measured the quantum yield of photochemistry and the fluorescence decay time of chlorophyll in phytoplankton. They collected 200,000 data sets at various locations in five oceans. Night time (dark-adapted) lifetimes ranged from 0.5 to 2.7 ns. From the data the authors calculated an average quantum yield of photochemistry of 0.35, and a average quantum yield of the fluorescence of 0.07. The remaining 58% of the absorbed energy is converted into heat.

Pavlinka et al. examined the responsiveness of *Dunallia tertiolecta* alga to Cadmium, Acidification, and laser-induced photobleaching [441]. They recorded FLIM in two channels, from 500 to 550 nm, and from 650 to 710 nm. Presence of cadmium caused an increase in the intensity in the 500-550-nm channel but no change in the fluorescence lifetime. Acidification caused an increase in the intensity accompanied by an increase in lifetime. An examination of the lifetimes of *Chlorella* sp. under different acidification [388] showed a decrease in intensity and a decrease in the lifetime, τ_1 , in the red channel with increasing acidity. Under severe acidification, also the lifetime, τ_2 , in the green channel changed but the change went toward longer lifetime.

In addition to chlorophyll, plant tissue contains an (often unknown) number of different fluorophores, namely carotenoids and flavinoids [633, 634]. Many of them are fluorescent but little is known about the fluorescence lifetimes and their dependence on environment conditions. FLIM measurements of plant tissue with the bh DCS-120 MP system and ultra-fast detectors have shown multi-exponential decay functions with extremely fast decay components, see Fig. 442. The fast decay component, τ_1 , at the cursor position has a lifetime of 33 ps, and it is present with 87% of the amplitude of the entire decay function!

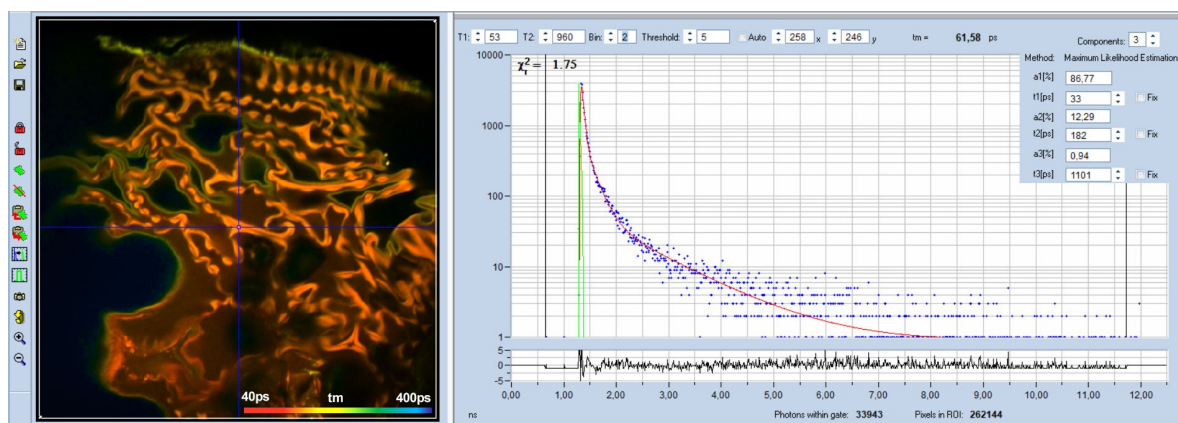


Fig. 442: Tissue in an anther of a green pine cone. Note the fast decay component, τ_1 , with 33 ps decay time. The component constitutes 87% of the amplitude of the entire decay..

Due to the large number of fluorophores which have to be expected it can be useful to record multi-wavelength decay or multi-wavelength FLIM data. A multi-wavelength FLIM image of plant tissue is shown in Fig. 443. The fluorescence of chlorophyll is detected above 680 nm, at the red end of the spectrum. At wavelength below about 650 nm fluorescence from flavines is detected. The intensity of the flavin fluorescence is much lower than that of the chlorophyll fluorescence. The intensity in Fig. 443 therefore was normalised to the brightest pixel of each wavelength channel. The decay times are generally short, and strongly multi-exponential.

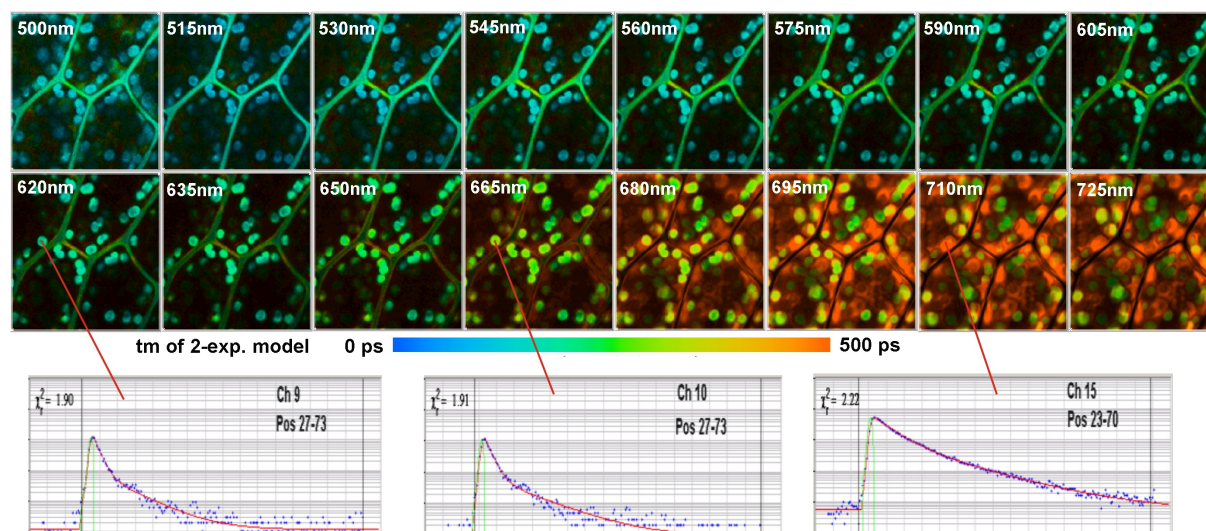


Fig. 443: Multi-spectral FLIM of plant tissue. Moss leaf, LSM 710 NLO, two-photon excitation at 850 nm. Amplitude-weighted mean lifetime of double-exponential fit. Intensity normalised to brightest pixel of each wavelength interval. Decay curves displayed for indicated spot of the 620nm, 665nm, and 710nm images.

Chlorophyll Transients

The fluorescence of chlorophyll competes with the energy transfer into the photosynthesis channels. The photosynthesis rate changes with the light intensity and the time of exposure. Therefore, the fluorescence lifetime of the chlorophyll in live plant tissue not only depends on the state of the plant but also on the light intensity, and the time of exposure.

The changes in the fluorescence intensity of chlorophyll with the exposure to light have already been found 1931 by Kitschy and Hirsch [311]. The effects have been called fluorescence induction, fluorescence transients, or Kautsky effect [258, 259, 391]. When a dark-adapted leaf is exposed to light the intensity of the chlorophyll fluorescence starts to increase. After a steep rise the intensity falls again and finally reaches a steady-state level. The rise time is on the range from a few milliseconds to a second, the fall time can be from several seconds to minutes.

The initial rise of the fluorescence intensity is attributed to the progressive closing of reaction centres in the photosynthesis pathway. Therefore the quenching of the fluorescence by the photosynthesis decreases with the time of illumination, with a corresponding increase of the fluorescence intensity. The fluorescence quenching by the photosynthesis pathway is called 'photochemical quenching', and the associated change in the fluorescence intensity 'photochemical transient'. The slow decrease of the fluorescence intensity at later times is termed 'non-photochemical quenching'. Both the photochemical and the non-photochemical transient are accompanied by a corresponding change in the fluorescence lifetime [11, 254, 405].

These light-induced changes in the fluorescence lifetime in live plants are often considered an obstacle for the use of the fluorescence lifetime in chlorophyll research. The effects make it

necessary that measurements are performed under exactly defined illumination, and under steady-state conditions. Researchers in the chlorophyll field therefore often consider lifetime measurements too difficult and resort to intensity measurements. However, the chlorophyll transients can well be recorded by advanced TCSPC technique. We believe that the amplitude and the speed of the chlorophyll transients are an even better indicator of the plant state than fluorescence intensity or decay measurements under steady-state conditions.

Single-Point Measurement

Both the photochemical and the non-photochemical quenching transients can be measured by time-series TCSPC. A dark-adapted leaf is put in a standard fluorescence-decay measurement setup. The laser is switched on, and a series of decay recordings is started. A non-photochemical fluorescence transient of a leaf recorded this way is shown in Fig. 444. The time of the series runs from the back to the front. From left to right, the behaviour of a fresh leaf, a faded leaf, and a dried leaf is shown. The differences between leaves of different health state are striking. In fact, the amplitude and the speed of the non-photochemical transient are a much better indicator of the health state of the plant than the (average) lifetime itself. The figure also shows that a measurement of a single decay function of the chlorophyll fluorescence delivers unreliable results: Unless the measurement is performed under steady-state conditions, i.e. when the transient is complete, the measured decay curve would be just an average of decay profiles of an undefined time interval within the transient. Most likely, the results would depend on the acquisition time and the excitation power. More important, it would miss the most important effect, which is that the decay profile is *changing*, and that the change bears information of the health of the plant.

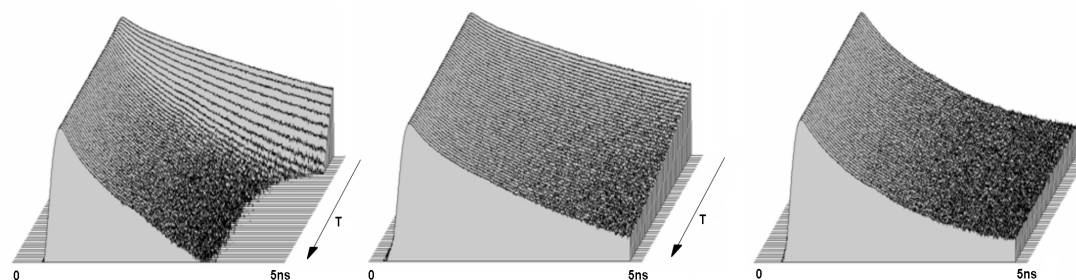


Fig. 444: Sequences of fluorescence decay curves measured at a leaf after start of illumination. Left to right: Fresh leaf, faded leaf, dried leaf. Decay time axis 0 to 5 ns, time per curve 2 seconds, logarithmic intensity scale. The sequence starts from the back.

The decay curves in Fig. 444 were recorded at speed of 2 seconds per curve. There is no problem to record enough photons for high accuracy decay analysis within this time. To record the 'photochemical transient' a much faster recording speed has to be used. The problem is that the acquisition time per curve becomes too short to record enough photons. The solution is triggered accumulation of the sequence: The light is periodically turned on and off, a sequence of decay curve is recorded over the time of the 'ON' phases, and the photons of many on/off periods are accumulated [77].

An example for the recording of a photochemical transient is shown in Fig. 445. The time per curve is 100 μ s, 10,000 on/off periods were accumulated. The time of the sequence runs from the front to the back. It can clearly be seen how the fluorescence lifetime increases over the time into the laser-on period.

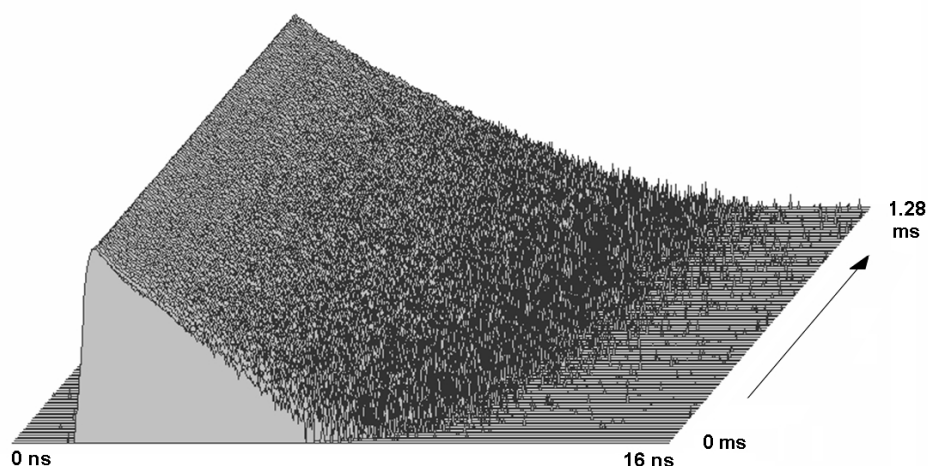


Fig. 445: Photochemical quenching transient of chlorophyll in a fresh leaf. The sequence starts at the front. Time per curve 100 μ s, transient-time scale 0 to 12.8 ms into laser-on periods. 10,000 on/off cycles accumulated.

FLIM of the Non-Photochemical Transient

The non-photochemical transient can be recorded by FLIM. Even with a conventional record-and-save procedure a time series can be recorded at a speed of one image per second. An example is shown in Fig. 446. The acquisition time per image was 1 second, the image size 256 x 256 pixels x 64 time channels. It can clearly be seen that the fluorescence lifetime becomes shorter over the time of exposure. Other examples have been shown in [67, 291, 310]. Holub et al. have recorded a FLIM series of the non-photochemical transient by wide-field frequency domain FLIM [283] at a rate of a few seconds per image. With the DCS-120 scanner TCSPC FLIM reaches at least the same speed, with the advantage that depth resolution is available.

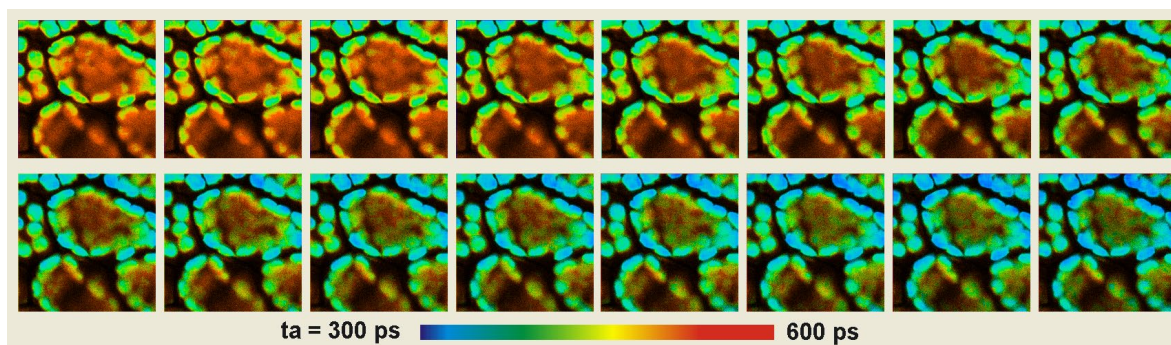


Fig. 446: Change of the fluorescence lifetime of chlorophyll with time of exposure. Moss leaf, 1-photon excitation at 445 nm, 256x256 pixels. SPC-150 autosave time-series, 1 image per second.

Temporal Mosaic FLIM

A more advanced way of recording time-series of non-photochemical chlorophyll transients is by temporal mosaic FLIM. Time series recording is performed over the elements of a mosaic of FLIM images. Because the entire mosaic is kept in the memory the sequence is not slowed down by save operations. Please see section ‘Time-Series FLIM by Mosaic’, page 151. A chlorophyll series recorded by temporal mosaic FLIM is shown in Fig. 447.

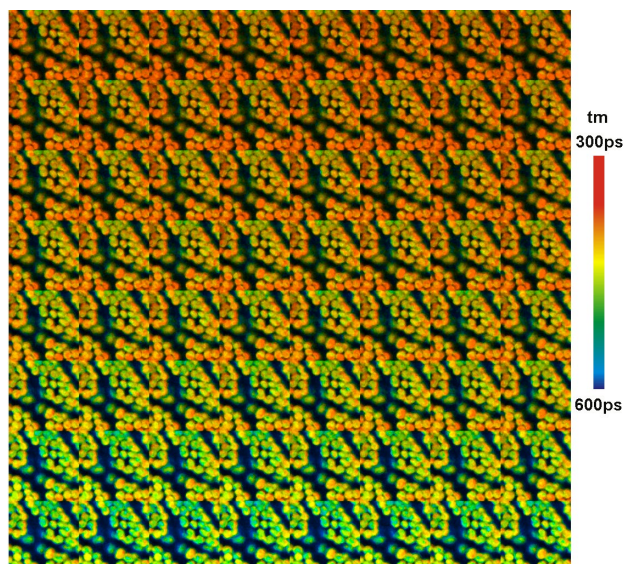


Fig. 447: Non-photochemical transients in the chloroplasts of a moss leaf, recorded by temporal mosaic FLIM. Time runs from lower left to upper right. Red means long lifetime. DCS-120 confocal scanning system.

The sequence contains 64 images (or 64 mosaic elements) for consecutive times after the turn-on of the excitation light. The acquisition time per element was 1 second, the total time of the sequence 64 seconds, the image size of each element 128 x 128 pixels, with 256 time channels per pixel.

Recording the Chlorophyll Transients by FLITS

A second way to record the chlorophyll transients with spatial resolution is by using the 'FLITS' technique [74, 76, 229], see 'FLITS: Fluorescence Lifetime-Transient Scanning', page 164. FLITS records the changes in the decay profile of a sample over time and along a one-dimensional scan. For technical details please see page 164 of this handbook. An example for the non-photochemical transient is shown in Fig. 448. It shows decay curves for one selected pixel within the line scan for times of 0.5 s, 7.5 s, and 13.4 s after the turn-on of the laser. The mean lifetimes, t_m , obtained from a double-exponential fit are 560 ps, 385 ps, and 311 ps, respectively. The changes in the decay profiles are clearly visible.

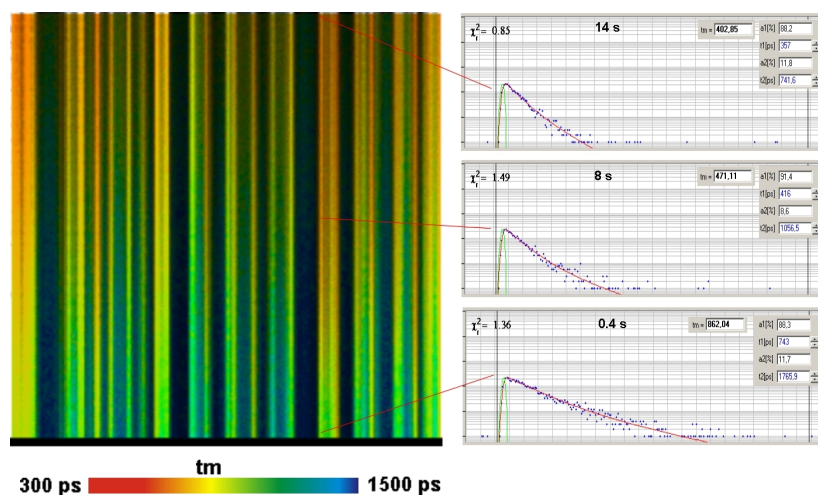


Fig. 448: FLITS of chloroplasts in a plant leaf. Non-photochemical transient, decay curves for a selected pixel within the line for times of 0.5 s, 7.5 s, and 13.4 s after the turn-on of the laser. Lifetimes are amplitude-weighted averaged of a double exponential fit to the decay data. The vertical stripes are the lifetime traces of individual chloroplasts over the transient-time.

With FLITS, a sequence fast enough for the photochemical transient can be recorded. To obtain a sufficiently high number of photons per time interval accumulation over a larger number of on/off periods must be used. An example is shown in Fig. 449.

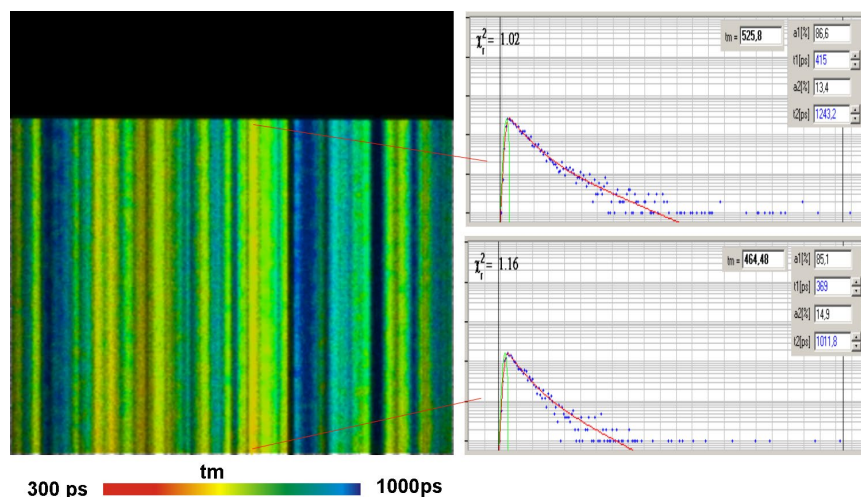


Fig. 449: FLITS of chloroplasts in a plant leaf. Photochemical transient, triggered accumulation, decay curves for a selected pixel within the line for times of 3 ms and 191 ms into the laser-on periods. Amplitude-weighted lifetime of double-exponential decay.

Other Chlorophyll Lifetime applications

The results presented above show that lifetime changes induced by physiological effects in plants can be recorded by various operating modes of the bh TCSPC modules. Nevertheless, very little has been published in this area. Broess recorded FLIM data of single chloroplasts [130]. Teplicky et al. [581] recorded FLIM of *Chlorella* algae, aiming on a possible use of the chlorophyll lifetimes for environment monitoring. Gilmore et. al [254] showed the fluorescence lifetime changes during the non-photochemical transient, Nozue et al. [424] recorded FLIM for different laser power, and the associated changes in the chlorophyll decay parameters. Lambrev et al. [355] recorded spectrally resolved intensity changes during the transient. Miloslavina et al. [405] used TCSPC to record decay curves of dark-adapted cells, light-adapted cells, and light-adapted cells under the influence of DCMU (3-(3',4'-dichlorophenyl)-1,1-dimethylurea). Lifetime imaging of plant tissue during the non-photochemical transient was used by Holub et al. [283]. The authors used a wide-field frequency-domain technique that acquired images within 3.9-second intervals during the non-photochemical transient. Iwai et al. [291] showed that there are distinct peaks in the lifetime distribution that change their relative amplitudes during exposure to light. Other applications aim on the construction of chlorophyll-based light harvesting complexes with of higher absorption coefficients. Grayson et al. [260] designed a YFP-enhanced reaction centre, Szalkowski et al. [574] used chlorophyll assemblies on nanowires.

SPCImage NG Data Analysis Software

Overview

SPCImage NG is a new generation of bh's TCSPC-FLIM data analysis software. It combines time-domain and frequency-domain analysis, uses a maximum-likelihood algorithm to calculate the parameters of the decay functions in the individual pixels, and accelerates the analysis procedure by GPU processing. In addition to FLIM data, SPCImage NG processes single-curve decay data, multi-wavelength data, excitation-multiplexed data, PLIM data, mosaic FLIM data, and other multi-dimensional TCSPC data sets. SPCImage NG provides decay models with one, two, or three exponential components, incomplete-decay models, and a shifted-component model. A global analysis function is available for analysing multi-exponential data with constant component amplitudes. Another important feature is advanced IRF modelling, making it unnecessary to record IRFs for the individual FLIM data sets. 1D and 2D parameter histograms are available to display the distribution of the decay parameters over the pixels of the image or over selectable ROIs. Image segmentation can be performed via the phasor plot or the 2D parameter histograms. Pixels with similar phasor or 2D parameter signature can be combined for high-accuracy time-domain analysis, resulting in photon numbers known only from cuvette-based lifetime experiments. A batch-processing function and a batch export function are available for analysing a large number of FLIM data sets automatically and to convert them into bmp or tif images.

Main Panel

A typical main panel of SPCImage is shown in Fig. 450. It shows a lifetime image on the left, a parameter histogram in the upper right, and a fluorescence decay curve in the lower right.

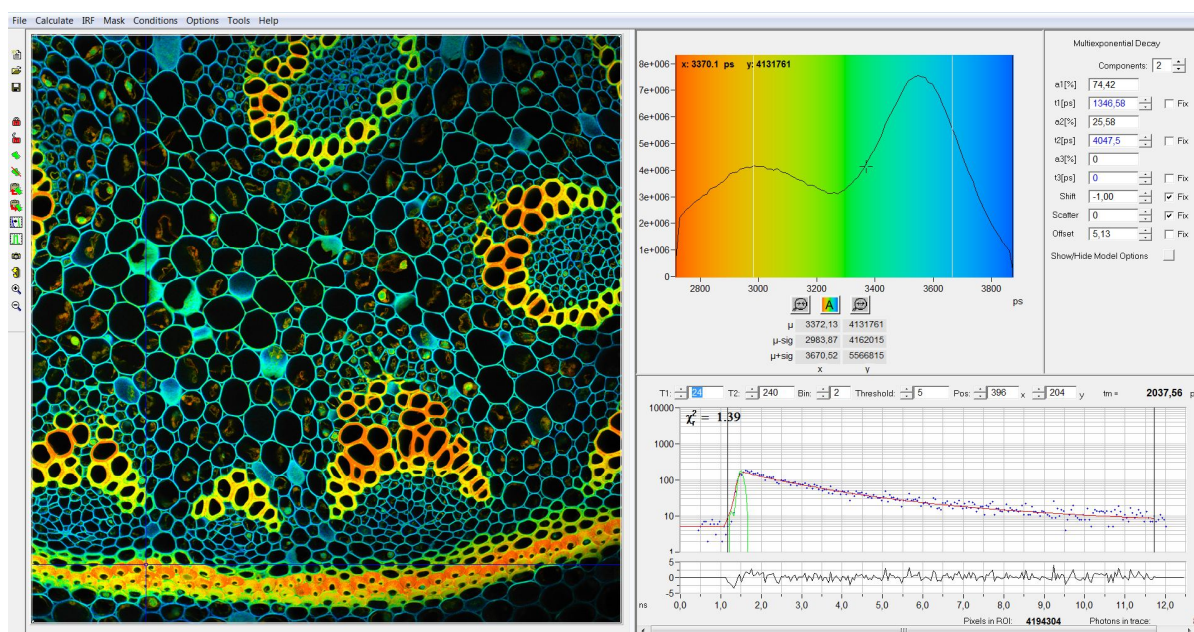


Fig. 450: Main panel of SPCImage NG

Since version 8.5 SPCImage NG is able to display two lifetime images simultaneously. Typically, these are images of the same sample in different wavelength intervals or for different excitation wavelength. An example is shown in Fig. 451.

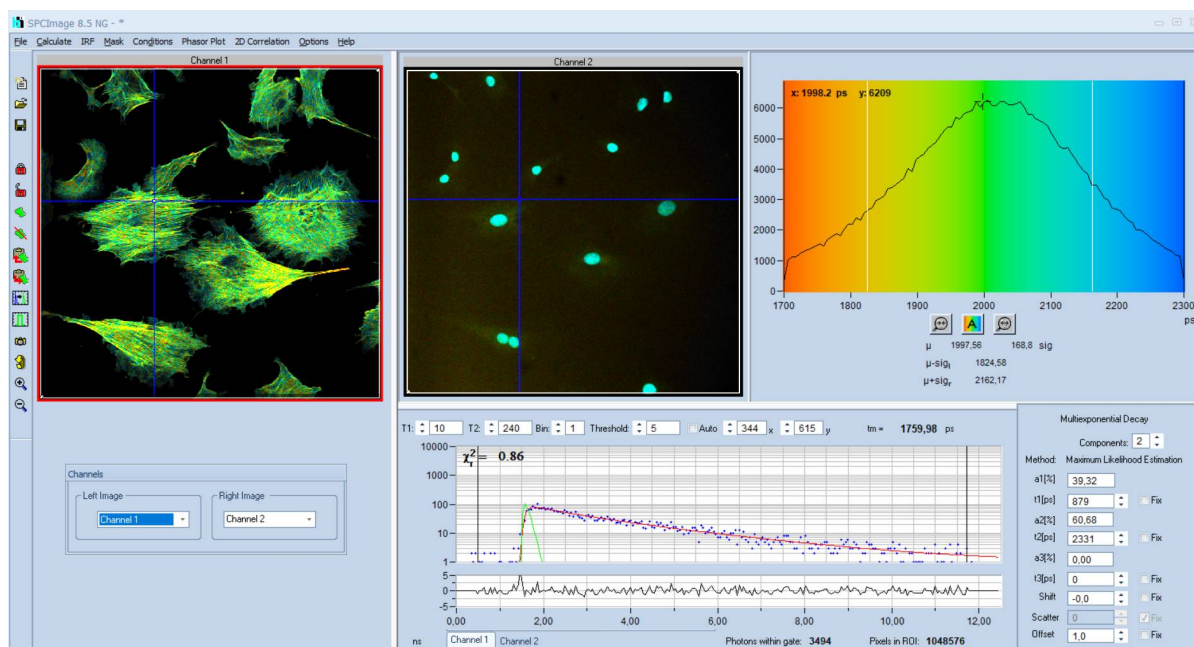


Fig. 451: SPCImage NG with two images in different wavelength intervals

The upper right part of the SPCImage NG panel can be replaced with a phasor plot. An example is shown in Fig. 452

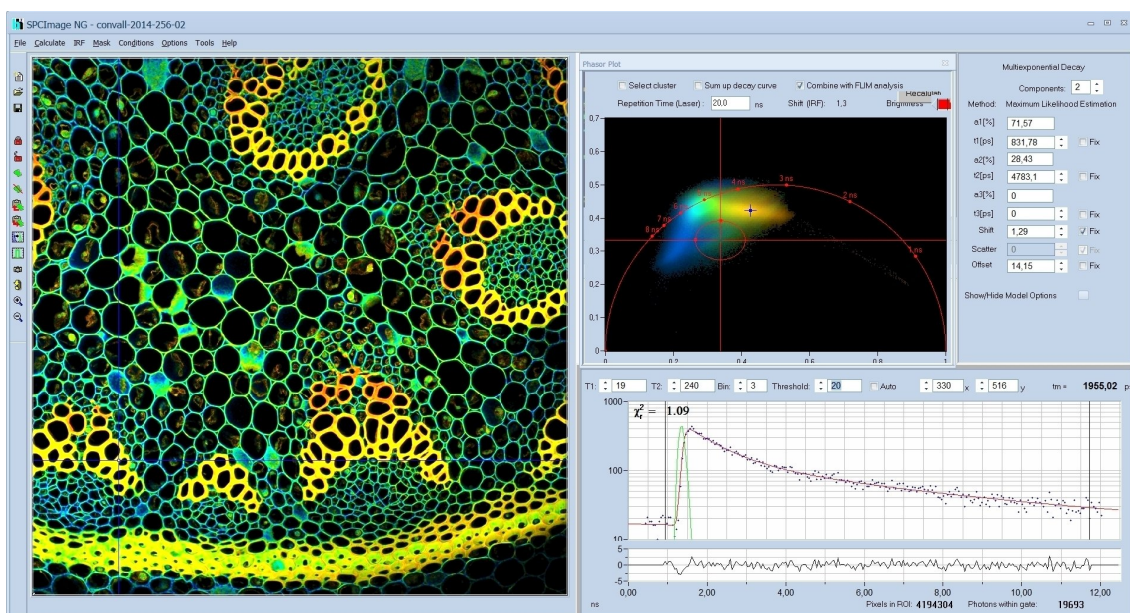


Fig. 452: SPCImage NG main panel with phasor plot

Other main panel configurations are possible. These include combinations with grey-scale images, with 2-D parameter histograms, and configurations for single-curve analysis. An example is shown in Fig. 453.

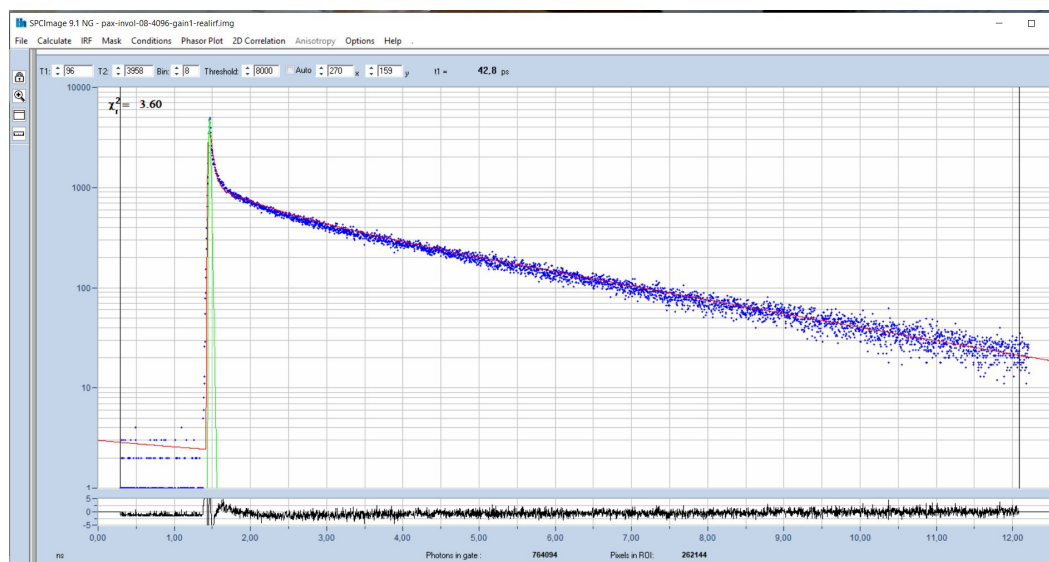


Fig. 453: Main panel for single-curve analysis

Images of Decay Parameters

In the simplest case, the result of FLIM analysis is the 'lifetime' of the decay functions in the individual pixels. However, in practice the decay functions are not single-exponential. Moreover, the desired biological information often is in the composition of the decay functions, not in the apparent lifetime. For complex decay functions SPCImage delivers the lifetimes and amplitudes of the decay components. SPCImage then creates colour-coded images of the amplitude- or intensity-weighted lifetimes in the pixels, images of the lifetimes or amplitudes of the decay components, images of lifetime or amplitude ratios, and images of other combinations of decay parameters, such as FRET intensities, FRET distances, bound-unbound ratios, or the fluorescence-lifetime redox ratio, FLIRR. Examples are shown in Fig. 454 through Fig. 457.

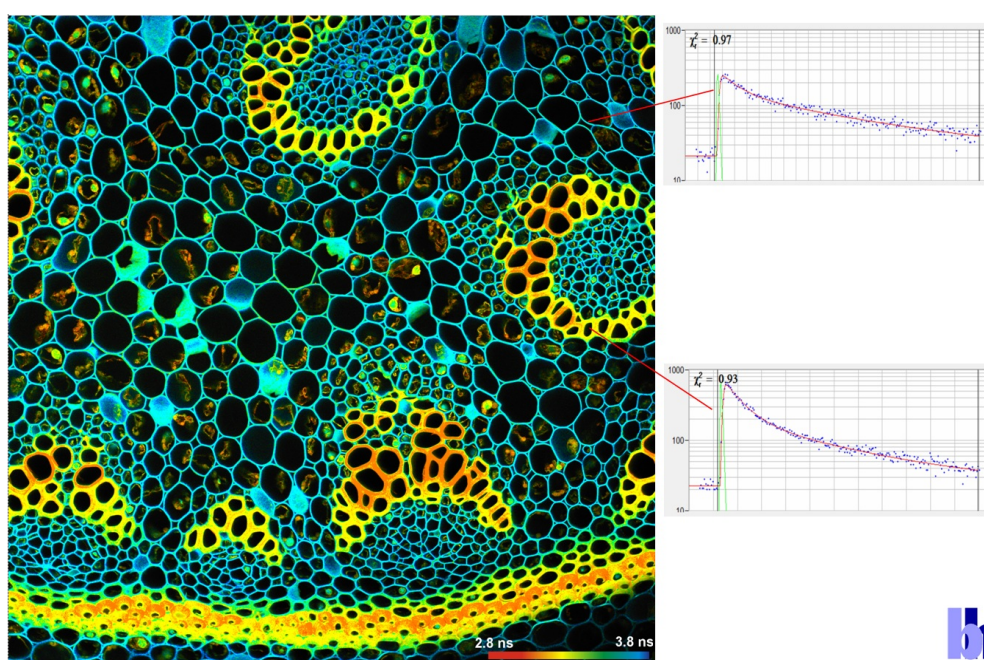


Fig. 454: Image of the amplitude-weighted lifetime, t_m , of a double-exponential decay. Right: Fluorescence decay curves in selected pixels.

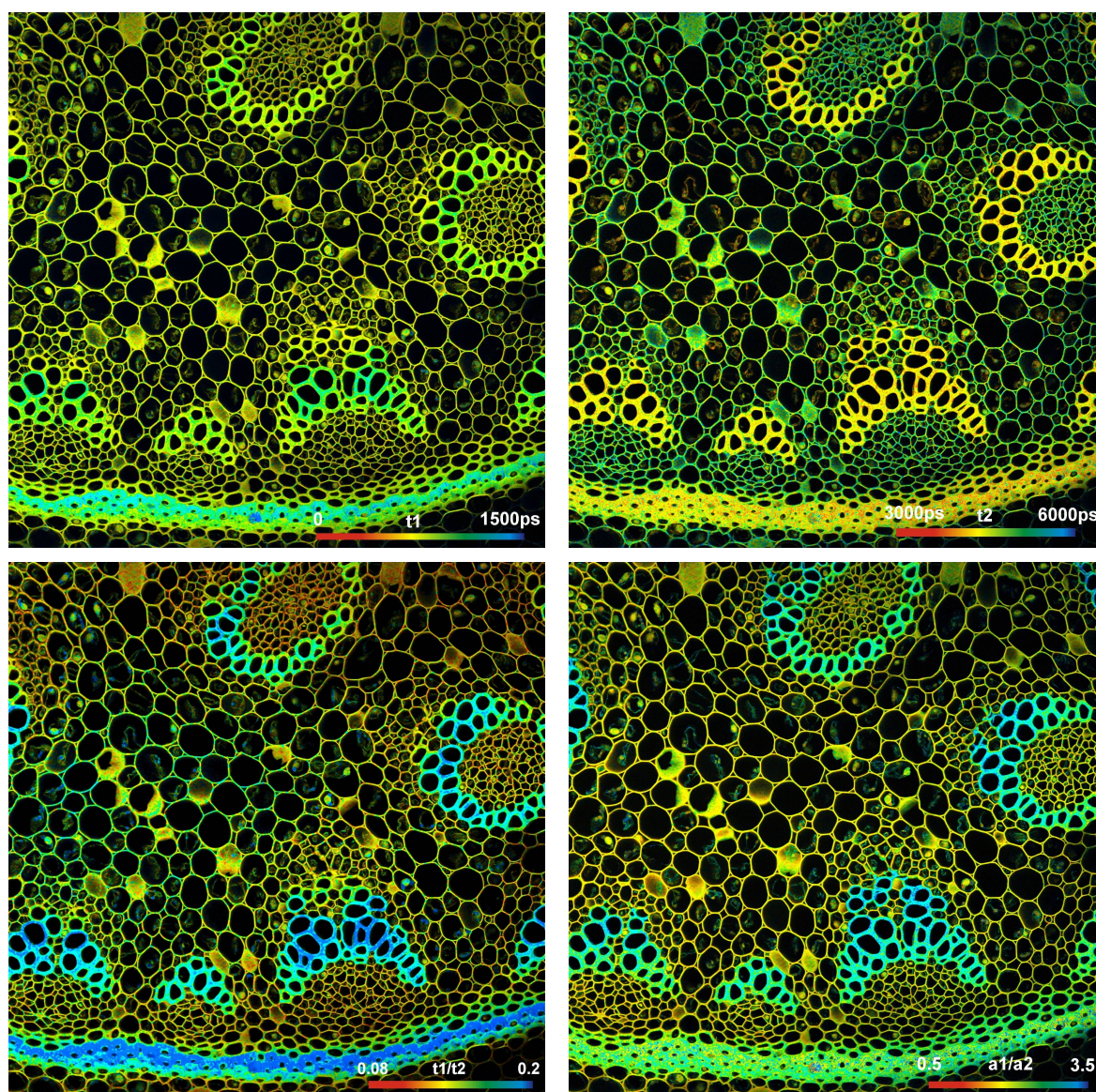


Fig. 455: Upper row: Images of the lifetimes of the fast component, t_1 , and the slow component, t_2 , of a double-exponential decay. Lower Row: Images of the amplitude ratio, a_1/a_2 , and the lifetime ratio, t_1/t_2 , of the fast and the slow decay component.

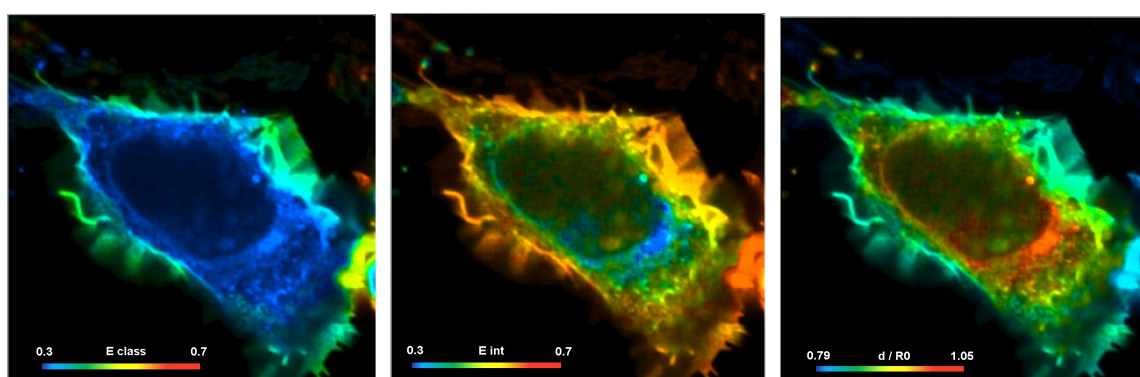


Fig. 456: Cell with interacting proteins, labelled with a FRET donor and a FRET acceptor. Left to right: Classic FRET efficiency, FRET efficiency of interacting donor fraction, FRET distance

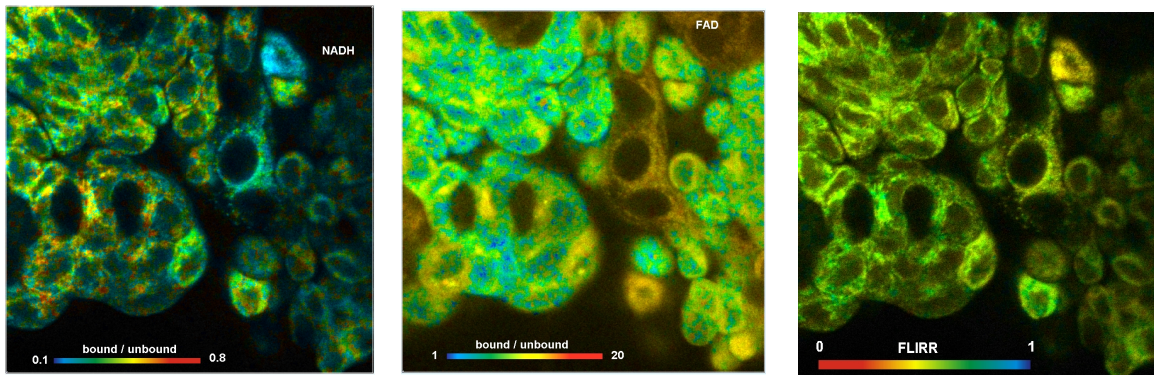


Fig. 457: Metabolic FLIM. Bound-unbound ratio of NADH, Bound/unbound ratio of FAD, Fluorescence-Lifetime Redox Ratio, FLIRR.

Phasor Plot

SPCImage FLIM analysis software combines time-domain multi-exponential decay analysis with phasor analysis. Phasor analysis expresses the decay data in the individual pixels as phase and amplitude values in a polar diagram, the 'Phasor Plot', page 390. Pixels with similar decay signature form distinct clusters in the phasor plot. Clusters of interest can be selected and back-annotated in the lifetime image for further processing or for combination of pixel data. An example is shown in Fig. 458.

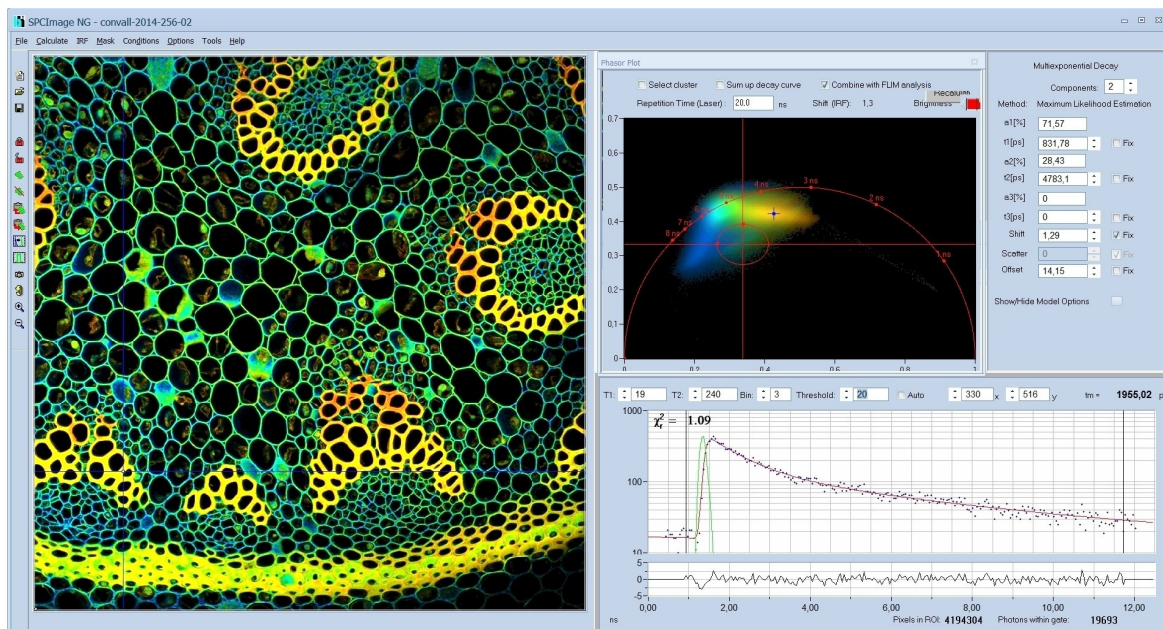


Fig. 458: Combination of time-domain analysis (left and lower right) and phasor plot (upper right)

Maximum-Likelihood Algorithm

SPCImage NG runs an iterative fit and de-convolution procedure on the decay data of the individual pixels of the FLIM images. Since version 8.5 SPCImage NG uses a maximum-likelihood estimation (MLE) process to determine the decay parameters in the pixels. In contrast to the frequently-used weighted least-square (WLS) fit, MLE is based on calculating the probability that the values of the model function correctly represent the data points of the decay function. Compared to the least-square method, the fit accuracy is significantly improved for multi-exponential decay functions. Moreover, there is no bias toward shorter lifetime as it is unavoidable for the least-square fit. Please see 'Fit Procedures', page 371.

Modelling of the Instrument-Response Function

Recording the 'Instrument Response Function' (IRF) is a permanent problem of time-resolved fluorescence spectroscopy. Recording the IRF in a FLIM system is difficult, and often impossible. As a result, there is rarely an IRF that was recorded in a FLIM system and represents the temporal behaviour of the system correctly. Therefore, SPCImage NG does away with IRF recording altogether. Instead, the IRF is extracted from the FLIM data themselves. Earlier SPCImage versions had an 'Auto IRF', which was derived from the rising edge of the fluorescence decay function. The Auto IRF has been used successfully for more than 20 years. It works well for decay functions which are not too far from a single-exponential function but has deficiencies if very fast decay components are present. A new approach introduced by SPCImage NG is the 'Synthetic IRF'. It is created by modelling the IRF with a generic function. The exact parameters of this function are determined by fitting it to the FLIM data together with the selected decay model, see 'Model Functions', page 309. The results of this procedure are so good that an accurate IRF is obtained even for decay functions containing ultra-fast components, see Fig. 459.

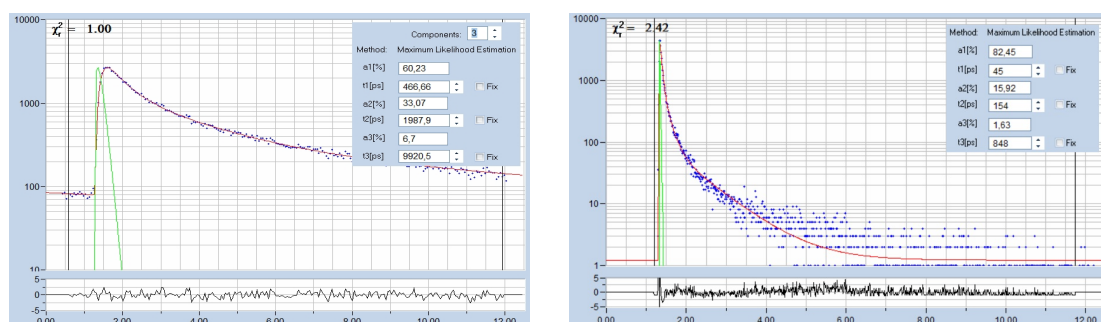


Fig. 459: Analysis with synthetic IRF. Left: Fluorescence excited by diode laser. Right: Ti:Sa laser, sample with extremely fast decay component. Green curve IRF, blue dots data points, red curve fit with triple-exponential decay model.

Decay Models

SPCImage NG provides single-, double-, and triple-exponential decay models. An 'Incomplete Decay' option is available to determine long fluorescence lifetimes within the short pulse period of the Ti:Sa laser of a multiphoton system. SPCImage NG provides also a 'Shifted-Component' model. In this model, the decay components of a multi-exponential model functions can be shifted in time by predefined values. The model is used for ophthalmic FLIM, where different decay components come from different depth within the eye. For details please see bh TCSPC Handbook [77].

GPU Processing

Data recorded with bh FLIM systems can contain an enormous number of pixels and time channels. Images with 1024 x 1024 or even 2048 x 2048 pixels are not uncommon, and time-channel numbers of 1024 are routinely used in combination with fast HPM detectors. Processing such amounts of data by the CPU of even a fast computer would take tens of minutes, and a fit with global parameters would be entirely out of reach. SPCImage NG therefore runs the data analysis on a GPU (Graphics Processor Unit). The image data are transferred into the GPU, which then runs the de-convolution and fit procedure for a large number of pixels in parallel. With the GPU, data processing times are thus massively reduced. The image shown in Fig. 460 was calculated on an NVIDIA GPU within five seconds.

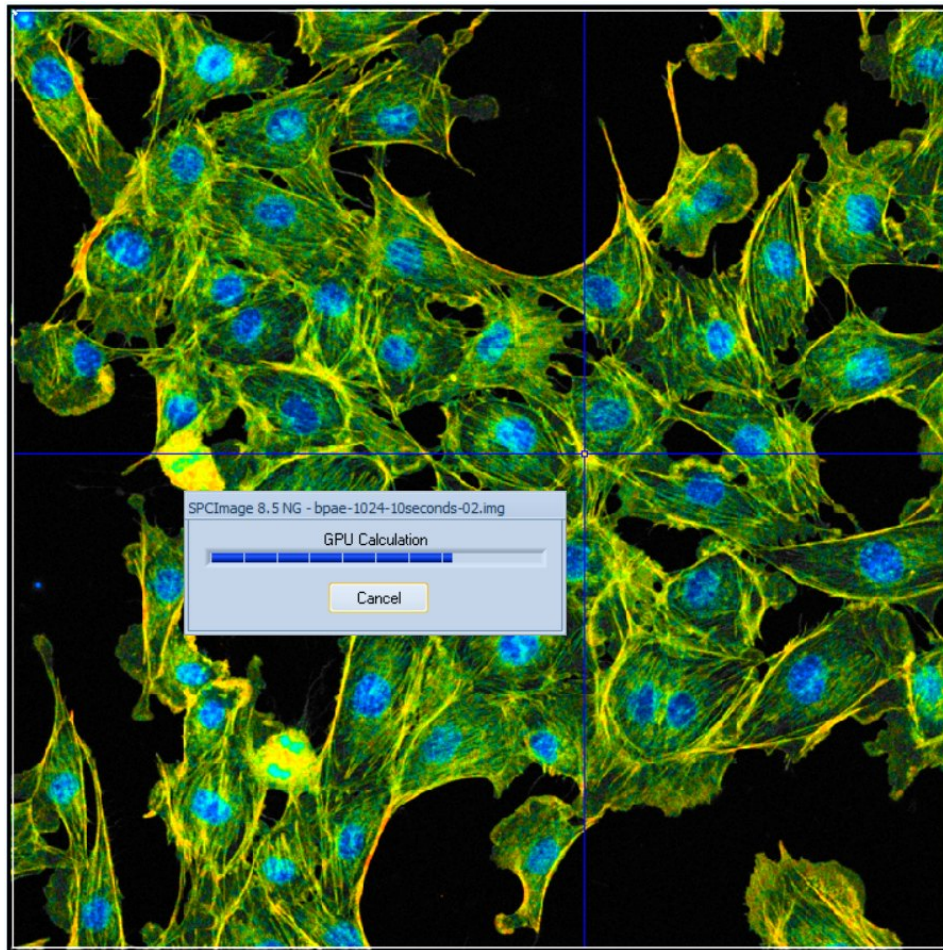


Fig. 460: A lifetime image with 1024 x 1024 pixels and 1024 time channel per pixel. The image was calculated on an NVIDIA GPU in 5 seconds.

Parameter Histograms

SPCImage has histogram functions for the decay parameters. The histogram shows how often pixels of a given parameter value occur in the lifetime image. The histogram refers either to a selected region of interest or, if no ROI was defined, to the entire lifetime image. Together with the various options to select decay parameters and combinations of decay parameters a wide variety parameter histograms can be obtained. Two examples are shown in Fig. 461.

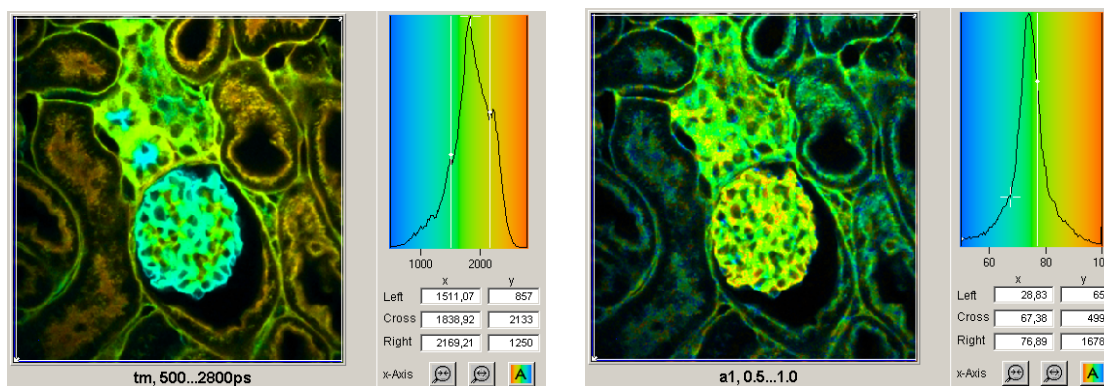


Fig. 461: Histograms of the mean (amplitude weighted) lifetime of double-exponential fit (left) and of the amplitude of the fast decay component, a_1 (right)

2-D Histograms

2D histograms present density plots of the pixels over two selectable decay parameters. The decay parameters can be lifetimes, t_1 , t_2 , t_3 , or amplitudes, a_1 , a_2 , a_3 , of decay components, amplitude or intensity-weighted lifetimes, t_m or t_i , or arithmetic conjunctions of these parameters. An example is shown in Fig. 462. A histogram of the amplitude, a_1 , of the fast decay component versus the amplitude-weighted lifetime, t_m , has been created. Cursors in the histogram are available to select special parameter combinations and back-annotate the corresponding pixels in the lifetime image.

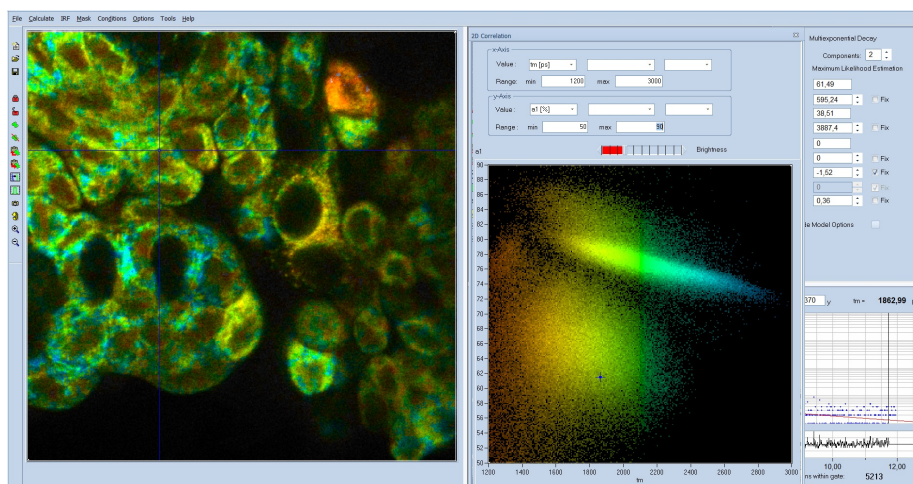


Fig. 462: 2-D histogram showing density plot of pixels over amplitude-weighted lifetime, t_m , and amplitude of fast component, a_1 .

ROIs

SPCImage NG allows the user to define ROIs in the images. Both rectangular and polygonal ROIs can be defined. Parameter histograms are displayed for the selected ROI, see Fig. 463.

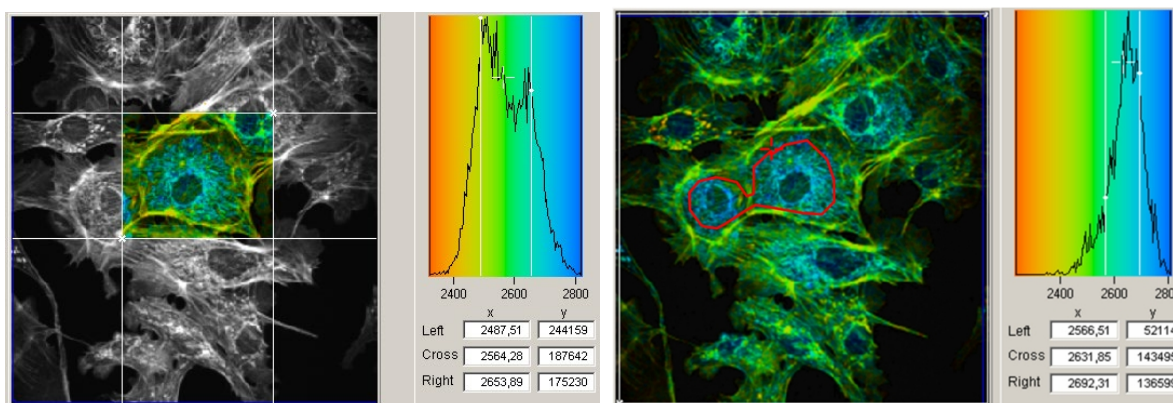


Fig. 463: ROI Definition. Left: Rectangular ROI. Right: Polygonal ROI

Several polygonal ROIs can be defined, and the corresponding parameter histograms be selected via the buttons on top of the histogram window. Please see Fig. 464.

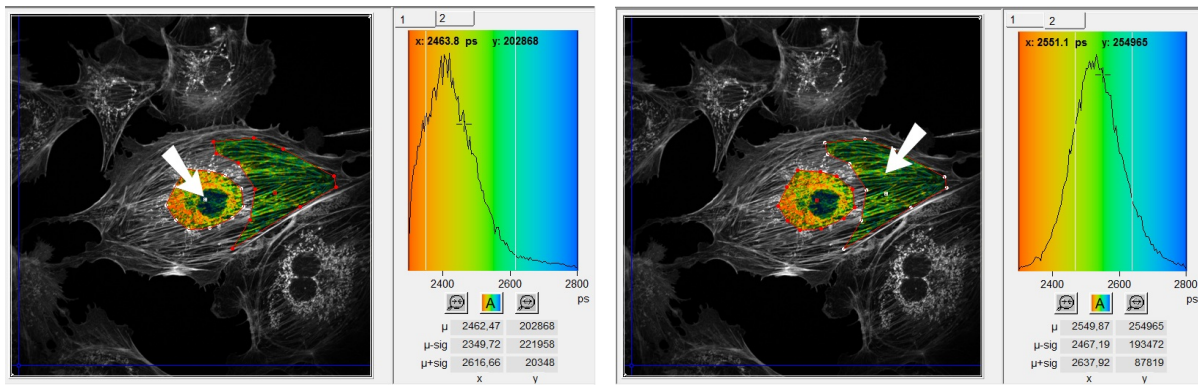


Fig. 464: Multiple ROIs, with selection of parameter histogram.

Image Segmentation

Images taken at high pixel numbers and Mosaic FLIM images can contain a large number of cells. In these cases, it is time-consuming, if not impossible, to manually select regions of interest for each of the cells in the image. SPCImage NG therefore provides automatic image segmentation functions via the phasor plot and the 2D histograms. Areas with different decay signature form separate clusters in these presentations. Interesting clusters can be selected and back-annotated in the images. An example is shown in Fig. 465. The image area contains a large number of cells. A phasor plot of the image was calculated, the phasor range of the cell nuclei selected, and the corresponding pixels back-annotated in the lifetime image. The decay data of these pixels were combined. The result is single decay curve, containing an enormous number of photons. This curve can be analysed at high precision with double- and triple-exponential decay models, see Fig. 465, bottom right. For details please see 'Phasor Plot', page 339.

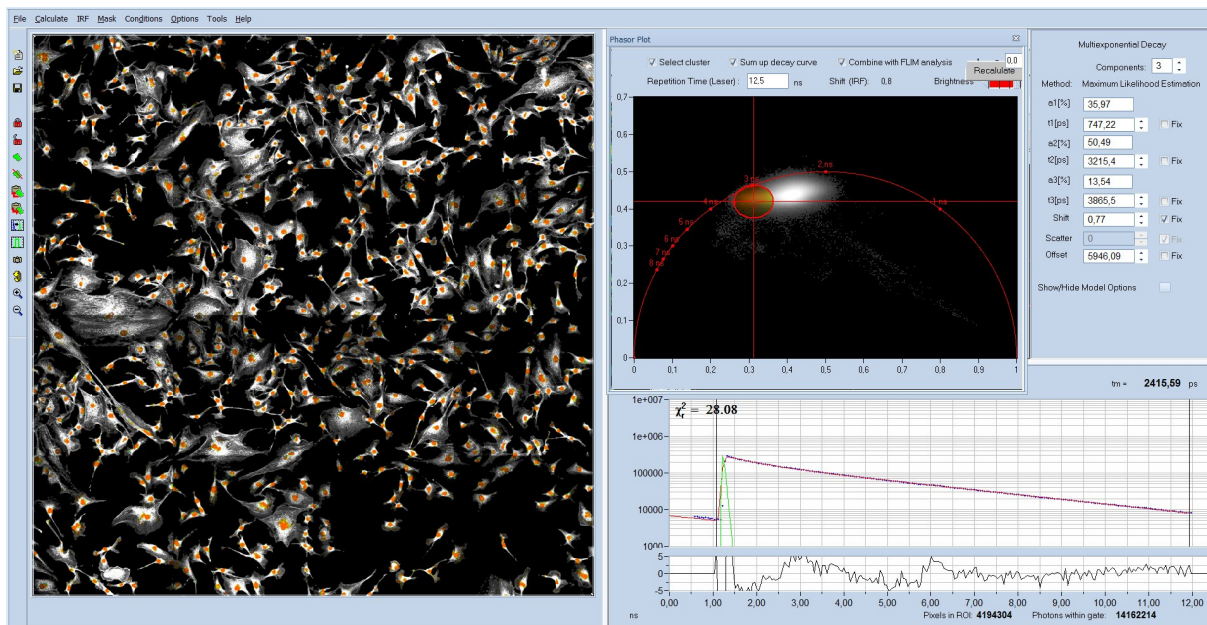


Fig. 465: The phasor range of the nuclei of the cells has been selected by the 'Select Cluster' Function. The decay-parameter histogram (shown right) refers to the selected pixels. A combined decay curve for the selected pixels is displayed by the 'Sum up decay curves' function.

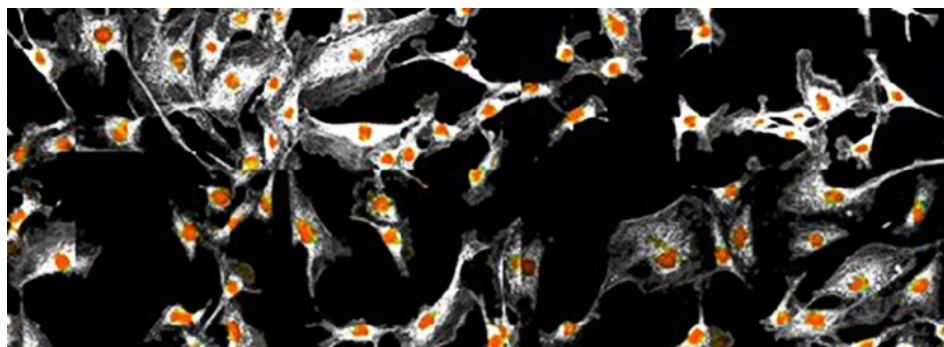


Fig. 466: Detail from Fig. 465. The nuclei have been selected by phasor-segmentation.

Analysing FLIM Data with SPCImage

Loading the FLIM Data into SPCImage NG

There are several ways to load FLIM data into SPCImage. You can use the 'Send Data to SPCImage' function of the SPCM software, import a .sdt TCSPC data file, or load .img data previously analysed by SPCImage.

Loading of SPCImage Files

Data previously analysed and saved by SPCImage data analysis (.img files) are loaded via the 'Open' function. Click into 'Main', 'Open' as shown in Fig. 467.

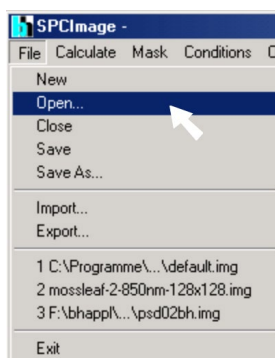


Fig. 467: Opening .img files generated by SPCImage

After loading the data SPCImage will come up in a configuration as shown in Fig. 468.

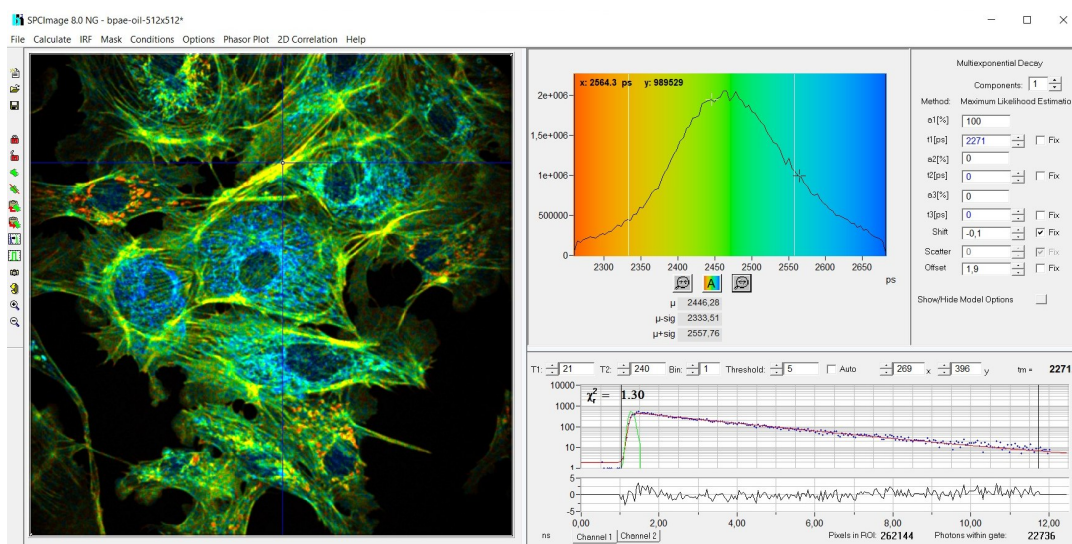


Fig. 468: SPCImage NG main panel after loading .img data

Importing Data From SPCM by Send Data Function

Raw data can be send directly from SPCM to SPCImage. The 'Send Data to SPCImage' function of the SPCM software is illustrated in Fig. 469. The function automatically opens SPCImage and transfers the data. In the 'application options' of the SPCM software you can select whether you want to transfer the data of all active display windows or only the data of the selected one. To select a display window, first click into the image that you want to analyse, see Fig. 469, left. Then transfer the data by clicking into 'Main', 'Send data to SPCImage', see Fig. 469, right.

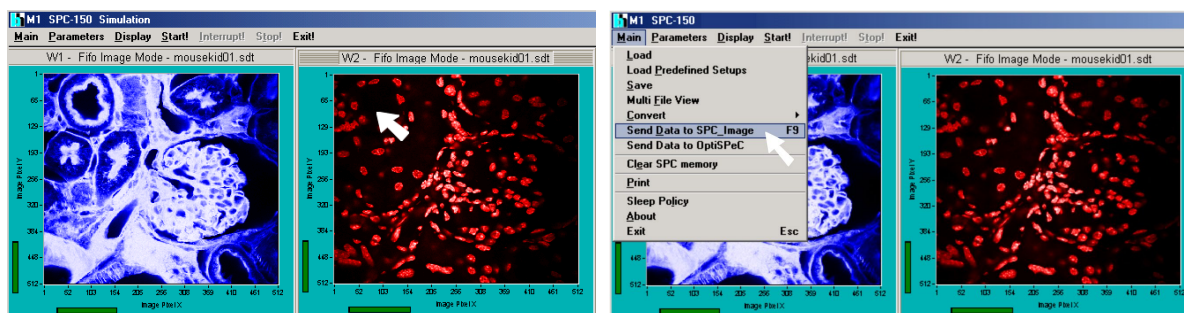


Fig. 469: Sending data from SPCImage into SPCImage. Left: Selection of the data to be analysed. Right: Sending the selected part of the data to SPCImage

Import of .sdt Files

Files from SPCM (.sdt format) are loaded via the 'Import' function. A click into 'Main', 'Import', (Fig. 470, left) opens a file selection panel (Fig. 470, right). Select the desired file from this panel and click on 'Open'. SPCImage then examines the selected file and opens the Import Options panel for the type of data found in the file.

The 'Import Options' panel for FLIM images is shown in Fig. 471, left. Top left, it shows the general structure of the data. In the example shown the file contains two FLIM images from different TCSPC / FLIM modules. Upper right, the import panel shows information on the images, such as pixel number and number of time channels. At the bottom of the panel, you can specify subsets of the data for import. For standard applications we recommend to select 'All', and then click on 'OK'. When the import is completed the image and a decay function will show up in the SPCImage main panel. It can happen, however, that the .sdt data contain combinations of modules, routing channels and page numbers which you do not want to import. In that case, you can disable or enable modules, and select ranges of routing channels which you want to import. There is also an option to combine the data of all modules and channels, or to add or subtract data from the data already loaded. These options should be handled with care. They require that all data are recorded with the same time scale, time-channel number, IRF position and IRF shape.

The Import Options for files that contain decay curves are shown in Fig. 470, right. The file structure window (upper left) indicates that the file contains single decay curves in different 'Traces' of SPCM. The Measurement Info window upper right shows basic information about the data, such as number of time channels and time range. 'Import as' allows you to import the data as normal decay curves or to import a single curve as an Instrument Response Function (IRF). At the bottom of the import panel you can select which curves you want to be import. If in doubt, just select 'All' and proceed by clicking 'OK'.

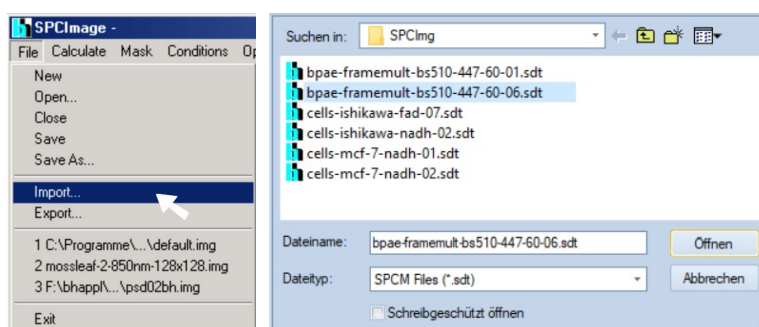


Fig. 470: Import of FLIM data. Left: Select Import function in 'File' menu. Right: Select the file to be imported.

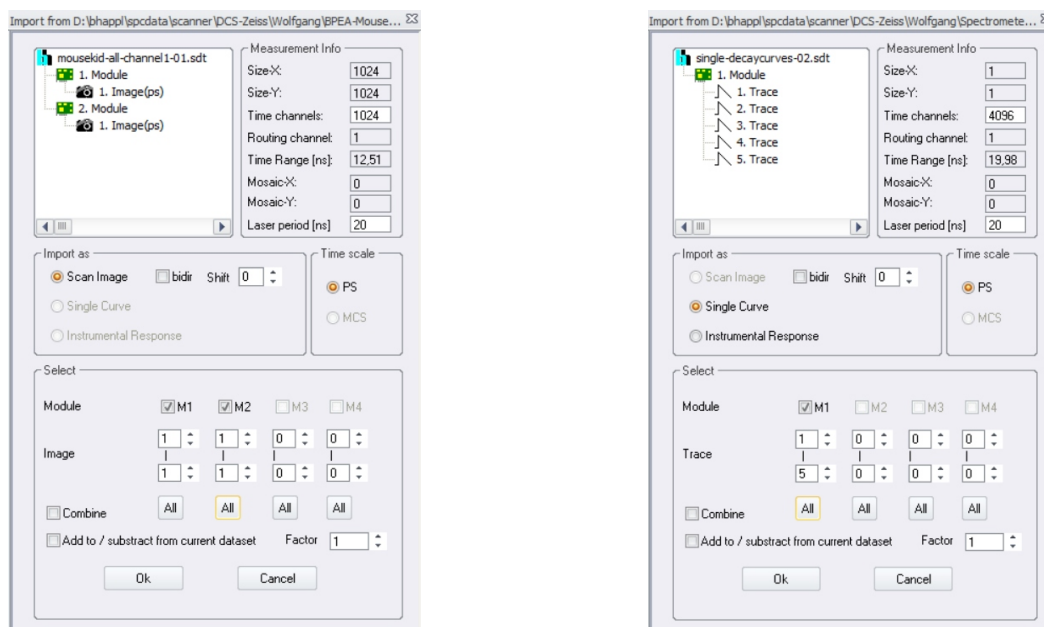


Fig. 471: Import Options panel for image files (left) and single-curve files (right)

SPCM Panel after Import of Data

After importing .sdt data into SPCImage the main panel comes up as shown in Fig. 472 or Fig. 473. Fig. 472 shows the main panel when image data were imported. An intensity image is shown left, a decay curve at the cursor position bottom right. The image shows only a grey-scale image because these have not been calculated yet. A decay-parameter histogram is shown upper right. It is empty because no decay analysis has been performed yet. In the upper right, the decay model can be specified. After loading new data the model function is single exponential (Components=1), which is the default.

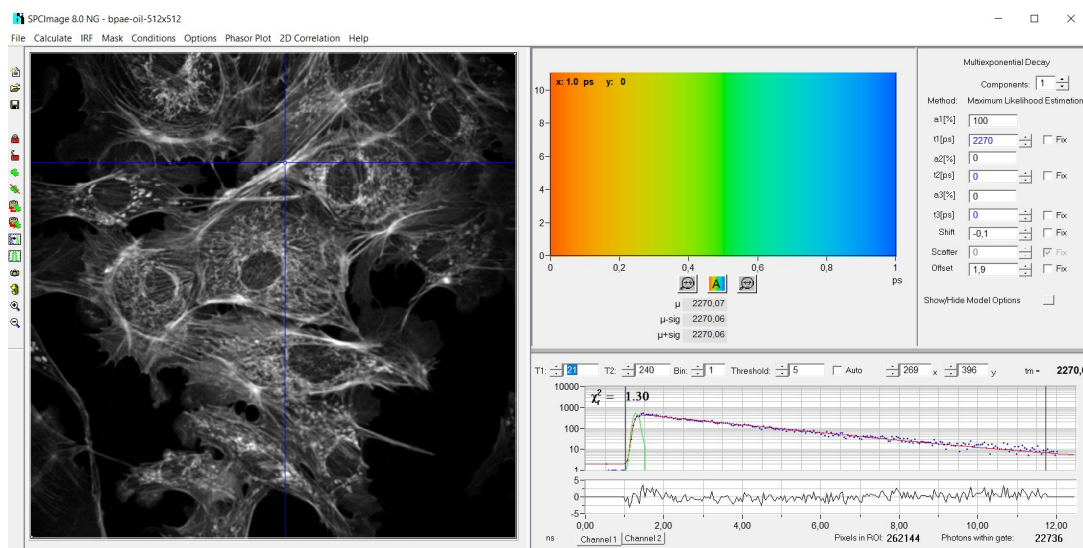


Fig. 472: SPCImage main panel after importing image sdt data

When single-curve data were imported the main panel comes up as shown in Fig. 473. The decay-curve window shows a decay curve, the decay parameters are shown upper right. The decay model is single-exponential. If several decay curves were imported these are accessible through the tabs below the decay-curve window. The image window (top left) and the histogram window (top middle) remain empty because the imported file did not contain suitable data for these windows.

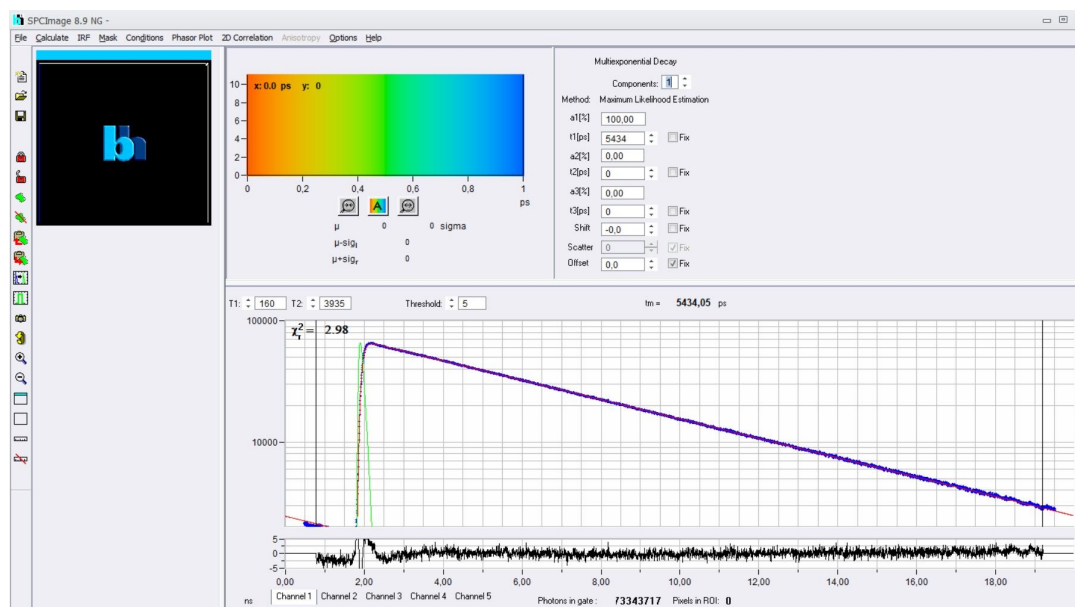


Fig. 473: SPCImage main panel after importing single-curve sdt data

Initial Check of Decay Data

After importing or loading data you should give the decay data a brief check for integrity. The decay curve should be correctly placed in the decay window (the observation-time interval), as shown in Fig. 472 and Fig. 473. The rising edge must be in the decay window, and there should be a few (5 to 10%) of the time channels left of the rising edge. Some unfavourable situations are shown in Fig. 474 and Fig. 475. These situations are not necessarily fatal but suggest corrections in the measurement procedure, the measurement setup or the measurement parameters.

In Fig. 474, left, the decay curve does not contain enough photons. The signal-to-noise ratio of lifetimes obtained from such data is low, and usually not sufficient for the intended application. Increase binning, or record new data with more photons. The decay curve shown in the Fig. 474, middle, contains large background. SPCImage will extract correct decay parameters from such data, but the signal-to-noise ratio will be sub-optimal [91]. Therefore, find and remove the source of the background. The curve shown in Fig. 474, right, is not placed well in the observation-time interval. Also here, data analysis will deliver correct data, but with a signal-to-noise ratio below the theoretical limit [91]. Therefore the curve should be shifted in the correct position by changing the TCSPC parameters or the SYNC delay [77].

The situations shown in Fig. 475 are more serious or even fatal. In Fig. 475, left, the decay curve is clipped at the left end, and the rising edge is not in the observation time interval. SPCImage may deliver a reasonable single-exponential lifetime in this case, but multi-exponential decay parameters derived from such data will be entirely wrong. There is no way around taking another measurement with corrected TCSPC system parameters. In Fig. 475, right, the decay curve does not contain any reasonable decay data at all. The source of the problem is usually that wrong filters had been used, and the FLIM system is detecting scattered laser light.

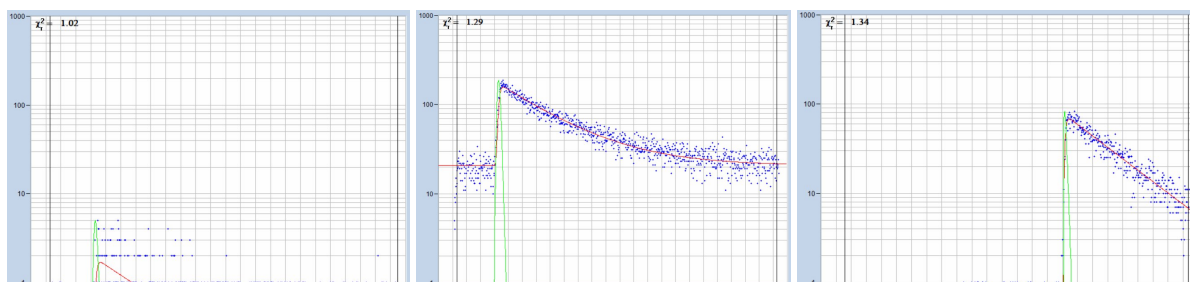


Fig. 474: Situations where correction is indicated. Left: Not enough photons. Middle: High background. Right: Decay curve shifted in observation-time interval.

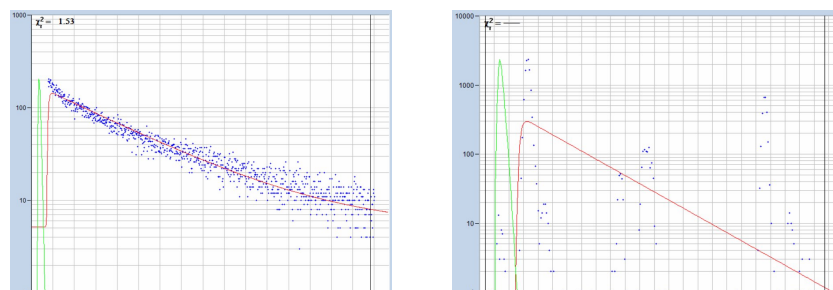


Fig. 475: Situations which are fatal to FLIM analysis. Left: Decay data clipped at left and, rise of fluorescence not recorded. Right: The recorded signal is scattered laser light.

Calculating the Lifetime Image

Starting the Calculation

In principle, you can start a lifetime analysis immediately from the state shown in Fig. 472. (There is even an option to do this automatically, see Preferences, page 345.) To start the analysis, click into 'Calculate', 'Decay Matrix', 'Selected channel' or, if you have loaded data with several channels 'all channels'. This starts the fit process. A progress bar shows the advance of the calculation as the procedure runs through the pixels. If there is a GPU in the computer the calculation will complete within a few seconds, if there is no GPU it can take several minutes. What you get is a colour-coded lifetime image calculated with default parameters of SPCImage. The decay model will be single-exponential, the IRF will be 'Auto', and the lifetime and intensity ranges will be set automatically.

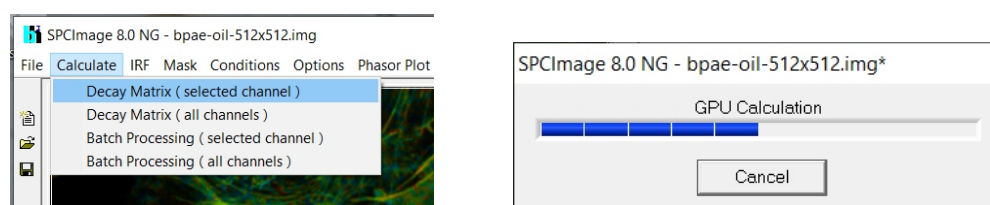


Fig. 476: Starting the fit procedure for all pixels of the image

Single-Exponential Analysis

The number of decay components used in the analysis is defined in the upper right of the SPCImage panel. FLIM analysis with a single-exponential model (or with default parameters) is shown in Fig. 477. Number of 'Components' is 1. The procedure delivers a single lifetime, τ_1 . This lifetime is used for colour-coding the image. The lifetimes in the pixels will be correct and reasonably accurate. However, the fluorescence decay in biological objects is rarely single-exponential. Normally there are several decay components in each pixel, either from different fluorophores, or from one fluorophore in different molecular environment. Often the information is in the composition of the decay rather than in an average ('apparent') lifetime.

Single-exponential analysis is therefore unlikely to deliver the maximum of information you can obtain from your raw data.

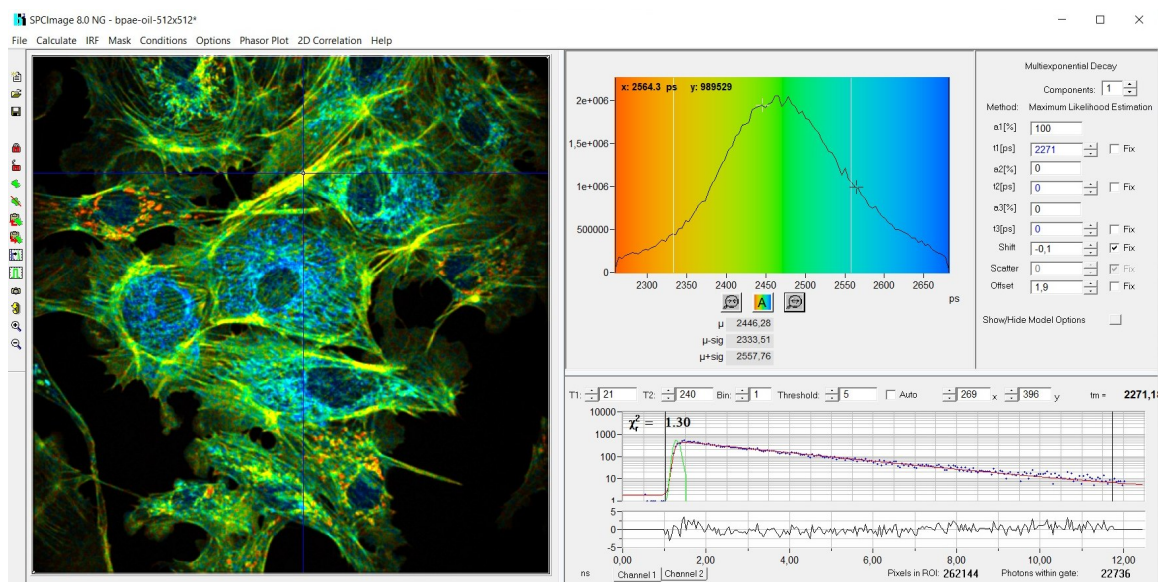


Fig. 477: SPCImage panel after calculating the lifetime image with the default settings

Multi-Exponential Analysis

Multi-exponential decay analysis is shown in Fig. 478. In the upper right, analysis with three exponential components has been selected. For every pixel, the analysis procedure delivers three lifetimes, t_1 , t_2 , t_3 , and three amplitudes, a_1 , a_2 , a_3 , for the three decay components. The display routine of SPCImage can display each of these parameters, ratios of the parameters, and intensity- or amplitude-weighted averages of the component lifetimes. The default is the amplitude-weighted mean lifetime, t_m . The display routine has functions to further refine the images, for example by manually adjusting the colour scale and the intensity scale, or by creating time-gated images. Please see page 'Display of Colour-Coded Images'.

Also the model function can be further refined, such as by the 'Incomplete Decay' or the 'Shifted Component' option. It is also possible to fix one or two of the decay components to values which are a priori known. For details please see 'Model Functions', page 309 and 'Model Parameters', page 342.

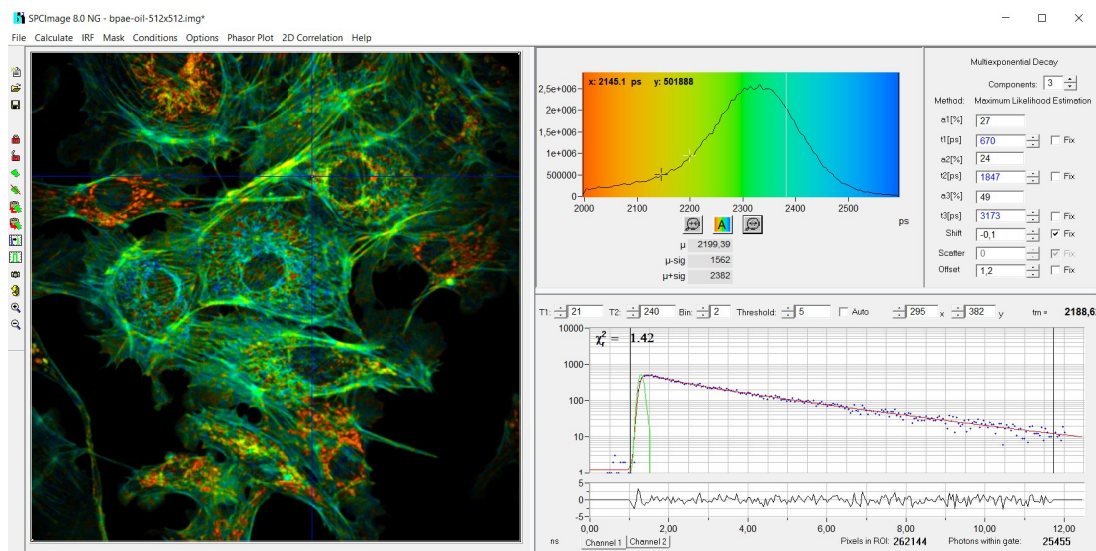


Fig. 478: Triple-exponential decay analysis. Model selection and decay parameters at cursor position shown in the upper right. Analysis with three exponential components has been selected, the amplitude-weighted lifetime, t_m , is shown.

Parameter Histogram

A histogram of the selected decay parameter over the pixels of the image is shown above the decay-curve window. It shows how frequently a given value of the parameter occurs in the image. The parameter is the same that was selected for colour-coding the image. It can be the amplitude-weighted lifetime (as in Fig. 478), the intensity-weighted lifetime, a component lifetime, a component amplitude, or a ratio of two of these parameters. The parameter histogram can be displayed for selected regions of interest, please see 'Decay-Parameter Histograms in ROIs', page 335. The parameter histogram has two cursors, which interact with the display function (see 'Interaction with the Colour Parameters', page 329). In combination with the , , and buttons a desired parameter range can be selected, and the colour scale adjusted accordingly. Please see 'Parameter Histogram', page 329.

Model Functions

Basic Decay Models

The fluorescence decay function obtained from a homogeneous population of molecules in the same environment is a single exponential. Decay functions of mixtures of different molecules or of molecules in inhomogeneous environment are sums of exponential functions of different decay time. The basic model functions used in SPCImage are therefore sums of exponential terms:

Single-exponential model: $f(t) = e^{-t/\tau}$

Double-exponential model: $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$

Triple-exponential model: $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$

The models are characterised by the lifetimes of the exponential components, τ , and the amplitudes of the exponential components, a . In principle, models with any number of exponential components could be defined. However, higher-order models become so similar in curve shape that the amplitudes and lifetimes cannot be obtained at any reasonable certainty. Therefore, SPCImage NG FLIM analysis does not provide model functions with more than three components.

Single, double, and triple-exponential models are selected via the decay-parameter panel on the right of the decay-curve window, see Fig. 479.

Multexponential Decay (1 Component)

Method: Maximum Likelihood Estimation

a1[%]: 100.00

t1[ps]: 1654 ☐ Fix ☐ Float

a2[%]: 0.00

t2[ps]: 0 ☐ Fix ☐ Float

a3[%]: 0.00

t3[ps]: 0 ☐ Fix ☐ Float

Shift: -0.04 ☒ Fix ☐ Float

Scatter: 0 ☒ Fix ☐ Float

Offset: 2.74 ☐ Fix ☐ Float

Multexponential Decay (2 Components)

Method: Maximum Likelihood Estimation

a1[%]: 49.39

t1[ps]: 477 ☐ Fix ☐ Float

a2[%]: 50.61

t2[ps]: 2274 ☐ Fix ☐ Float

a3[%]: 0.00

t3[ps]: 0 ☐ Fix ☐ Float

Shift: -0.04 ☒ Fix ☐ Float

Scatter: 0 ☒ Fix ☐ Float

Offset: 0.84 ☐ Fix ☐ Float

Multexponential Decay (3 Components)

Method: Maximum Likelihood Estimation

a1[%]: 58.47

t1[ps]: 697 ☐ Fix ☐ Float

a2[%]: 32.25

t2[ps]: 2200 ☐ Fix ☐ Float

a3[%]: 9.27

t3[ps]: 4349 ☐ Fix ☐ Float

Shift: -0.04 ☒ Fix ☐ Float

Scatter: 0 ☒ Fix ☐ Float

Offset: 0.01 ☐ Fix ☐ Float

Fig. 479: Selection of basic single, double, and triple exponential models

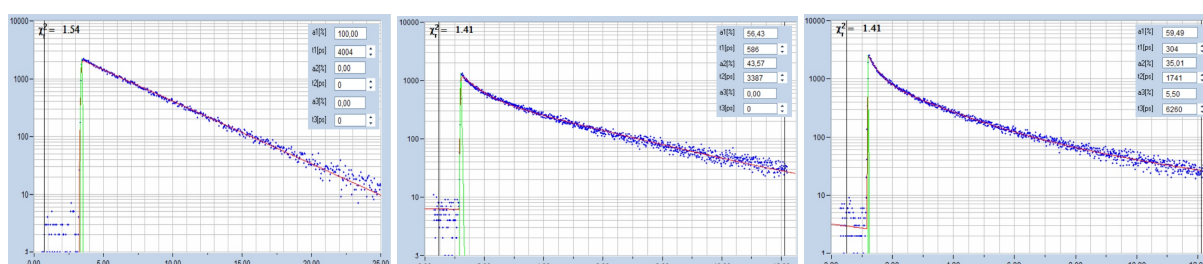


Fig. 480: Left to right: Fit with Single-, double-, and triple-exponential models. The blue dots are the data in the time channels, the red curve is the model function. The decay parameters are shown upper right.

A shift parameter can be included in the fit procedure. It shifts the IRF in a temporal position that yields the best fit of the decay functions. However, a floating shift decreases the lifetime accuracy and increases the calculation time. We recommend to determine the 'Shift' parameter before starting the analysis and then fix it, or let the analysis procedure fix it automatically when it starts the calculation. Please see Model Parameters, Fig. 537.

Typical examples of fits with different models are shown in Fig. 481. The single exponential model (left) does not fit the data. This can be seen from the differences of the model function and the data and from the systematic variation in the residuals. The double-exponential model (middle) fits well, the triple-exponential reveals a weak third component of long lifetime. However, the residuals (the curve below the decay function see 'Fit Quality Indicators', page 319) do not improve substantially for the triple-exponential model. The decay parameters derived by the triple-exponential fit may therefore not exactly represent the real composition of the decay curve.

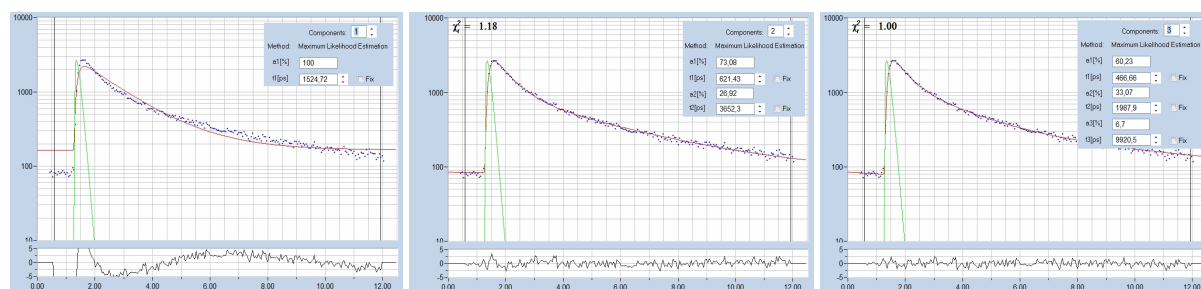


Fig. 481: Fit of decay data with a single, double, and triple-exponential model.

Incomplete-Decay Model

The basic decay models can be combined with model options provided by the 'Model' parameters, see page 342. The 'Incomplete Multiexponentials' option is used to account for re-

sidual fluorescence from previous laser pulses. The corresponding section of the Model Parameters is shown in Fig. 482. The incomplete decay model needs the period of the excitation pulses, which must be specified in the upper right.



Fig. 482: Incomplete-decay option in the model parameters.

Fig. 483 gives a comparison of the ordinary multi-exponential model (left) and the incomplete-decay model (right). The ordinary model interprets the intensity left of the rising edge of the decay curve as offset, the incomplete-decay model fits it correctly with fluorescence from the previous pulses.

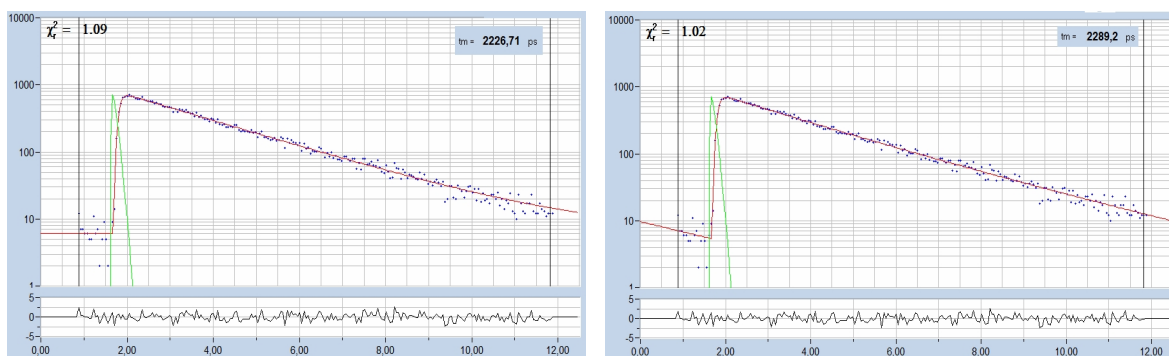


Fig. 483: Fit of the fluorescence decay of a Calcium sensor, lifetime 2.29 ns, excitation with Ti:Sa laser at 80 MHz. Left: ordinary double-exponential model. Right: Incomplete decay model.

The result is a different lifetime, t_m . The difference is not large as long as the lifetime is short compared to the laser pulse period. The difference can, however, be substantial if the decay time is longer. Fig. 484 shows an example for a 5.95-ns decay measured with the 12.5 ns repetition time of a Ti:Sa laser. A fit with the conventional model is shown left. The fit delivers a lifetime of 4.05 ns. The large residuals indicate that the model is not able to fit the data correctly. The fit with the incomplete-decay model is shown on the right. The model delivers a perfect fit, and a lifetime of 5.95 ns. The example shows that the use of the incomplete-decay option is mandatory for lifetimes larger than 25% of the laser repetition time.

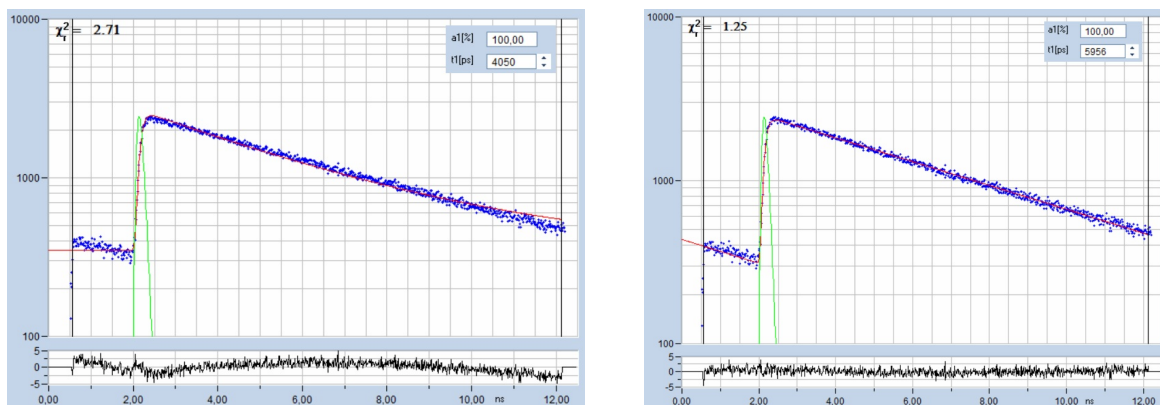


Fig. 484: A 5.95-ns decay recorded in a two-photon microscope. Laser repetition rate 80 MHz. Left: Fit with conventional model. It does not fit the data correctly. Right: Fit with incomplete-decay model. The incomplete-decay model not only fits the data perfectly but also delivers the correct decay time.

Shifted-Component Model

In clinical FLIM it happens that one or several decay components are shifted in time. A typical example is ophthalmic FLIM (FLIO) where fluorescence from the lens of the eye interferes with fluorescence of the fundus. The lens fluorescence appears about 150 ps before the fundus fluorescence. The shifted-component model takes this shift into account [85, 86].

A demonstration is given in Fig. 485 and Fig. 486. A FLIO decay curve together with the model definition is shown in Fig. 485. A triple-exponential model is used; the lens component is modelled by the third decay component and shifted 150 ps towards earlier times. As a result, the model fits the lens component correctly, including the kink in the rising edge caused by the early arrival of the lens fluorescence.

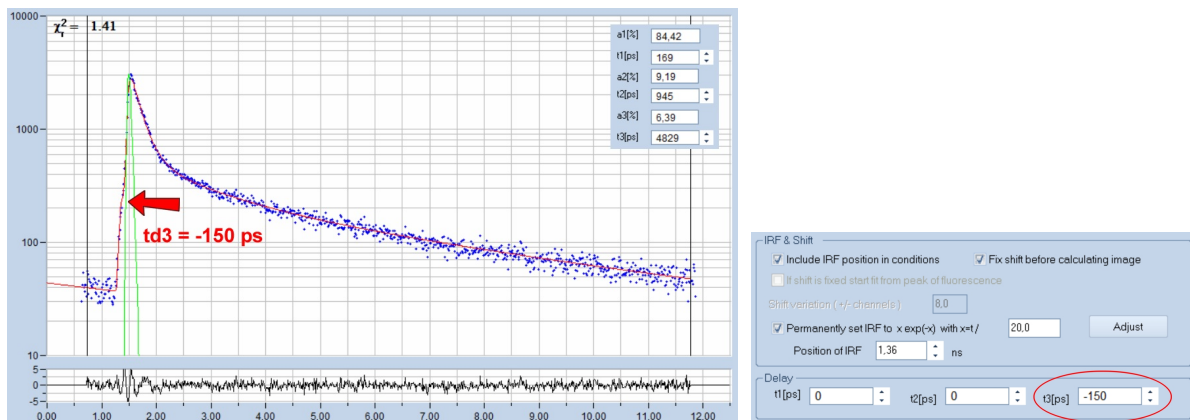


Fig. 485: Decay curve from FLIO data. Fit with shifted-component model, third decay component shifted by 150 ps to earlier time.

FLIO lifetime images obtained by the ordinary multi-exponential model and by the shifted-component model are compared in Fig. 486. For the ordinary model, the lens fluorescence causes a substantial shift of the mean lifetime, t_m , to longer values. The shifted-component model is able to deliver an image which contains only the fundus fluorescence, modelled by the components t_1 and t_2 . The corresponding image of the lifetime t_{m12} is shown in Fig. 486, right. It shows the correct lifetime of the fundus of the eye [85, 86].

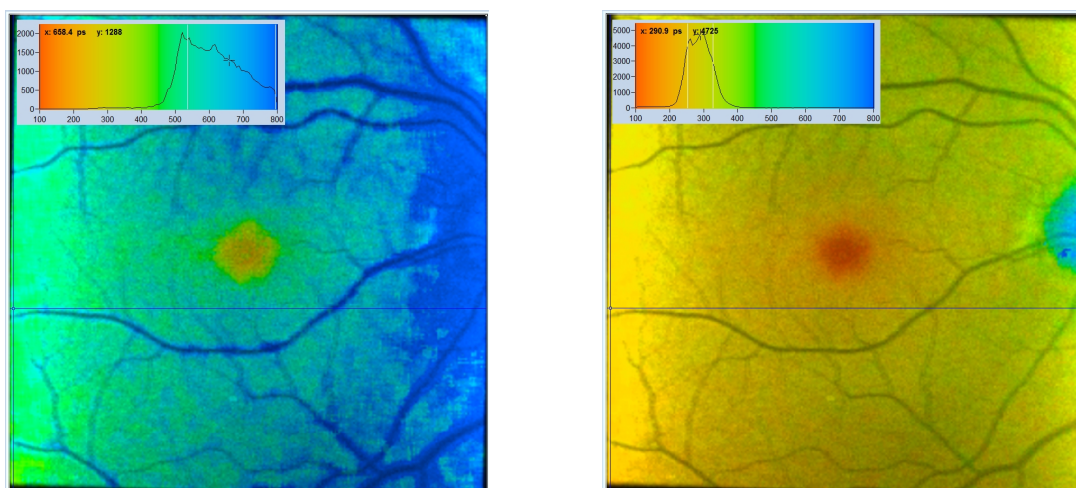


Fig. 486: Comparison of FLIO analysis with the ordinary 3-component model (left) and with the shifted-component model (right). Due to the contribution of the lens fluorescence, the ordinary image is biased towards long lifetime. The delayed-component model delivers a t_{12} image that does not contain the lens fluorescence, showing the correct lifetime of the fundus of the eye.

Analysis with fixed or global parameters

The lifetimes of the individual decay components can be fixed to known values or defined as 'Global'. The 'Global' option fits the decay data under the assumption that the component lifetimes are unknown, but otherwise the same in all pixels of the image. Analysis with fixed or global component lifetimes can increase the statistical accuracy of the fit results considerably. It requires, however, that the lifetimes are accurately known or constant over the entire image. For details, please see 'Global Analysis', page 351 The definition of the parameter status is shown in Fig. 487.

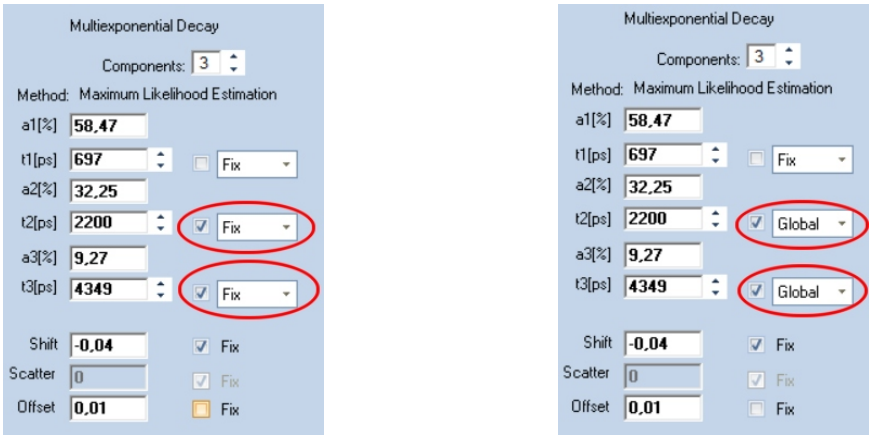


Fig. 487: Definition of lifetime components as 'Fixed' (left) and 'Global' (right)

IRF Options

The fluorescence waveform the FLIM system records is the convolution of the true fluorescence decay profile with the instrument response function, or 'IRF'. The IRF is the function the FLIM system would record when it detected the laser pulse directly. Decay parameters are derived from the recorded waveforms by convoluting a model function with the IRF, and fitting the result to the data, see 'The Convolution Integral', page 366. Thus, at least an approximate IRF is needed to derive fluorescence decay parameters from the detected fluorescence waveforms. It is often believed that the IRF has to be measured before data can be analysed. However, in a FLIM system this can be difficult or even impossible. SPCImage therefore offers several options to *generate* an IRF from the FLIM data themselves.

Selection of IRF Type

The different IRF types are defined by clicking into 'IRF' in the top bar, and selecting the desired option, see Fig. 488. The effect of the different options is shown in Fig. 489.

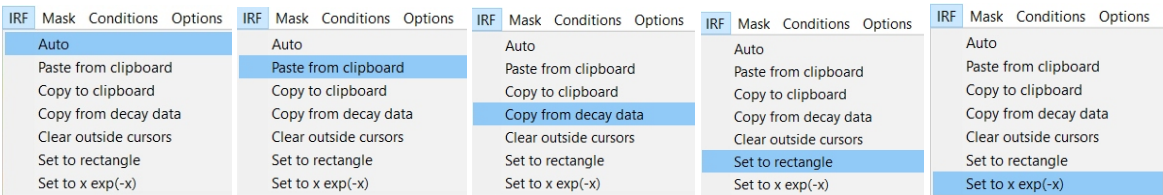


Fig. 488: Options to generate the IRF

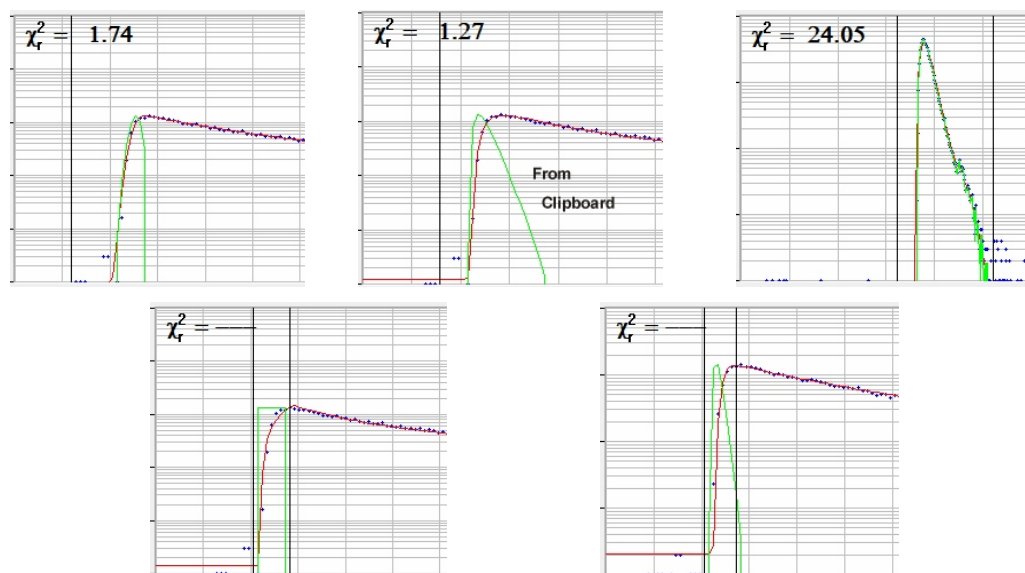


Fig. 489: Effect of the IRF options. Upper row, left to right: Auto IRF, IRF from Clipboard, IRF copied from recorded data. Lower row left: Rectangular IRF, Right: IRF of type $\text{irf}(t) = t/t_0 e^{-t/t_0}$.

'Auto' calculates an IRF from the rising edge of the decay data. The calculation is performed on data from an area around the brightest spot in the image. When 'Auto' has been set the IRF calculation is done automatically after loading data.

'Paste from clipboard' recalls an IRF which has been copied by 'Copy to Clipboard' before.

'Copy from decay data' uses decay data from a selected spot in an image as an IRF. The selected spot can either be a spot in the current image where the decay function is dominated by SHG, or an image that contains only SHG or fast scattering data. The IRF is generated from the data inside the decay cursor interval. Data points outside this interval are set to zero. To transfer the data into a different measurement data set, use 'Copy to clipboard' and, after loading the measurement data file, 'Paste from clipboard'. You can save the IRF by 'Save IRF' in the model parameter panel.

'Set to rectangle' sets a rectangular IRF. Width and location are determined by the cursors in the decay window. The function is often used for PLIM, where the IRF is close to a rectangle.

'Set to x exp (-x)' sets an IRF to a function of the type $\text{irf}(t) = t/t_0 e^{-t/t_0}$. The width and the location are selected by the cursors in the decay window, see Fig. 489, right. You can declare this IRF a permanent one in the 'Model' parameters, and further refine it by an automatic optimisation procedure, see page 377.

Modelling of the Synthetic IRF

Defining a synthetic IRF only by the cursors of the decay window is not quantitative, and leaves room for subjective judgement. SPCImage NG therefore has a function to model the synthetic IRF by fitting it to the recorded decay data. The procedure is described in detail in section 'Instrument Response Function' page 377. Briefly, the width parameter, t_0 , of the generic IRF function, $\text{irf}(t) = t/t_0 e^{-t/t_0}$ is optimised until the best fit of the convolution integral of the IRF with the selected model to the recorded fluorescence data is obtained. The adjust procedure for the width parameters is accessed under 'Model', section 'IRF & Shift'. A click on the upper 'Adjust' button, Fig. 490, left, starts the procedure.

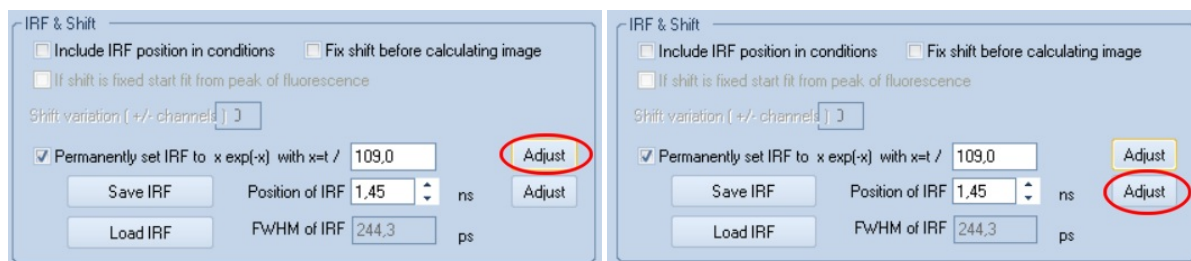


Fig. 490: IRF Adjust for width (left) and position of IRF (right)

The result of the operation is an IRF with a correct shape, but not necessarily in the correct temporal position. To get the IRF in the correct position, click on the Adjust button for 'Position of IRF', see Fig. 490, right. The effect of the two adjust operations is demonstrated in Fig. 491.

Both adjust operations should be performed with a reasonably selected decay model. The reason is obvious: If the model is not able to fit the decay data correctly the IRF adjust procedure compensates for the deficiencies of the model with a change in the IRF. The result may be a reasonably good fit of the decay data but with wrong decay parameters.

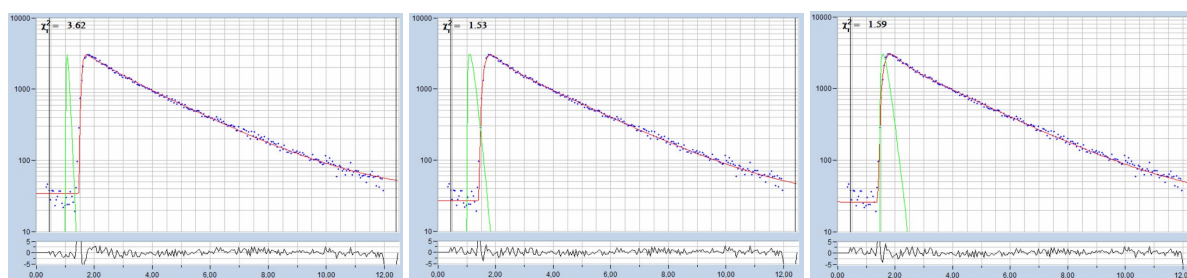


Fig. 491: Left to right: IRF (green curve) before adjust, after width adjust, and after position adjust.

Permanent IRF

A synthetic IRF, once created, can (and should) be declared 'permanent', see Fig. 492. The IRF parameters are then taken over in the default parameters of SPCImage, and can be used for other data recorded with the same FLIM system.

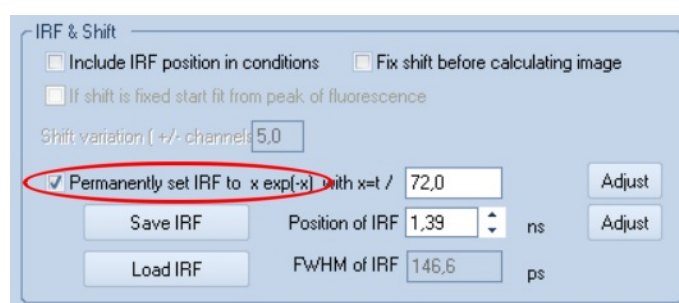


Fig. 492: Declaring a synthetic IRF 'Permanent'

Saving and Loading IRFs

A synthetic IRF can be saved for future use in a file, and loaded from this file when needed. The function is initiated via the 'Save IRF' and 'Load IRF' buttons (see Fig. 492). This opens a file selection panel in which the name of a new IRF can be defined or from which an existing IRF can be selected. Please see Fig. 493.

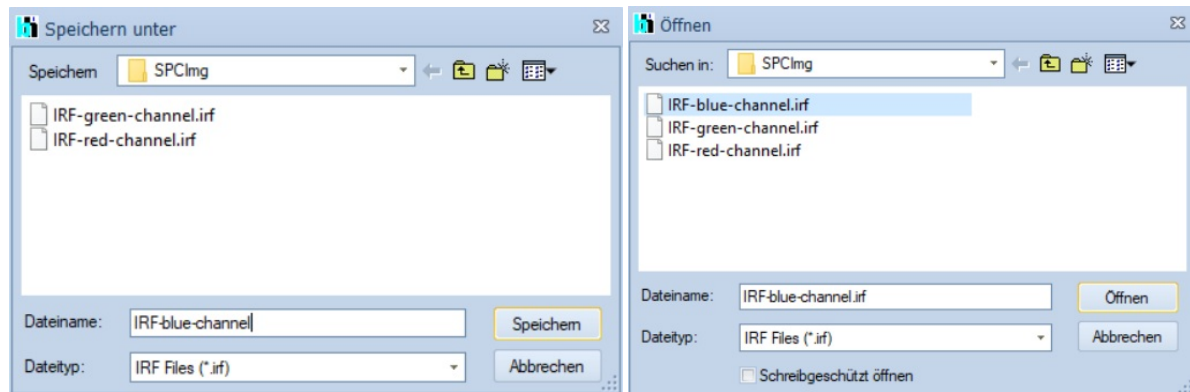


Fig. 493: File selection panel for saving (left) and loading IRFs (right)

Fit Algorithm

SPCImage NG has three different fit algorithms. 'Weighted Least Squares' (WLS) is the conventional algorithm, based on a minimisation of the sum of the squared differences between the data points and the points of the model function. 'Weighted' means that the differences are weighted with the reciprocal photon number, $1/(n+1)$. WLS works well for high photon numbers but has deficiencies when the photon number is low.

'Moment' (MOM) is a calculation based on the first moment of the decay curve. MOM is a simple calculation, not a fit algorithm. It is fast and delivers the maximum possible lifetime accuracy. However, it delivers only a single-exponential approximation of the lifetime, and the lifetimes become systematically biased when the tail of the decay function is not entirely in the observation time interval or the data contain background counts [91].

'Maximum-Likelihood Estimation' (MLE) is based on calculating the probability that the values of the model function correctly represent the data points of the decay function. Compared to the least-square method, the fit accuracy is improved especially for low photon numbers, and there is no bias toward shorter lifetime as it is unavoidable for the WLS fit. MLE uses GPU processing, which makes the calculation by a factor of 10 to 100 faster than by WLS. For a detailed description of the algorithms please see 'Fit Procedures', page 371 in section 'Supporting Information'.

The algorithm is selected in 'Algorithmic Setting', in the 'Model' parameters, see Fig. 494, The default algorithm is MLE.

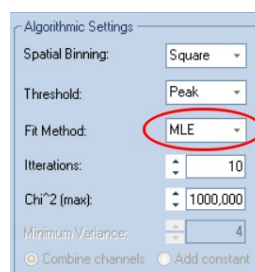


Fig. 494: Selection of the fit algorithm in the Model Parameters

Fit Control Parameters

Fit Interval

The fit of the decay data is performed in the interval between the cursors in the decay window. Normally, the fit should be performed over the entire interval where the decay function has valid data, i.e. the cursors should be placed on the first and the last data point, see Fig.

495, left. After importing data SPCImage runs a check on the data and suggests reasonable cursor positions.

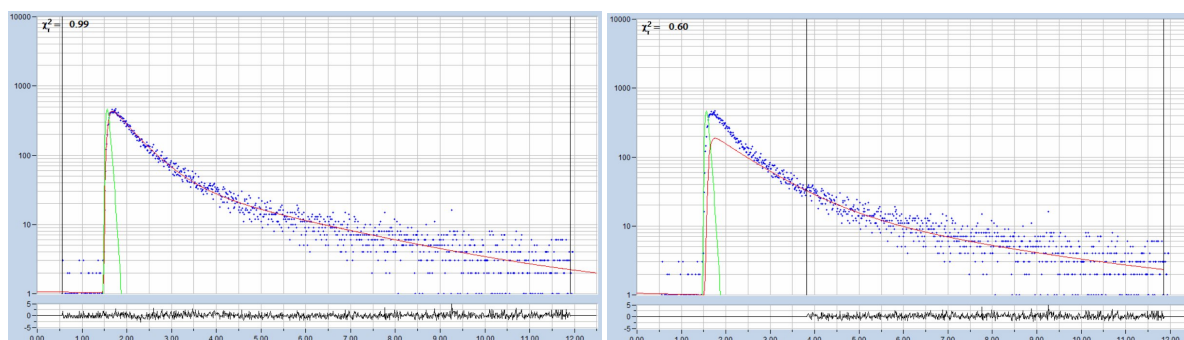


Fig. 495: Effect of cursors in the decay window. Left: Fit within entire range of valid temporal data. Right: Fit of late part of decay data only. The fast decay component is not correctly reproduced.

The fit can be restricted to a part of the data if necessary, as shown in Fig. 495, right. However, this should be done as an emergency solution only, e.g. if the decay data contain artefacts. In fact, restricting the fit to the later part of the fluorescence is common practice to solve the problem of a wrong IRF. A wrong IRF leads to large residuals in the rising edge of the decay curve. It is therefore sometimes attempted to exclude this part from the fit. However, excluding the first part of the curve does not make the result any better. On the contrary, the analysis routine is likely to miss fast decay components which may be contained in the data. An example is shown in see Fig. 495, right. The fit perfectly reproduces the later part of the decay curve but entirely misses the fast decay component. Another problem of the 'Tail Fit' is that the 'Shift' parameter cannot be reliably determined. The reason is that the shape of later part of the curve is virtually independent of the temporal location of the IRF. Therefore, a tail fit, if ever necessary, must be performed with fixed shift.

Binning

Correct spatial binning is key to good lifetime images [91]. Virtually all microscopy images are over-sampled to obtain maximum spatial resolution. Oversampling means that the point spread function is sampled by several pixels in x and y. Typical (linear) oversampling factors are around 5, that means the point-spread function is sampled by about 25 pixels. Of course, these pixels contain virtually identical lifetime information. Analysing them individually would result in low photon number per pixel, and in unnecessarily high noise in the decay parameters.

The solution to the problem is binning. In SPCImage binning is performed by combining the decay data from a specified binning area and assigning the net decay curve to the central pixel. The process is executed for all pixels of the original image. That means the binning areas overlap, and there is no reduction in the number of effective pixels. For details please see 'Spatial Binning' in section 'Supporting Information'.

Binning is controlled by the 'Bin' parameter on top of the decay-curve window. The parameter should be matched approximately to the radius of the point-spread function, expressed in pixels. An example is shown in Fig. 496. Two images were recorded with the same count rate and acquisition time. The left image was recorded with 128 x 128 pixels and analysed with bin=0. The right image was recorded with 512 x 512 pixels, and analysed with bin = 3. Due to the binning, the right image has the same number of photons per pixel and the same lifetime accuracy. However, the definition in the image on the right is much better.

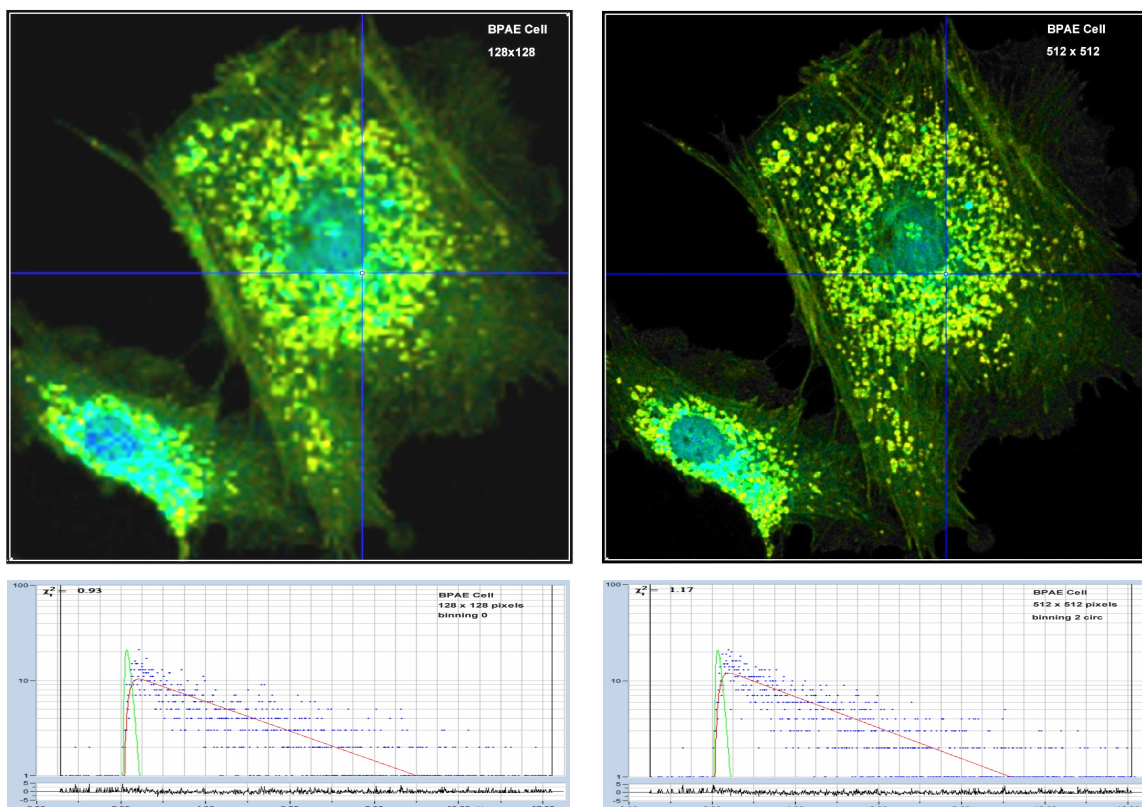


Fig. 496: Effect of spatial binning. Left: 128 x 128 pixels, bin=0, Right: 512 x 512 pixels, bin=3

The conclusion is that FLIM images should be recorded at sufficiently high pixel numbers to provide spatial resolution, and the resulting decrease in photon numbers per pixel be compensated by binning.

Threshold

The 'Threshold' parameter is used to suppress the analysis of dark pixels. It is located on top of the decay-curve windows, see Fig. 497. Pixels with photon numbers lower than 'Threshold' are not analysed by the fitting procedure, and do not get a colour assigned in the image. This not only accelerates the calculation process, it also avoids that the parameter histogram is distorted by invalid values from dark pixels. (see 'Parameter Histogram', page 335). Suppression of dark pixels is also essential for global analysis. The 'Threshold' value either refers to the maximum of the decay curve or to the total number of photons in it. A selection can be made in the 'Algorithmic Settings' part of the 'Model' panel, see Fig. 537, page 342.

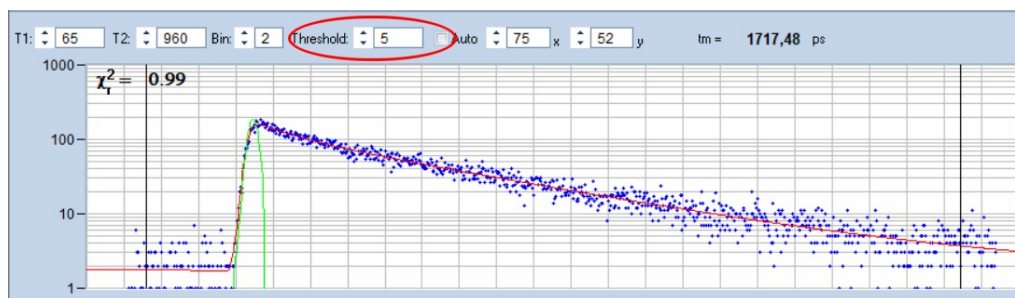


Fig. 497: Threshold parameter

Fit Quality Indicators

Residuals

The residuals are the differences between the values of the model function (convoluted with the IRF), $f_{mod}(t)$, and the data points of the recorded decay curve, $n(t)$ divided by the square root of $f_{mod}(t)$:

$$R(t) = \frac{f_{mod}(t) - n(t)}{\sqrt{f_{mod}(t)}}$$

The background of this expression is as follows. Provided the model function reproduces the shape of decay function properly the differences between $f_{mod}(t)$ and $n(t)$ can be considered the 'noise' in the data. The noise comes from the photon statistics. The average noise amplitude is statistically defined and is $\sqrt{n}$. If the model function is correct the denominator of $R(t)$ is equal to the expectation value of the photon number, $n(t)$. That means the residuals should not depend on the photon number in the respective time channel of the decay function, and vary randomly with a 'Sigma' of one. That means most of the $R(t)$ values are expected within the range from -1 to +1, and virtually all values should be within -5 to +5 (5 Sigma). Any deviation of the convoluted model function $f_{mod}(t)$ from the photon data, $n(t)$ causes systematic wobble in the residuals.

Fig. 498 shows an example of residuals for a near-ideal fit, a fit with a wrong IRF, and a fit with an inappropriate model function.

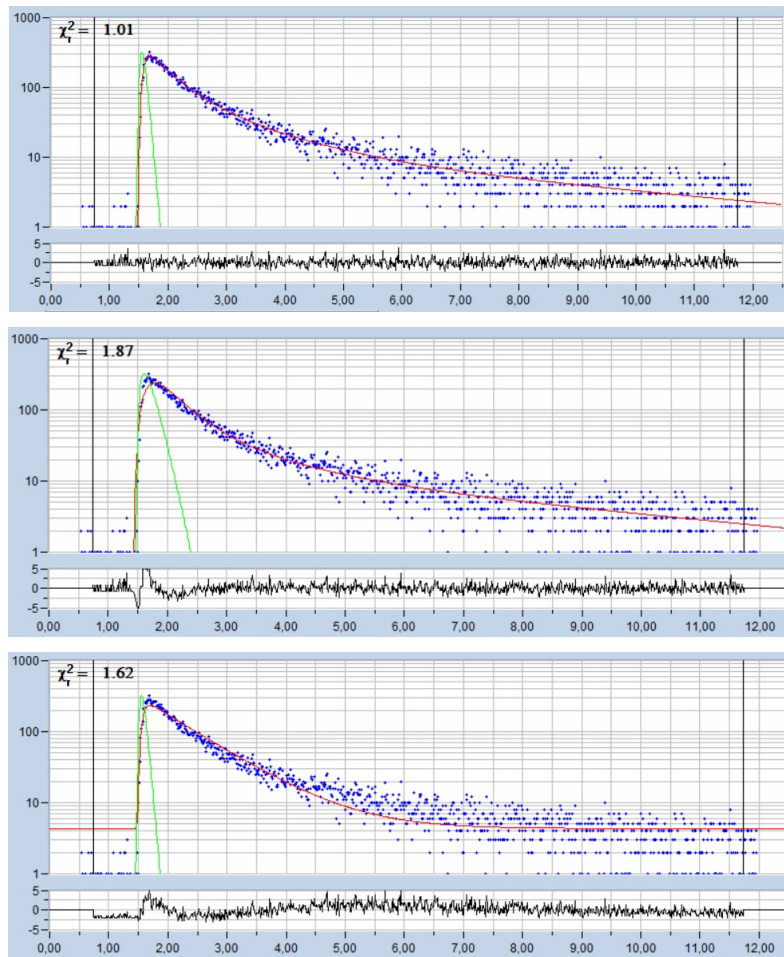


Fig. 498: Top to bottom: Residuals for a near-ideal fit, for a fit with a wrong IRF, and a fit with a wrong decay model.

There is a feature of the residuals which sometimes causes confusion. When the photon number gets very large the random variation remains in the range of -5 to +5. However, the systematic wobble increases. The reason is that the amplitude of systematic variations increases linearly with the photon number, n , whereas the random variations increase only with $\sqrt{n}$. The result is that residuals for decay data with a high number of photons can look ugly even though the quality of the data is good. Please remember this when you combine pixel data via the phasor plot or via the 'lock' function of SPCImage.

Reduced Chi-Square (χ^2)

The χ^2 parameter is another indicator of the fit quality. It is calculated by

$$\chi^2 = \sum \frac{(f_{\text{mod}}(t) - n(t))^2}{k(n(t) + 1)}$$

$f_{\text{mod}}(t)$ is the (convoluted) model function, $n(t)$ is the photon number in the time channel t , and k is the number of time channels. The background of the formula is similar as for the residuals. The numerator is the square of the difference between the model function and the photon number in the corresponding time channel. For large n , the denominator, $n(t) + 1$, is the square of the expected noise in the photon number. Consequently, an ideal fit should deliver a χ^2 of approximately one.

Unfortunately, the χ^2 has a two unpleasant features. The first one comes from the term $n + 1$ in the denominator. In fact, the correct term to describe the expected noise would be the photon number, n . However, $n + 1$ must be used to avoid a singularity for $n = 0$. The result is that χ^2 drops below one when the decay data contain time channels with low photon number or channels with $n = 0$. The other confusing feature is that χ^2 increases for large photon numbers. The reason is the same as for the residuals: The amplitude of systematic variations increases linearly with the photon number, n , whereas the random variations increase only with $\sqrt{n}$. The effect in SPCImage is that χ^2 increases with increased binning. This has led to the misconception that binning has a detrimental effect on the accuracy. This is, of course, not the case. χ^2 is simply not independent of n . It can be used to compare the fit quality for different models and different fit parameters for a given decay data set, but not to compare the quality of decay data which have different photon numbers.

Display of Decay Data

Decay Curve Window

The decay curve window of SPCImage displays the decay data (the photons in the subsequent TCSPC time channels) for a single pixel, the binning area around it, a region of interest, or an area defined by image segmentation. The decay-curve window with decay data is shown in Fig. 499. The blue dots are the photon numbers in the subsequent time channels, the green curve is the IRF (Instrument-Response Function, see page 377). The red curve is a fit with the model function, the numbers on the right show the fit results for the data in the decay window. The curve at the bottom are the residuals - the deviations of the fit from the real data.

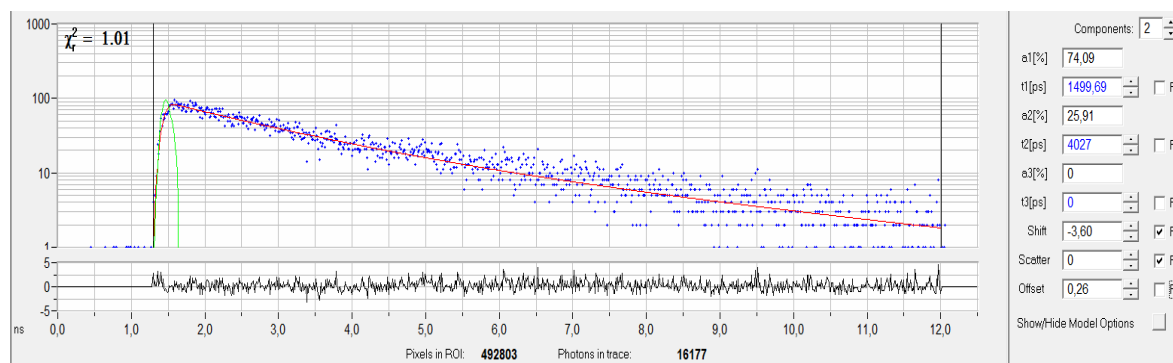


Fig. 499: Decay curve window of SPCImage

Decay Curve from an ROI

A decay curve can also be displayed for an entire ROI, both for a rectangular one or a polygonal one. To combine the decay data of an ROI into a single decay curve, click on the 'Lock' symbol on the left of the SPCImage main panel, see Fig. 500. A similar combination can be obtained via the phasor plot, see page 390. The result is a curve with a substantially larger number of photons than in a single pixel. Please don't get frightened by the residuals - they are normalised to the noise (see 'Fit Quality Indicators', page 319). The noise is small in the combined data, so that even minuscule systematic deviations stand out prominently.

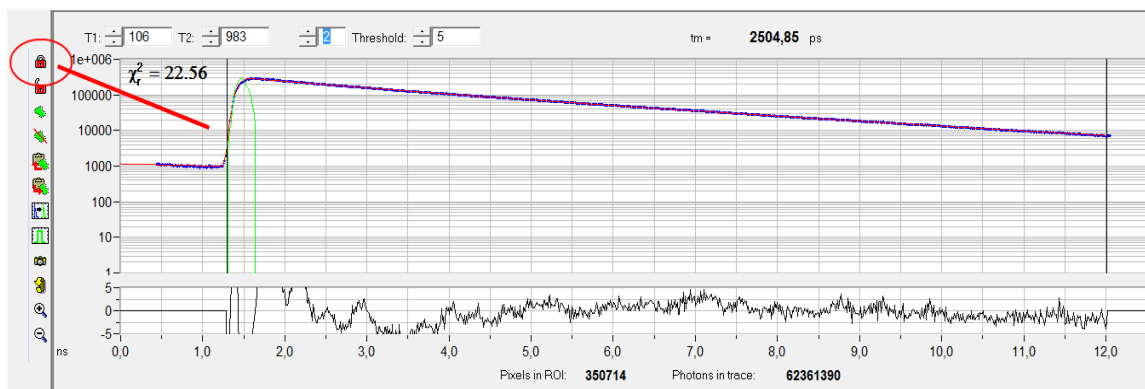


Fig. 500: Combination of the data of an ROI into a single decay curve

In combined decay data it can happen that the result of the combination exceeds the default Y scale of the decay window. In that case, you can change the scale by a right mouse click into the decay window, and select 'Scale'. This opens a panel in which you can change the x and y scale, as shown in Fig. 501.

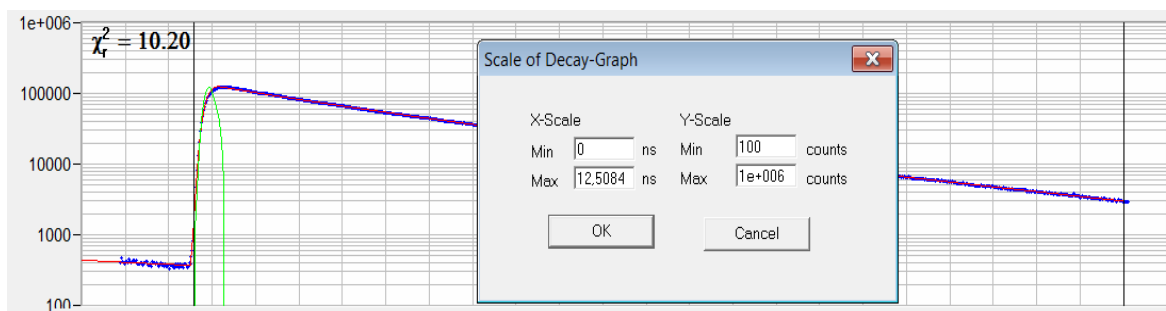


Fig. 501: Changing the scale of the decay window

Changing the Window Size

The size of the decay-curve window is variable. For FLIM analysis, the decay curve window is normally located under the parameter or intensity histogram and the fit parameter panel. To leave space for these items it is low and wide, as shown in the previous figures. It can, however, be resized to any size within the borders of the SPCImage panel. An example where the decay curve window fills the entire SPCImage panel is shown in Fig. 502.

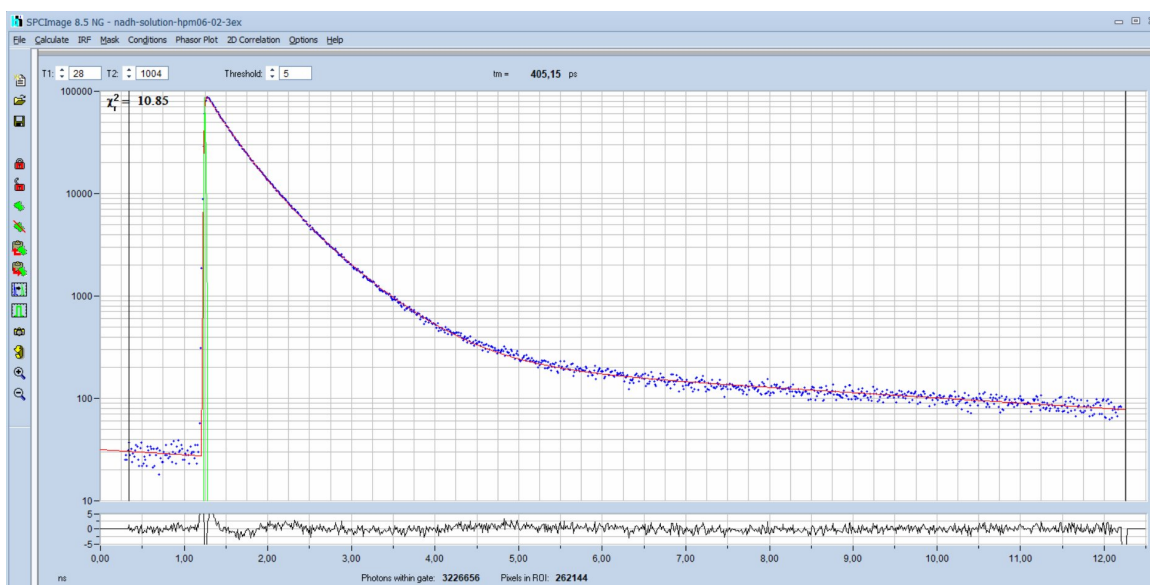


Fig. 502: Decay curve window, sized up to the entire area of the SPCImage software panel.

Display of Colour-Coded Images

General Configuration

The general configuration of the main panel is defined in the 'Preferences' panel, see page 345. For reasonably modern computers and computer screens we recommend to chose the 'Layout' and 'Lifetime Window' parameters as shown in Fig. 503. The display configuration then becomes as shown in Fig. 504. Of course, the colour-coded images does not show up until you have run 'Calculate Decay Matrix', see page 307. Before that, SPCImage displays an gray-scale intensity image, see Fig. 472, page 305.

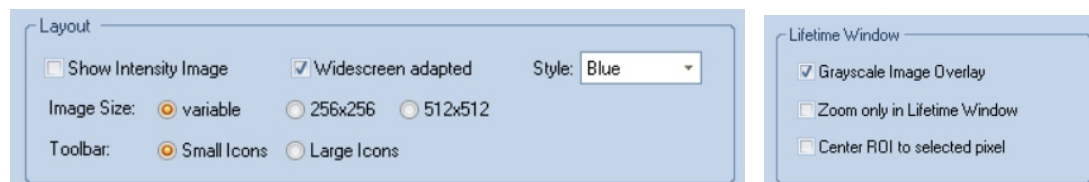


Fig. 503: Recommended 'Layout' and 'Lifetime Window' parameters in the 'Preferences' panel

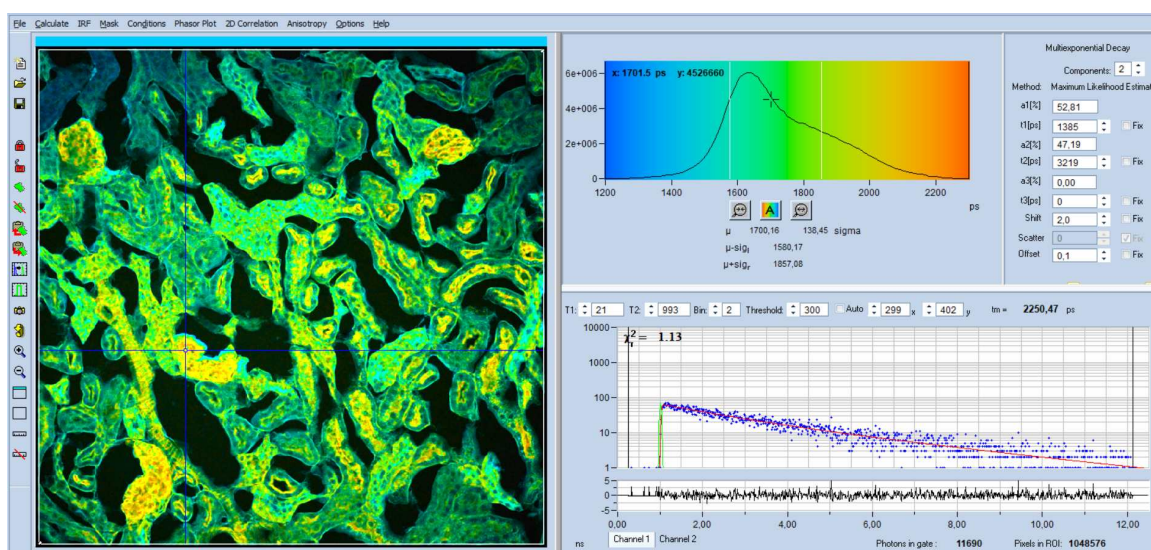


Fig. 504: Display configuration with the configuration parameters shown above

There are situations when a FLIM data set contains several images, e.g. from different wavelength channels. In that case, you can select the desired image by clicking on one of the tabs below the decay curve windows. Please see Fig. 505.

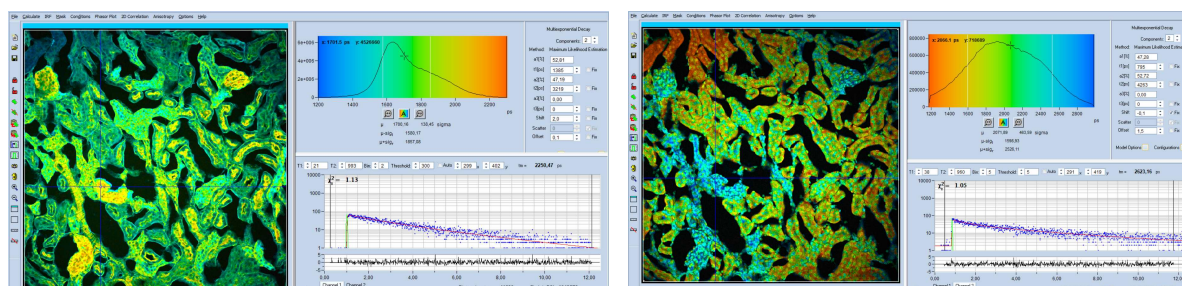


Fig. 505: Two FLIM images from the same FLIM data set but from different wavelength ranges. Selection by the 'Channel' tabs.

For direct comparison, and for cross-calculation of decay parameters two images can be displayed in one SPCImage main panel. Click into 'Options', 'Channels', and select 'side by side'. An example for simultaneous display of two channels is shown in Fig. 506.

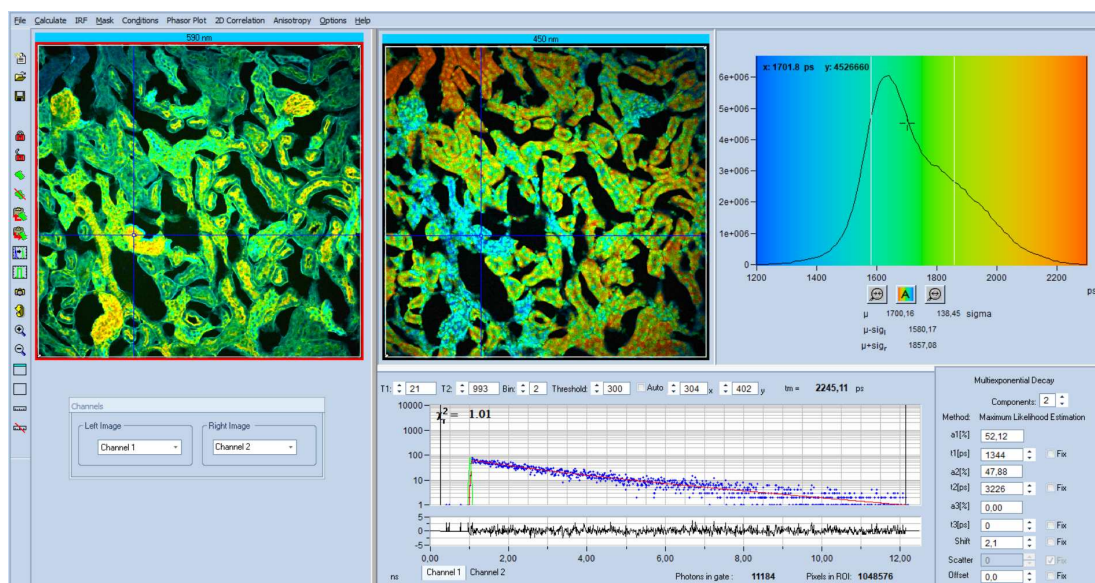


Fig. 506: Display of two images from two different recording channels

When two images are displayed the images can be given names. Click into the blue bar on top of the images, and type the desired text into the small panel that opens. The text is then displayed on top of the corresponding image, see Fig. 506.

Intensity Parameters

The display of the lifetime images themselves is controlled by the 'Intensity' parameters and the 'Colour' parameters. Both are accessible via 'Options' in the top bar of the main panel.

The intensity parameters are shown in Fig. 507. The parameter panel is shown on the right, the corresponding image on the left. There are separate sliders for contrast and brightness of intensity (grey scale) images and lifetime images. 'Intensity Overlay' defined which information is displayed as image intensity. Usually it is the photon number, but other parameters can be selected for special purposes. 'Scaling' is either 'autoscale' or a user-defined number of photons per pixel. 'Other' contains options to reverse images in x and y, to interpolate the intensity between the pixels of low-resolution images, and to time-gate images. Commonly used settings can be seen in Fig. 507.

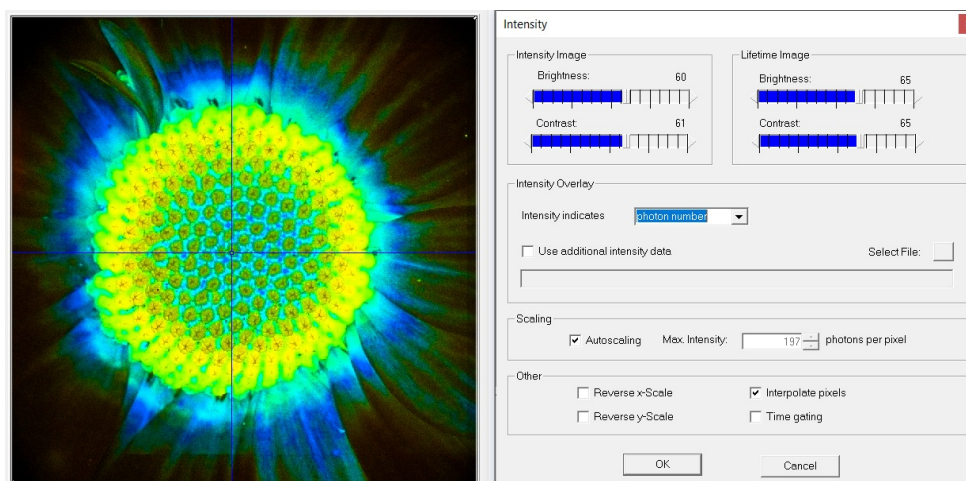


Fig. 507: Lifetime image with commonly used settings of the intensity parameters

Most of settings can be used more or less intuitively. Normally the images are displayed with autoscaling. The intensity scale then automatically adjusts to the photon number in the brightest pixel. The autoscaling may fail if there are overexposed spots in the image, such as specks of fluorescent dirt. In that case, turn off autoscaling and set a better intensity range manually or adjust the intensity range via the Intensity Histogram, see page 330.

An example for time-gating of the intensity is shown in Fig. 508. The time gate is defined by the cursors in the decay window, see Fig. 508, right. A late time window has been used for gating, therefore pixels with short lifetime appear dark.

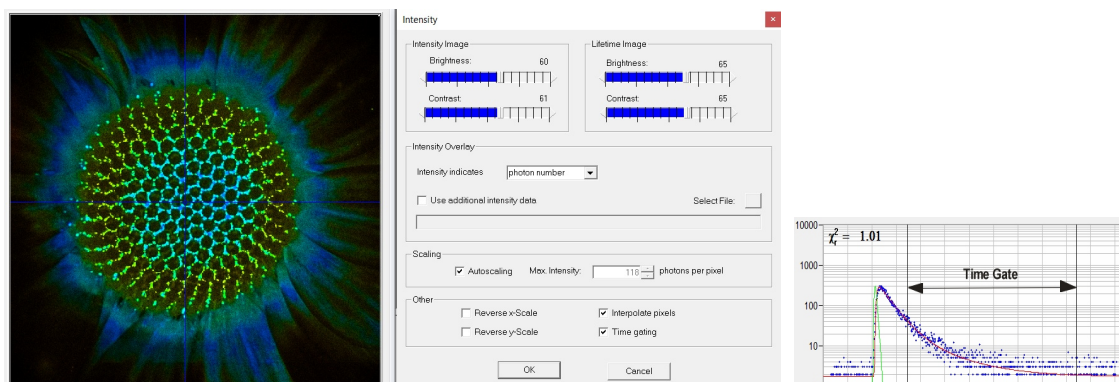


Fig. 508: Lifetime image with time-gated intensity. The time gate is defined in the decay curve window (right).

Colour Parameters

Colour Coding of Selected Decay Parameters

The 'Colour' parameters define the colour range into which the value of a selected decay parameter is converted. Three examples are shown in Fig. 509. The figure shows lifetime images of the mean lifetime, t_m , for different settings of the colour parameters. The left and middle image have a continuous colour scale from $t_m = 200$ to 1000 ps, but the colour scale goes in opposite directions. The image on the right has a discrete colour scale. The ranges for blue, green, and red are shown in the right part of the colour parameter panel.

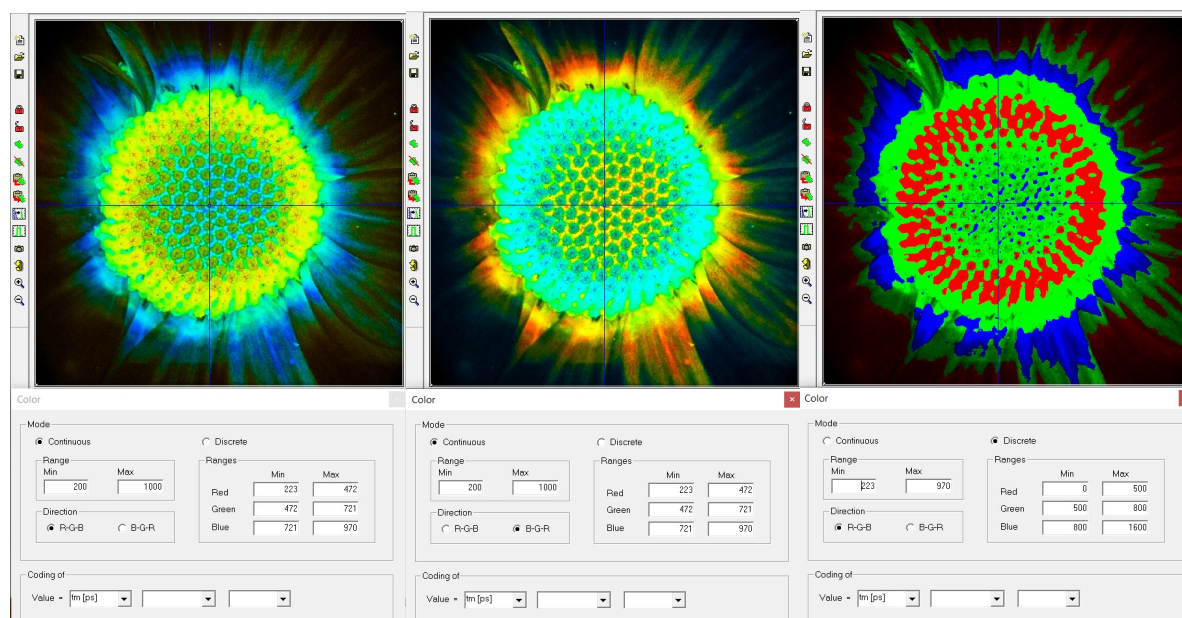


Fig. 509: Different colour scales. Left: continuous scale, red-green blue. Middle: continuous scale, blue-green-red. Right: Discrete colour scale.

Selection of Parameter for Colour-Coding

'Coding of' in the lower part of the colour panel selects the parameter to be colour-coded in the image. Available parameters are:

t_m	amplitude-weighted lifetime of all decay components enabled in general model parameters
t_{m12}	amplitude-weighted lifetime of the first two decay components of a triple-exponential decay. Used for FLIO analysis.
t_i	intensity-weighted lifetime of all decay components enabled in general model parameters
t_1, t_2, t_3	Lifetimes of the individual decay components
a_1, a_2, a_3	Amplitudes of the decay components. $a_1 + a_2 + a_3 = 1$
q_1, q_2, q_3	Relative intensity contributions of the decay components. Products of component amplitudes and lifetimes.
N	Number of photons in the pixel. N ratios are used for Redox-Ratio images
χ^2	χ^2 in the individual pixels
Sca	Amount of scattering or SHG, if scatter enabled in general model parameters
$Shift$	Shift parameter (if left floating in general model parameters)
E_{int}	FRET efficiency of interacting donor fraction, $E_{int} = 1 - t_1/t_2$
E_{class}	Classic FRET efficiency. $E_{class} = 1 - t_m/t_2$
$1-t_1/t_2$	Classic FRET Efficiency calculated from t_i . For comparison only.
r/r_0	Ratio of FRET donor-acceptor distance, r , and Förster radius, r_0
Offset	Offset parameter, see general model parameters

Three examples are shown in Fig. 510. The figure shows images of the fast decay component, t_1 , of the slow decay component, t_2 , and the amplitude of the fast component, a_1 , obtained by double-exponential analysis.

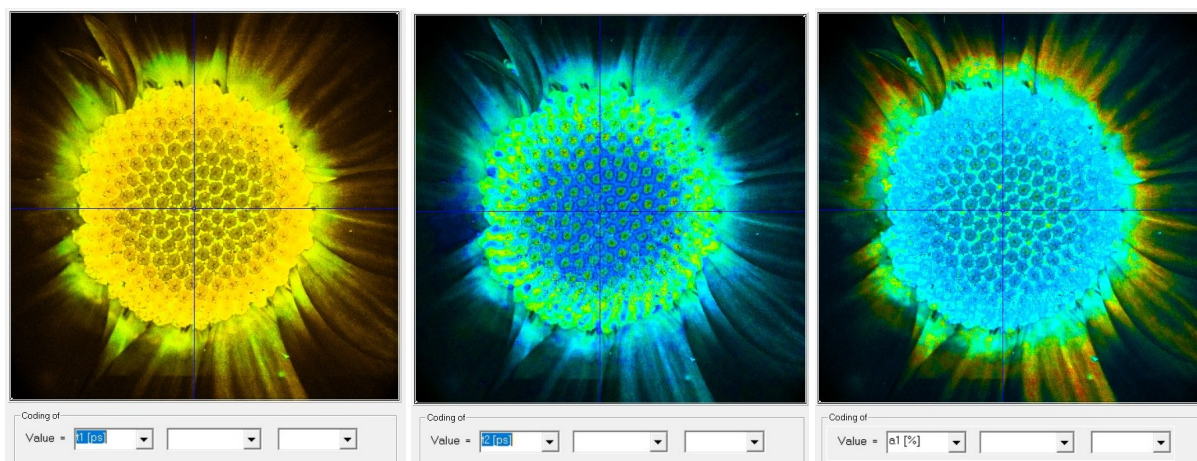


Fig. 510: Colour coding of t_1 , t_2 , and a_1 . Parameter ranges 0-2ns, 0-2ns, 0.5-1, respectively. Lower part of colour parameters shown only.

Arithmetic Expressions of two Parameters

Colour coding can be performed either by the decay parameters themselves or by arithmetic expressions of decay parameters. Examples are shown in Fig. 511. It shows the ratios, a_1/a_2 , t_1/t_2 , and q_1/q_2 , of the parameters of double-exponential decay.

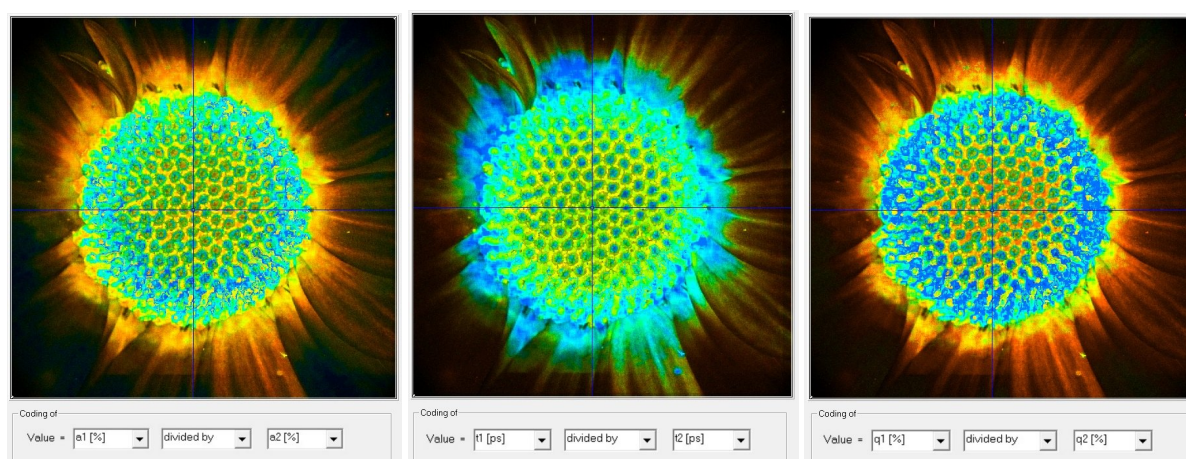


Fig. 511: colour coding of a_1/a_2 , t_1/t_2 , q_1/q_2 . Parameter ranges 1-20, 0.1-0.5, 1-5, respectively. Lower part of colour parameters shown only.

Linking Parameters from Different Images

If a FLIM data file contains images from two or more detection channels arithmetic expressions of parameters from different channels can be calculated and displayed. An example is shown in Fig. 512. The FLIM data set contains images from two TCSPC channels, indicated by the tabs below the decay curve window. With the Colour parameters shown SPCImage calculates a_2 from the selected image channel (Channel 2) divided by a_1 of Channel 1. The result is used as colour coding of the selected image channel (Channel 2).

In the example shown Channel 1 contains FAD data and Channel 2 NADH data. The selected expression represents the Fluorescence Lifetime Redox Ratio,

$$\text{FLIRR} = a_{2\text{NADH}} / a_{1\text{FAD}}$$

Please see 'Metabolic Imaging by NAD(P)H and FAD FLIM' page 248.

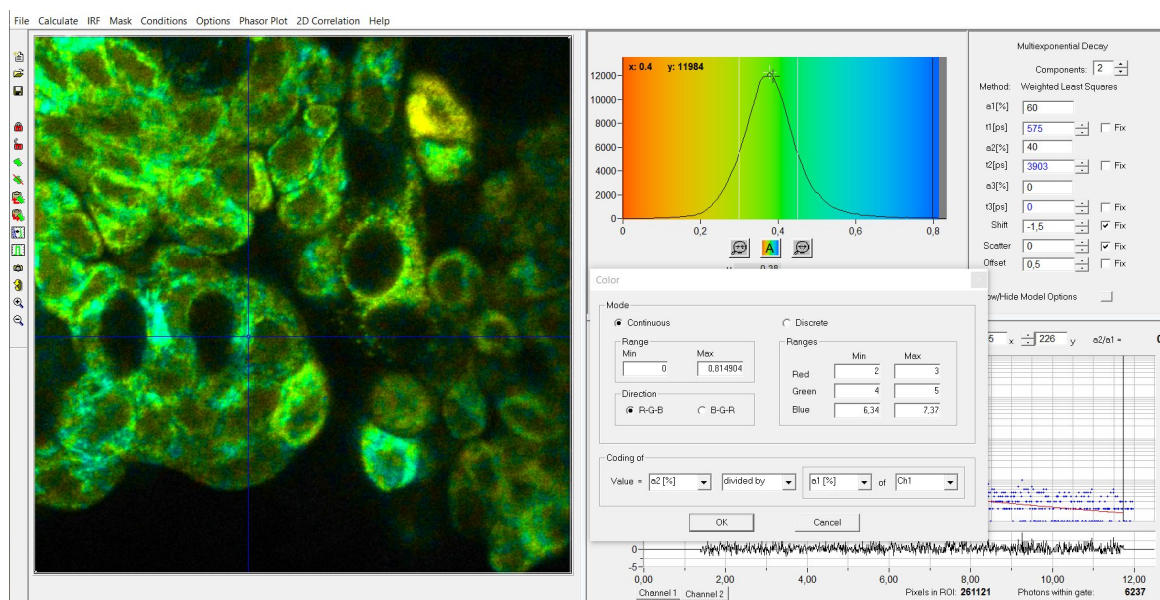


Fig. 512: Linking parameters from two image channels of one FLIM data set. The example calculates $a_{2\text{channel}2}$ divided by $a_{1\text{channel}1}$

Parameter Histogram

SPCImage displays a histogram for the decay parameter selected for colour-coding the lifetime image. The histogram shows how often pixels of a given parameter value occur in the lifetime image. Depending on the settings in 'Preferences', the histogram either displays the pure pixel frequency or the pixel frequency weighted with the pixel intensity, see 'Preferences', Fig. 541, upper right. The parameter histogram serves two different purposes.

Interaction with the Colour Parameters

The parameter histogram helps the user conveniently set an appropriate range of the colour parameters of the image. The histogram has two cursors, one for the upper end of the parameter range and one for the lower end, see Fig. 513. A change in the cursor positions in the histogram (middle) automatically changes the range in the colour parameters (right) and, consequently, the colour coding of the image.

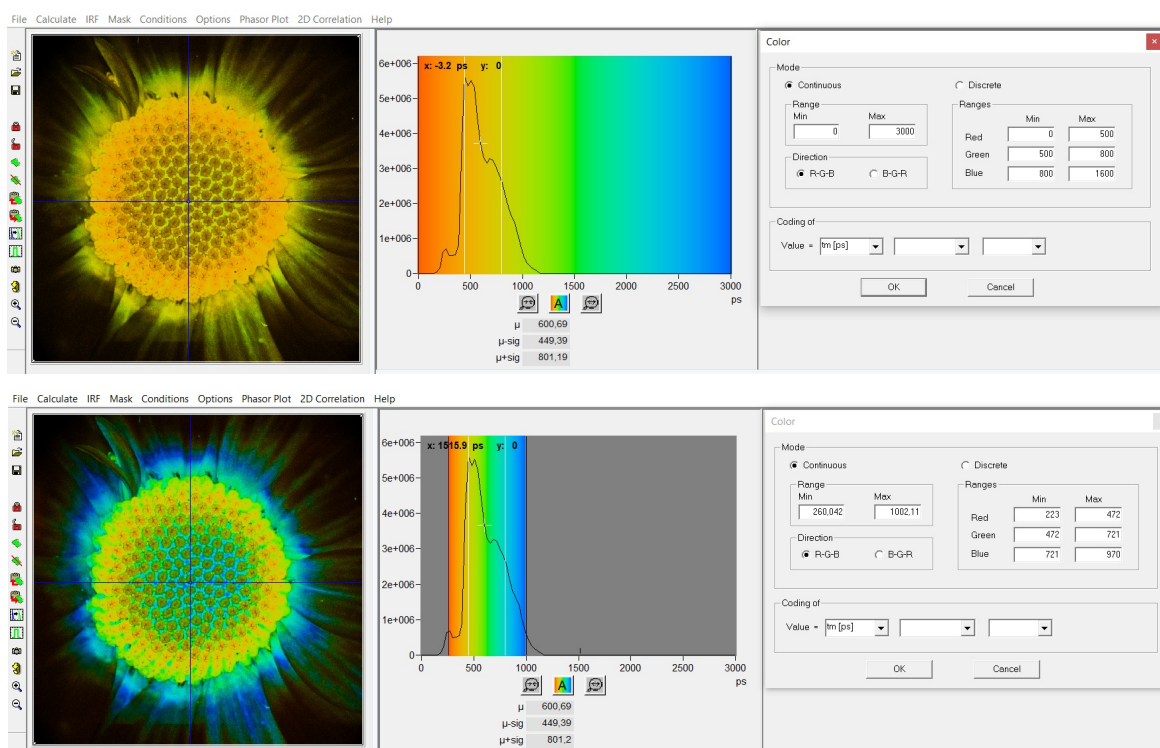


Fig. 513: Interaction of cursors in the parameter histogram (middle) with colour parameters (right) and colour-coding of the image.

The buttons underneath the histogram help the user define the parameter range. The button zooms the distribution into the selected parameter range, zooms out. The button sets the parameter range automatically.

Estimation of Decay Parameter Values

The parameter histogram shows the distribution of the selected decay parameter in the image. Information from the histogram is therefore more informative than numerical parameter values from a single spot of the image. By giving a graphical overview, the histogram shows what the most frequent value is, how broad the distribution is, and what the shape of the distribution is. The parameter histogram is thus an excellent tool to compare the results of different FLIM measurements. An example is shown in Fig. 514. The images show four FLIM data sets obtained from mushroom spores [93]. The images and histograms show the lifetime of the fast decay component, t_1 , of triple-exponential decay analysis. The data are for different

spore colour, from light brown (upper left) to black (lower right). Although lifetimes in the range of 20 ps (note the colour scale!) are necessarily a bit uncertain the histograms show a clear trend with the spore colour.

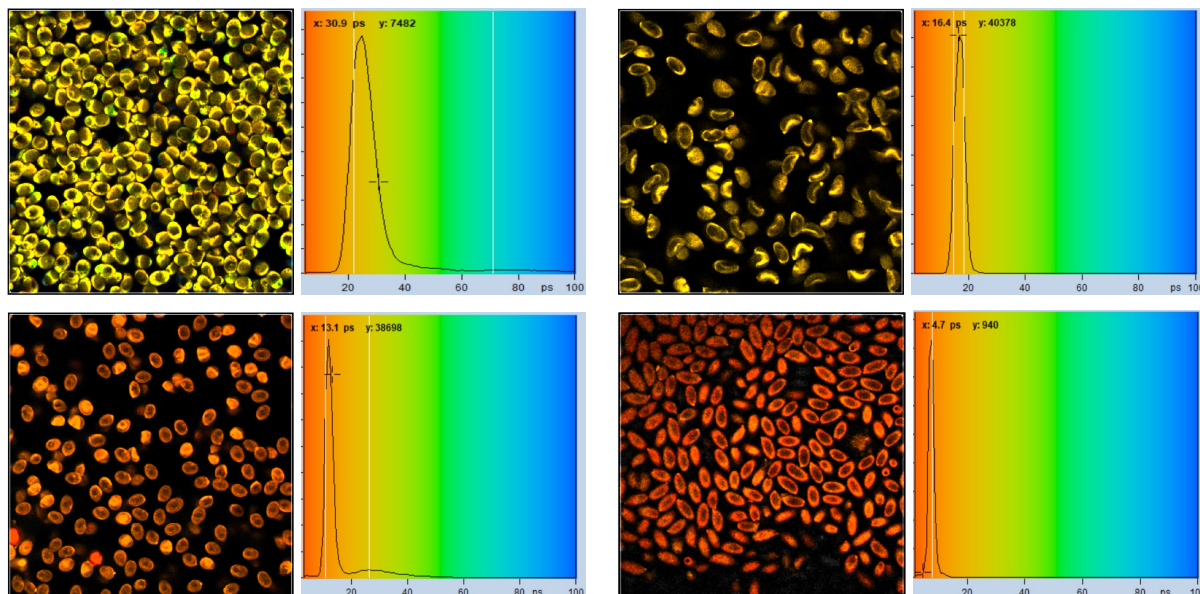


Fig. 514: t_1 images and t_1 histograms for mushroom spores of different colour. The histograms show a clear trend from upper left to lower right.

Intensity Histogram

Instead of the parameter histogram, SPCImage NG can also display an intensity histogram. The histogram type is switched by a right-mouse click into the image window. This opens a selection panel, from which either 'Parameter Histogram' or 'Intensity Histogram' can be selected, see Fig. 515. The parameter histogram is shown on the right in Fig. 515.

The intensity histogram interacts directly with the Intensity Parameters of the image display. The histogram therefore has two cursors by which a suitable intensity range can be selected. An example is shown in Fig. 515 and Fig. 516. The image includes a number of extremely bright objects, which are probably dead cells. The normal autoscale procedure (see 'Intensity Parameters', page 324) normalises the image brightness on these cells. The good cells are much dimmer and are therefore displayed at low brightness and contrast. One solution is to open the Intensity Parameter panel, turn off autoscale, and try with a new 'Max Intensity' value.

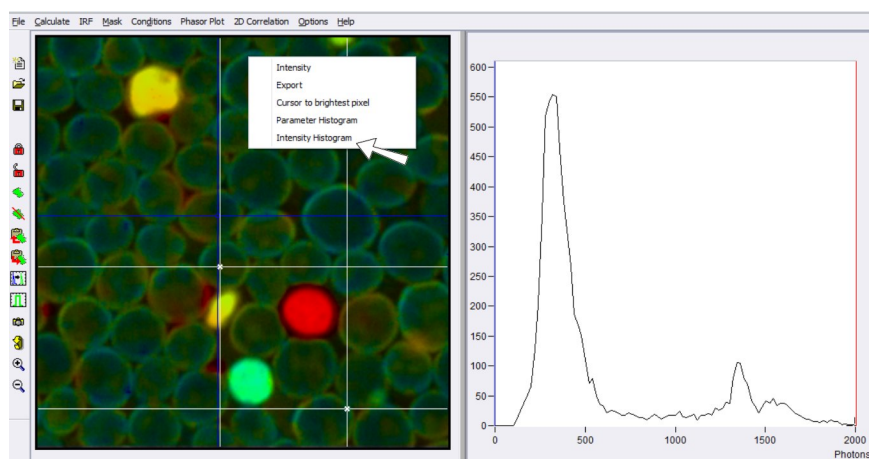


Fig. 515: Switching from the Parameter Histogram to the Intensity Histogram. Histogram shown on the right.

With the intensity histogram, the intensity range can be adjusted much more easily and quantitatively. Different features form different peaks in the histogram. It is thus easy to identify the peaks of different features, and adjust the intensity range to display the desired feature optimally. In Fig. 516 the right cursor has been set just above the peak that corresponds to the good cells. The result is that the cells are displayed at a much more favourable brightness.

With the adapted intensity range, it becomes obvious that the background of the image is not entirely dark. The reason in this case is that the image was recorded by simultaneous FLIM/PLIM, and the PLIM signal leaks in the FLIM recording. In Fig. 517, the background has been suppressed by pulling up the left histogram cursor. The image adjusted this way has a much more favourable appearance than the original image, compare Fig. 515.

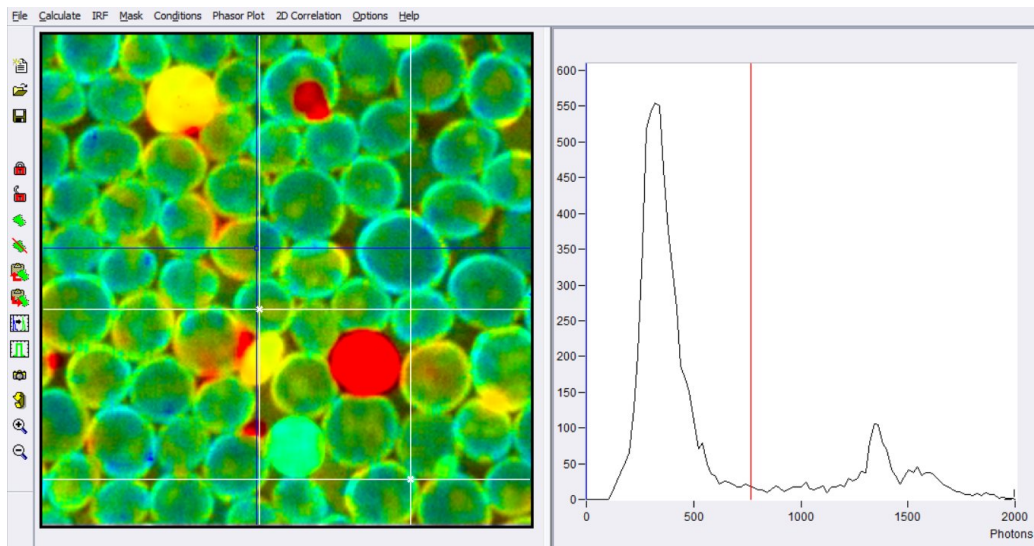


Fig. 516: Image brightness scale adjusted by histogram cursors. High-Intensity cursor adjusted to brightness peak of good cells.

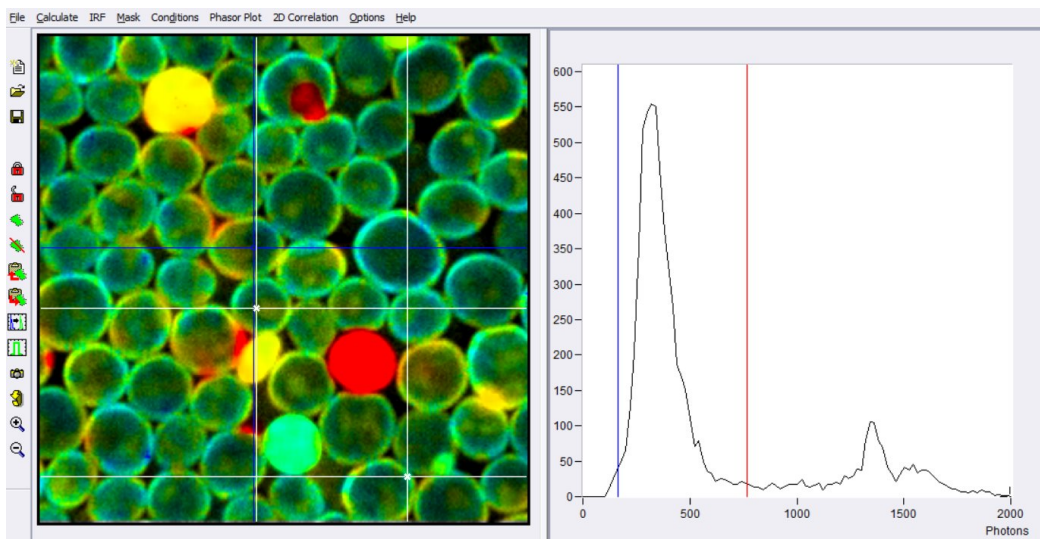


Fig. 517: Image brightness scale adjusted by histogram cursors. Low-Intensity cursor adjusted to brightness peak of good cells.

ROI Definition

SPCImage has several options to define ROIs. When an ROI is defined the parameter histograms are created from the pixels inside the ROI only. An ROI selection can therefore be used to obtain (and compare) lifetime histograms from different regions of the image. Moreover, decay data from all pixels inside an ROI can be combined into a single decay curve.

Rectangular ROI

The image window of SPCImage contains three cursors. The blue cursor is used to select a spot in the image for which a fluorescence decay curve is displayed. The white cursors are used for region-of-interest (ROI) definition. To define an ROI by the image cursors move the cursors in the desired position, see Fig. 518. The area between the cursors is the ROI. You can zoom into the selected area by the zoom button on the left of the SPCImage main panel.

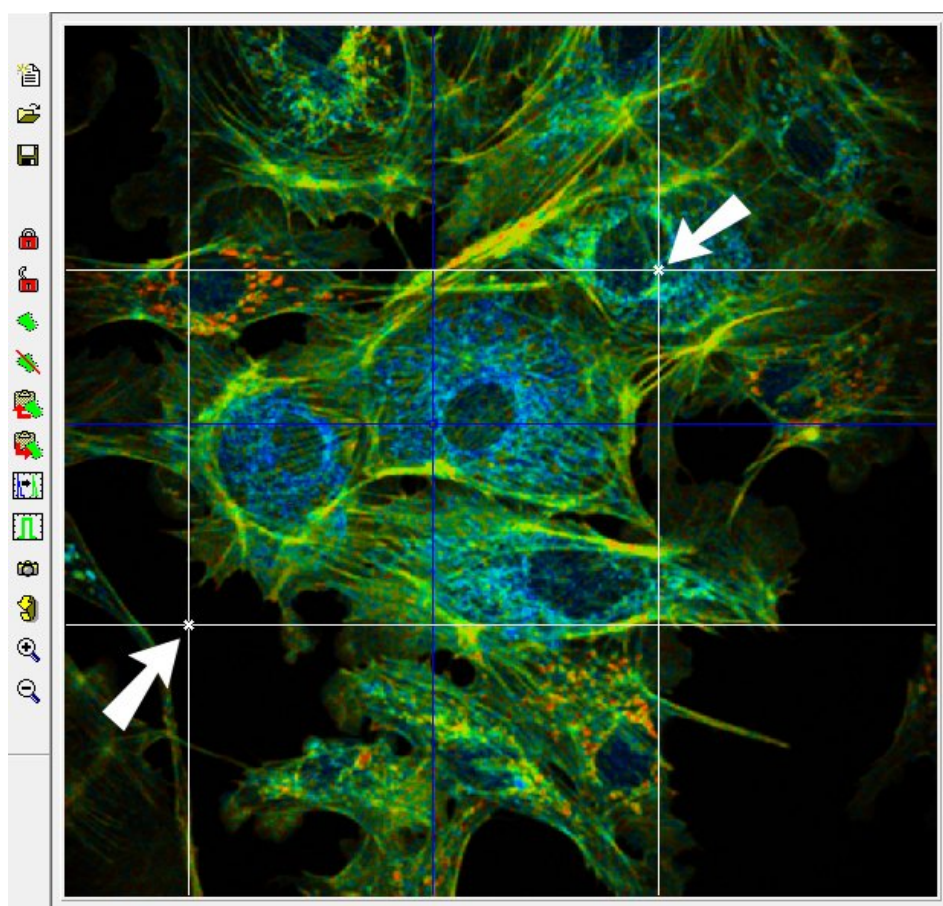


Fig. 518: Rectangular ROI, definition by image cursors

Polygonal ROI

A polygonal ROI is defined by clicking on the  ('Create ROI') button on the left of the image window. You can also choose 'Mask' in the top bar and select 'New'. This opens the panel shown in Fig. 519. Select 'Polygon Mask', and find look for the little red cross in the FLIM image.

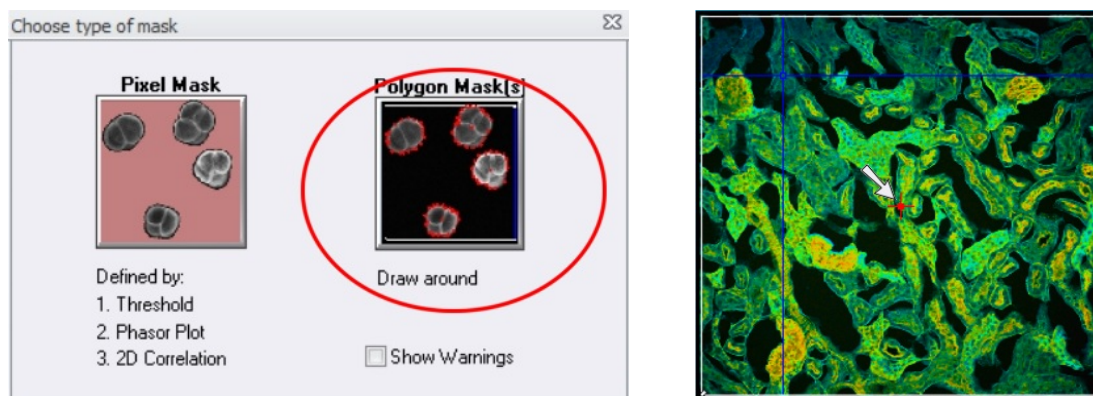


Fig. 519: Selection of 'Polygon Mask'

The ROI is created by shifting the red cross as shown in Fig. 520, left. Every mouse click adds a new point to the polygon in the spot where the cross has been placed. You can define several ROIs. To do so, click on the 'Create ROI' button again. This opens a new ROI, see Fig. 520, middle. To modify an ROI click on 'Mask' in the top bar and select 'Select/Modify'. Click on the ROI you want to modify and shift the points into the desired position, see Fig. 520, right.

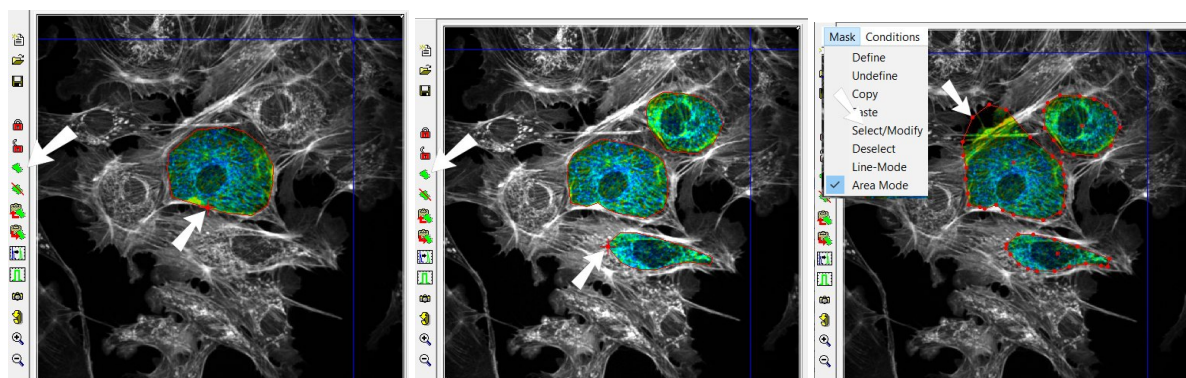


Fig. 520: Definition of a polygonal ROI. Left: Single ROI. Middle: Several ROIs, every click on the ROI symbol on the left opens a new ROI. Right Modification of an ROI.

Lines of Interest

SPCImage can define 'Lines of Interest'. Click on 'Mask', and select 'Line Mode' instead of 'Area Mode'. Instead of an Area, the 'Define' function then defines a line, e.g. along a cell membrane. Also in the 'Line' mode you can make several definitions in the same image, select individual histograms for the lines, and combine the decay data of each line in a single decay curve.

ROIs from 2D Histograms and Phasor Plots

ROIs can not only be defined manually but also be obtained from the 2D Histogram and the Phasor Plot of SPCImage, see page 337 and page 339. In these cases the areas are not defined in the images but in two-dimensional histograms of decay or phasor parameters. Areas defined by these functions can be much more complex than manually defined ROIs. In particular, they can contain multiple areas that are not connected to each other, or even disconnected pixels. The associated patterns are therefore also referred to as 'Masks', and the procedures to obtain them as 'Image Segmentation', please see Fig. 530 and Fig. 535.

Pixel Masks

Masks are, in principle, ROIs. However, there are differences: A mask can be much more complex than a normal RIO. In particular, it can contain multiple areas that are not connected to each other, or even disconnected pixels. Moreover, a mask can be defined in or derived from one image of a sample and then applied to another image or a series of images of the same sample. These images can be from a time series, from a Z stack, or a multi-wavelength image of the sample. To define a mask, click into 'Mask' in the top bar, and select 'New', see Fig. 521, left. This opens the selection panel in Fig. 521, right. A pixel mask can be defined by defining a 'Threshold' of the photon counts per pixel, by selecting a phasor range in the Phasor Plot, or a decay-parameter range in the 2D Correlation histogram.

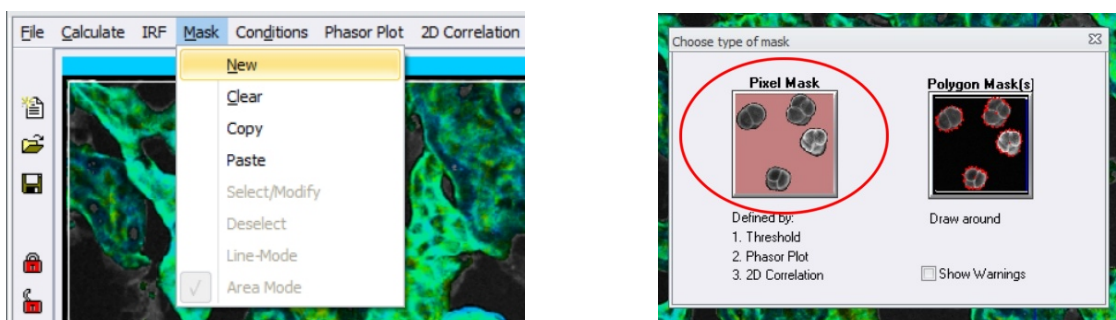


Fig. 521: Definition of a Mask

'Threshold' creates a mask that contains pixels with numbers exceeding a certain number of photons. The number of photons is defined by the 'Threshold' parameter above the decay curve window, see Fig. 522, left. The original FLIM image is shown in Fig. 522, middle. The mask created by a threshold of 3000 (photons in the decay curve of the pixel) is shown in Fig. 522, right.

Fig. 523 shows the mask definition via the Phasor Plot. The selection of the desired phasor range in the phasor plot is shown on the left, see small red circle. Fig. 523, middle shows the FLIM image with the pixels within the selected phasor range highlighted. A mask created from the highlighted pixels is shown on the right.

The mask definition via the 2D parameter histogram is shown in Fig. 524. The parameter histogram shows the amplitude of the fast decay component, a_1 , over the amplitude-weighted lifetime, tm . A parameter range covering large a_1 with long tm was selected. Fig. 524, middle, shows the FLIM image with the corresponding pixels highlighted. Fig. 524, right, shows the mask created from the selection.

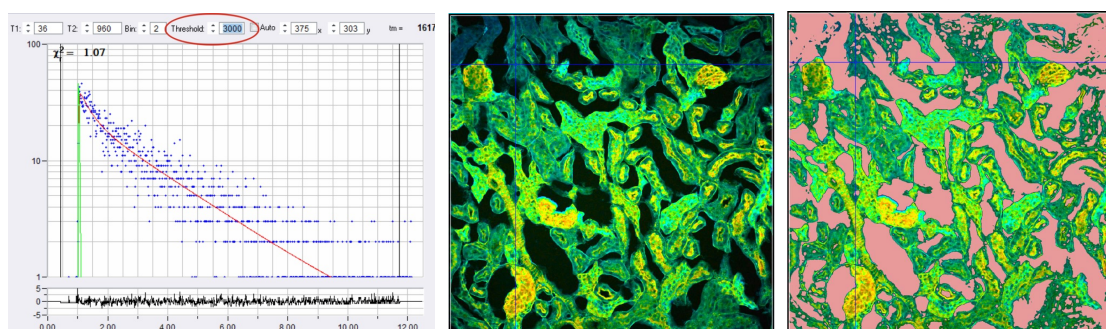


Fig. 522: Mask definition via 'Threshold'. Left: Threshold definition. Middle: FLIM image. Right: Mask created by Threshold = 3000 photons per pixel.

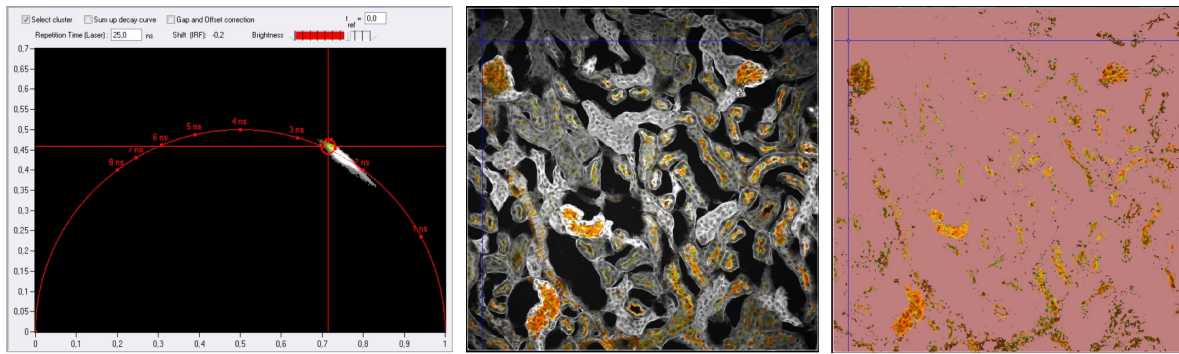


Fig. 523: Mask definition via Phasor Plot. Left: Selection of phasor range. Middle: Image areas highlighted for selected phasor signature. Right: Mask created from highlighted areas.

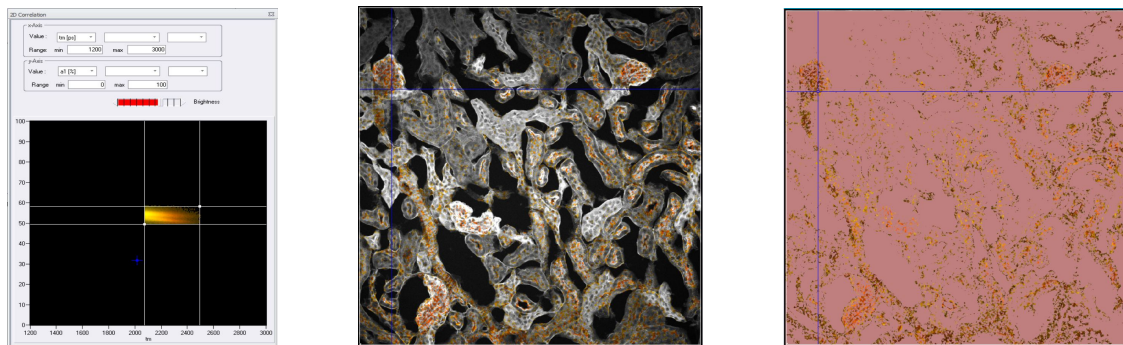


Fig. 524: Mask definition via 2D-Parameter Histogram. Left: Selection of parameter range, a1 over tm. Middle: Image areas highlighted for selected parameter range. Right: Mask created from highlighted areas.

Decay-Parameter Histograms in ROIs and Masks

If an ROI has been defined or a mask has been created the parameter histogram in the SPCImage main panel refers to the pixels within the ROI or the mask. An example for two different ROI definitions is shown in Fig. 525. If multiple ROIs have been defined the desired ROI is selected after opening 'Mask' in the top bar and selecting 'Select/Modify'. The ROI is selected either by clicking on the red dot in the centre of it or on one of the tabs on top of the histogram window, see Fig. 526.

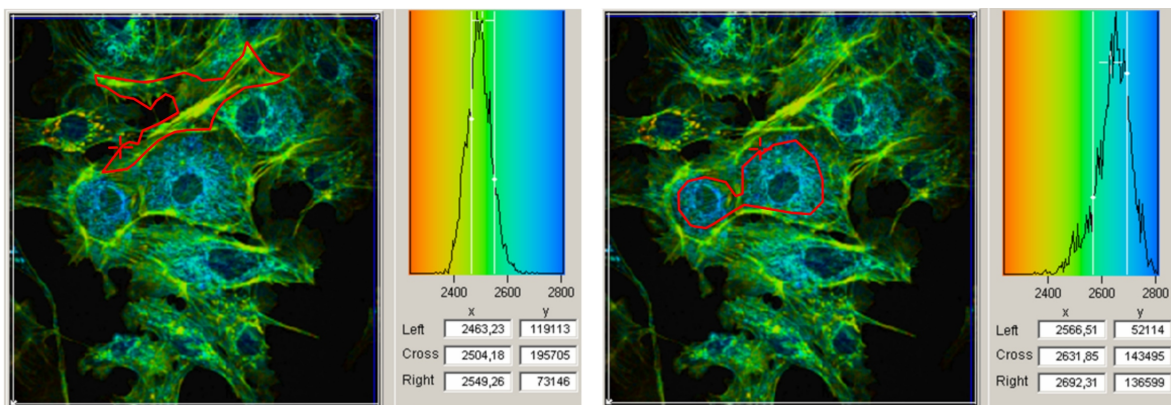


Fig. 525: Parameter histograms for different definition of the ROI

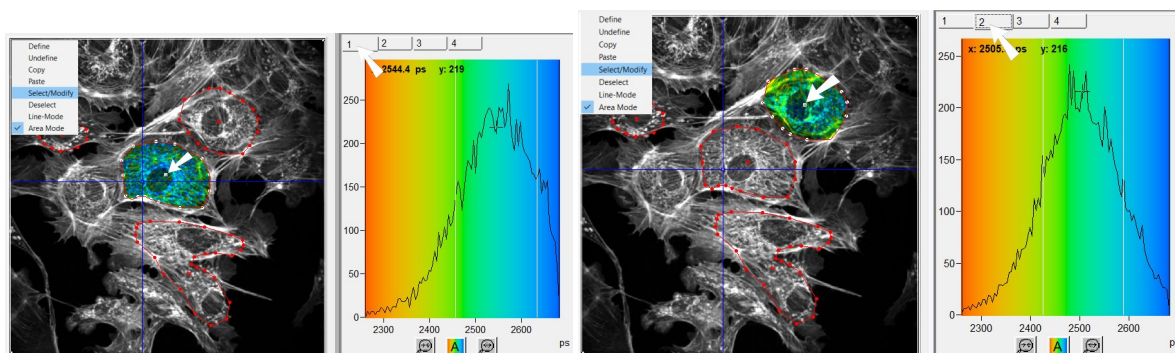


Fig. 526: Selection of one of several ROIs for display of the parameter histogram

The typical application of a pixel mask is shown in Fig. 527. The figure shows lifetime images from two wavelength channel of the same FLIM data set. A mask for pixels of long lifetime was created in one wavelength channel (450 nm), see Fig. 527, left. The mask was copied from this channel to the other (590 nm) by 'Mask', 'Copy', and 'Paste'. The lifetime histogram on the right is thus showing the distribution of the lifetimes of the pixels selected in the image on the left.

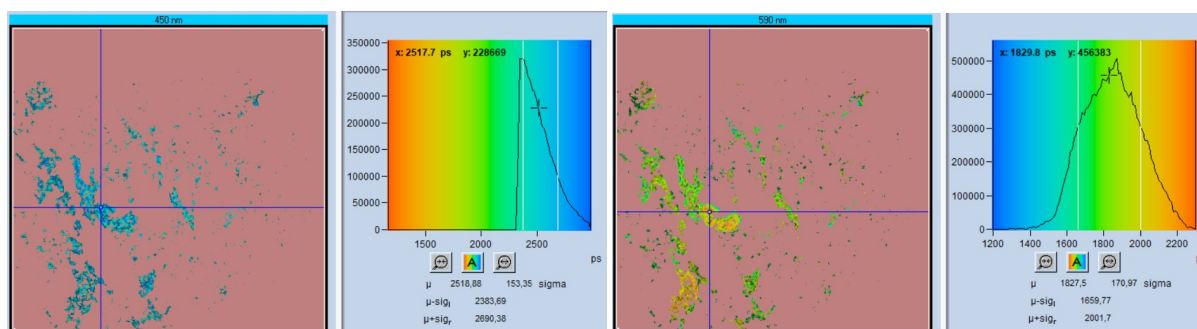


Fig. 527: Application of a pixel mask to images from different wavelength channels

Combination of Pixel Data into a Single Decay Curve

The  (lock) button in the task bar left of the lifetime image combines the decay data of all pixels within an ROI or a mask in a single decay curve. A decay curve for the ROI selected in Fig. 526, left, is shown in Fig. 528.

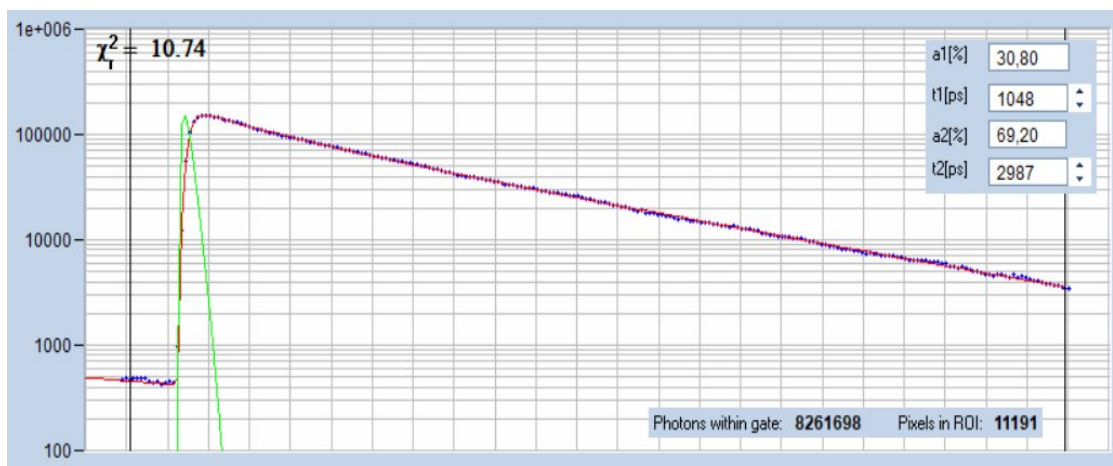


Fig. 528: Decay curve of combined pixels within ROI of Fig. 526, left

The resulting decay curve comes from a large number of pixels and thus contains a large number of photons. It can thus be analysed at an accuracy as it is normally achieved only in cuvette-based experiments.

2D Histograms

SPCImage has a two-dimensional histogram function. It displays a density plot of the pixels over two selectable decay parameters. These can be t_m , t_i , t_1 , t_2 , t_3 , a_1 , a_2 , a_3 , and arithmetic expressions of these parameters. The 2D histogram plot is opened via '2D Correlation' in the top bar. An example is shown in Fig. 529. The plot has been configured to show the values of t_2 over the values of t_1 , see upper part of the 2D histogram panel.

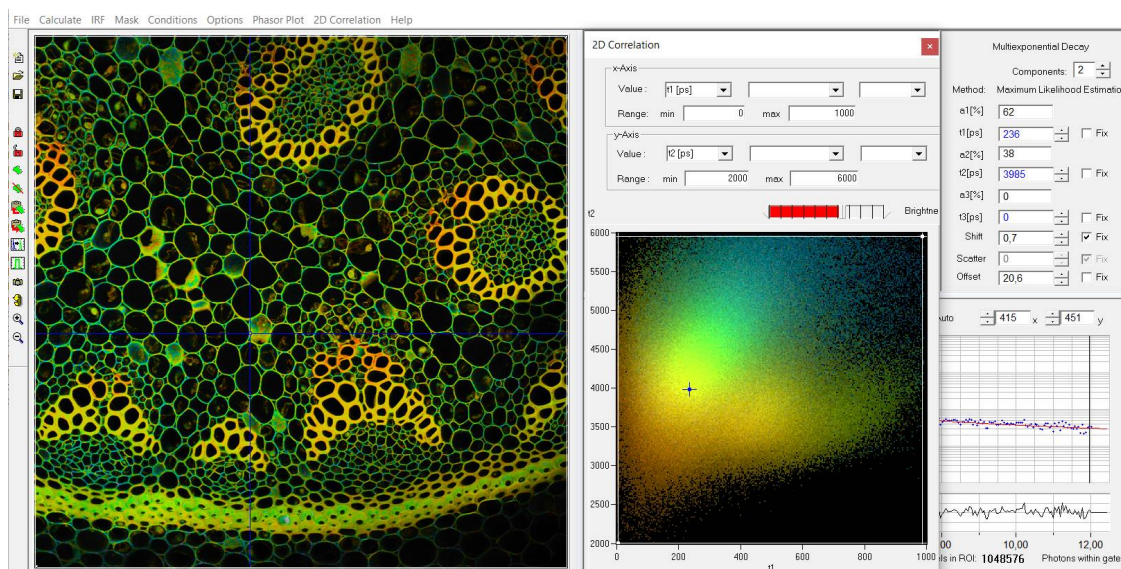


Fig. 529: 2D histogram of t_2 over t_1

Image Segmentation by 2D Histogram

Cursors in the 2D histogram can be used to select pixels of a specific decay signature and back-annotate them in the image. Pixels with a decay signature within the selected histogram area are shown in the image window normally, pixels outside the area are shown without colour, see Fig. 530. The selection also acts on the 1D parameter histogram. It is build up for pixels selected in the 2D histogram only.

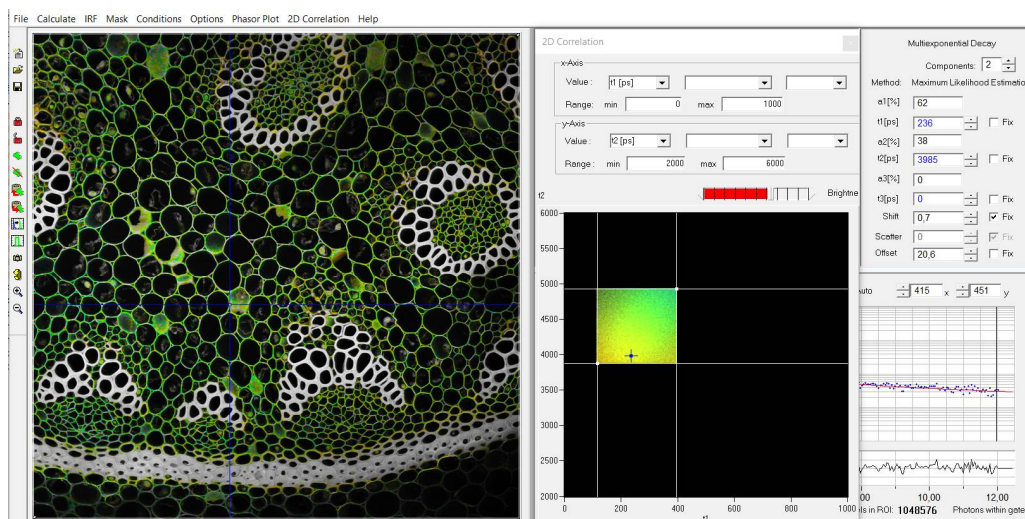


Fig. 530: Selection of pixels with a specific decay signature in the 2D histogram

Combination of Decay Data Within Selected Parameter Range

The decay data of the pixels within the selected pixels can be combined into a single decay curve. Click on the  (lock) button in the task bar left of the lifetime image. The decay curve of the combined pixels of the image features selected in Fig. 530 is shown in Fig. 531.

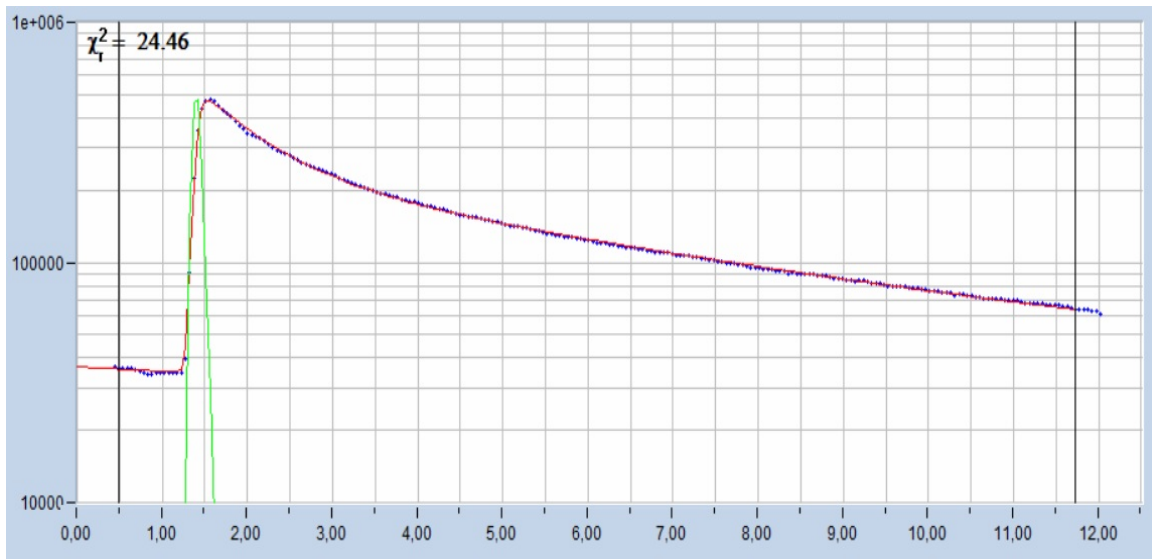


Fig. 531: Combined decay data from image features selected in Fig. 530

Phasor Plot

A click on 'Phasor Plot' in the top bar of SPCImage opens the phasor plot panel, see Fig. 532, right. Every pixel in the FLIM image (Fig. 532, left) is represented by a dot in the phasor plot (right). The location of the pixel in the phasor plot depends on the amplitude and phase of the decay function in the phasor space. To make the correspondence between the image and the phasor plot clearly visible SPCImage can assign the colours of the FLIM image to the dots in the phasor plot. This function is activated by selecting the 'Combine with FLIM analysis' option in the phasor plot panel, see Fig. 532, right.

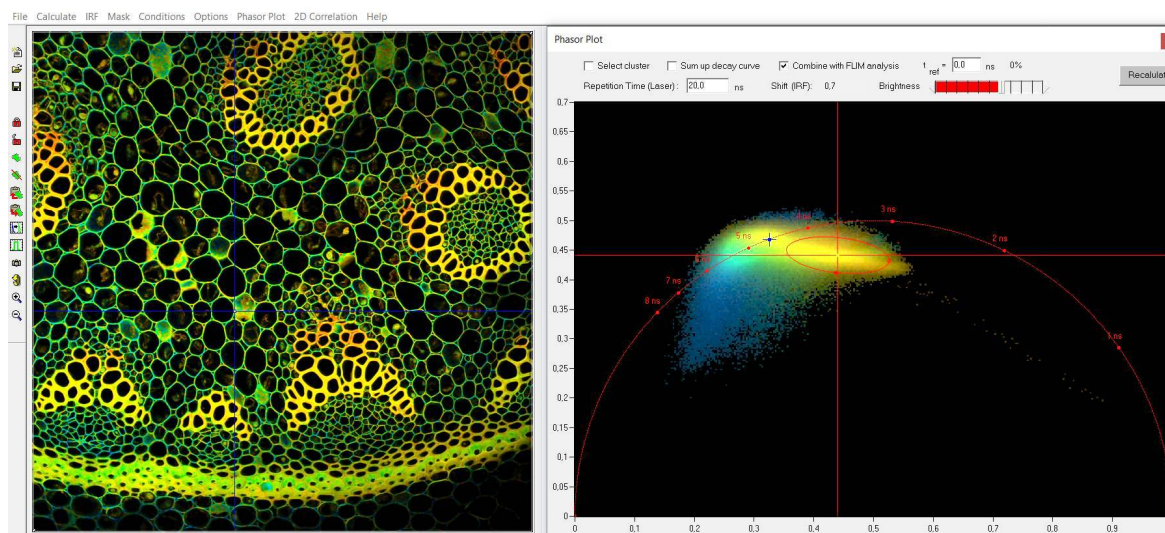


Fig. 532: Left: Lifetime image (left) and phasor plot (right)

A specific area in the phasor plot can be selected by a selection tool, see red ellipse in Fig. 532. By 'Select Cluster' the corresponding pixels are back-annotated in the FLIM image. Pixels outside the selected phasor range are displayed without colour, see Fig. 533.

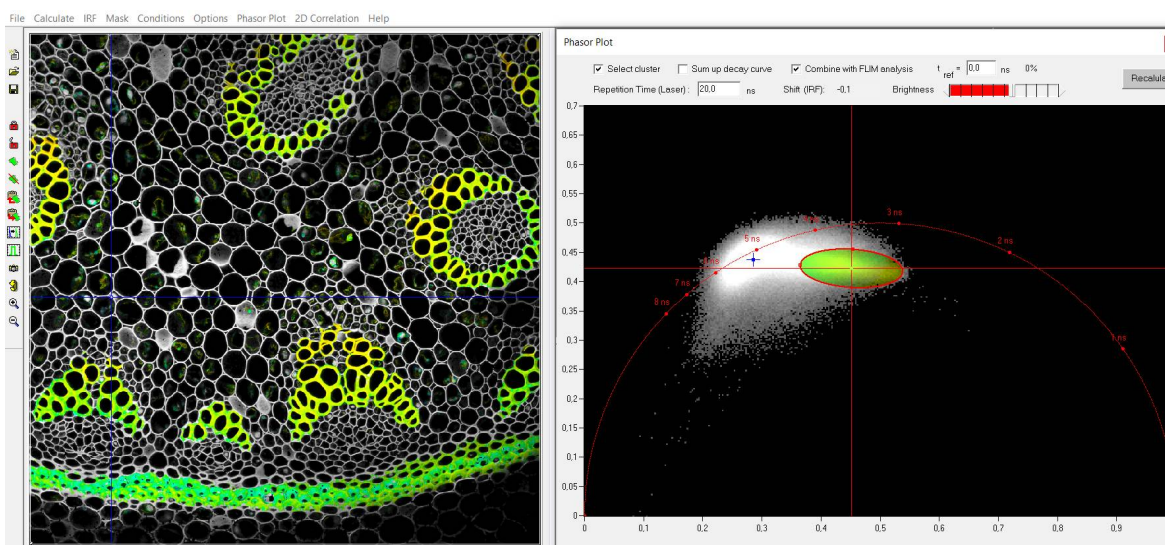


Fig. 533: Selection of a phasor range and back-annotation of the corresponding pixels in the image

Activation of 'Sum up decay curve' in the phasor plot combines the decay data of all selected pixels in a single decay curve, see Fig. 534. It represents an average decay curve of all pixels with a phasor signature inside the red ellipse. The curve can contain an enormously high number of photons (more than 88 million in the example, see 'Photons within gate' below decay curve window). It can thus be analysed at extremely high precision.

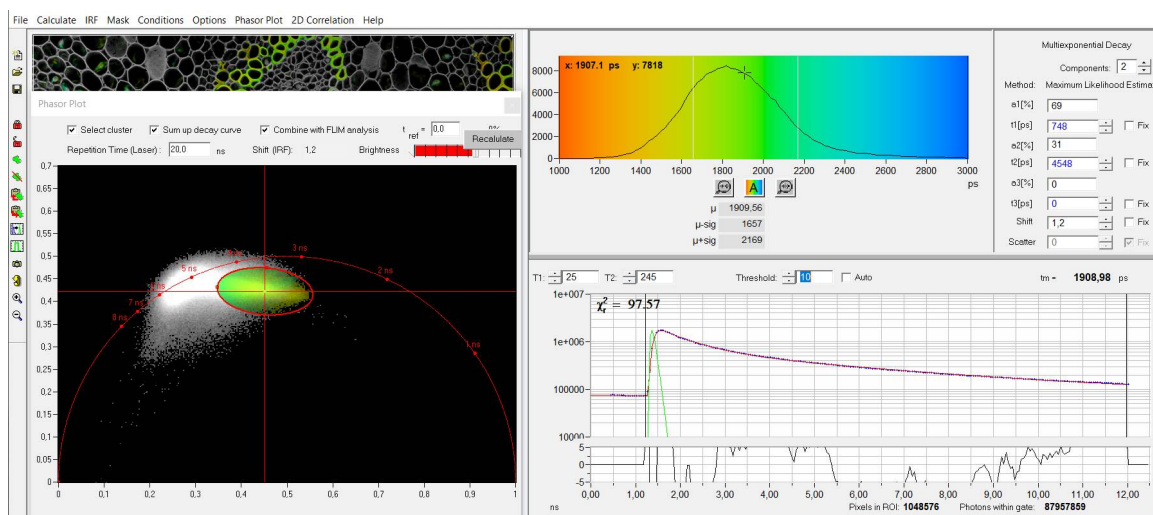


Fig. 534: Combination of the decay data of all pixels selected in the phasor plot in a single decay curve

Image Segmentation

Selecting a phasor range in the phasor plot and back-annotating the pixels in the FLIM image can be used as an automatic image segmentation function. It is equivalent to a manual ROI selection yet works with far less personal effort. Consider an image as the one shown in Fig. 535. You want to perform a heterogeneity study on the nuclei of the cells. Creating appropriate regions of interest around all the nuclei manually is almost impossible. But selecting the nuclei in the phasor plot is easy. The result of the selection is a histogram of the lifetime, t_m , of the nuclei, shown in the upper right are of the SPCImage panel.

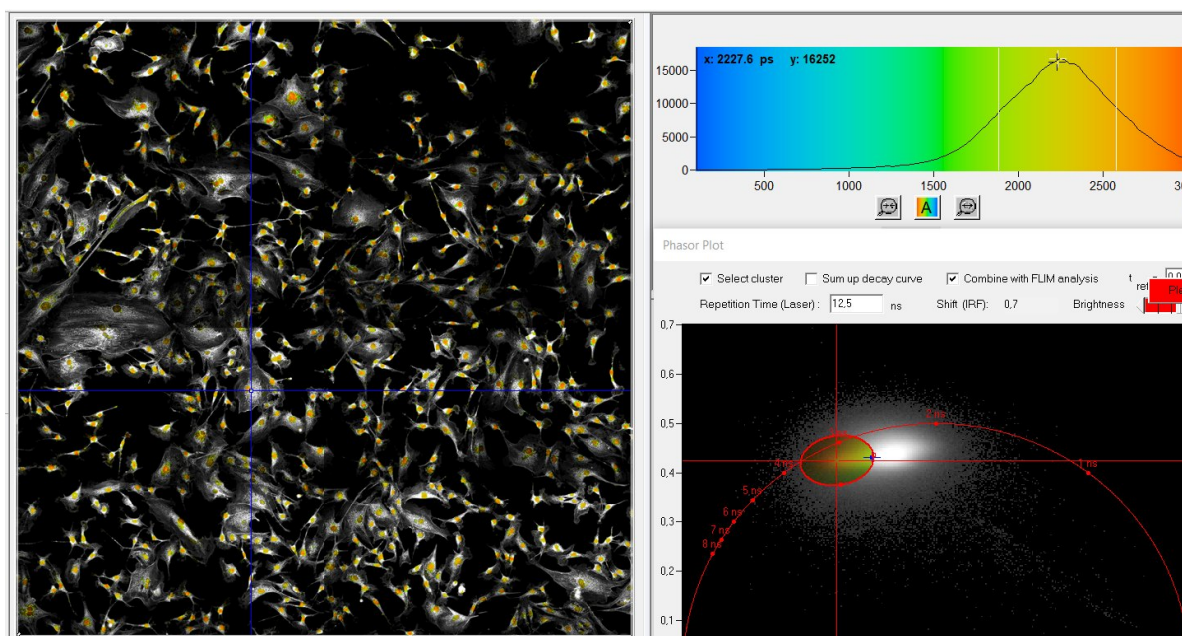


Fig. 535: Image segmentation by phasor plot.

Analysis of Moving Objects

Biological objects can move during the FLIM acquisition. The images, and, consequently, the fluorescence-decay information, is then smeared out over the a distance the object has moved during the acquisition time. A solution to this problem is provided by the Mosaic FLIM mode of the bh TCSPC modules. A large number of fast scans is performed, and the images are written in a Mosaic FLIM data array. Of course, the number of photons in the individual mosaic elements is too low to obtain precise lifetime information from them. However, if image

segmentation is applied to the entire mosaic, and decay data of the selected image details are combined, a decay curve with a high number of photons is obtained.

An example is shown in Fig. 536. The mosaic elements are 0.5-second scans of a water flea. The task is to obtain accurate decay parameters from the leg (yellow in the FLIM image) of the water flea. The leg is moving, and thus appears in different locations in the individual images. The number of photons contained in a single mosaic element is not enough for precise decay analysis, see decay curve in Fig. 536, top. To obtain precise fluorescence-decay information from the leg, load the mosaic in the phasor plot, and select a phasor range that corresponds to the decay signature of the leg, see Fig. 536, top. Then select 'Select Cluster' and 'Sum up decay curve', see Fig. 536, bottom. The result is a single decay curve containing an enormous number of photons. It can be analysed at high precision with a multi-exponential decay modes, as shown in the decay parameters in Fig. 536, bottom.

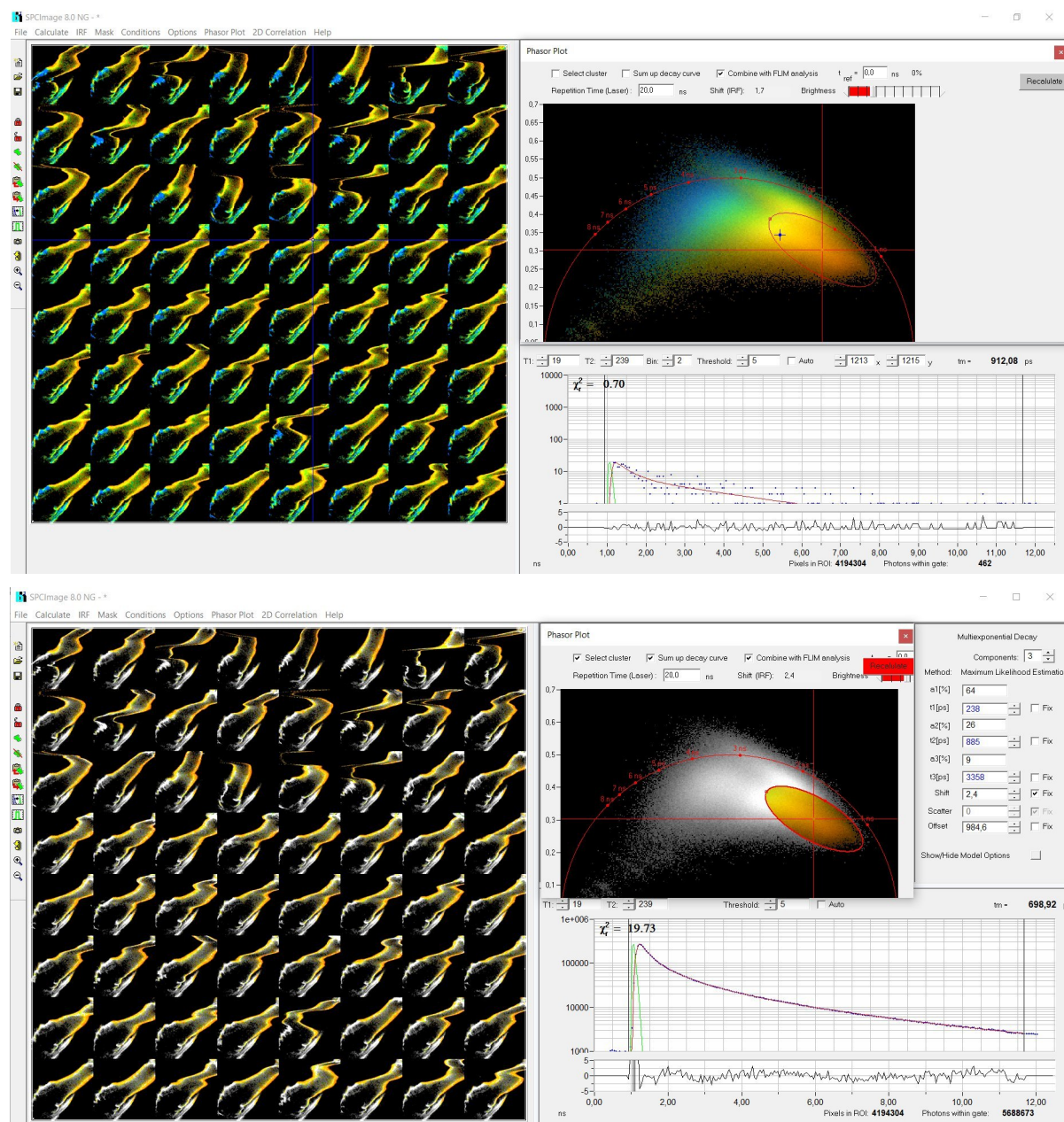


Fig. 536: Analysis of a moving object by Mosaic FLIM and image segmentation

Model Parameters

Details of the model functions and details of the algorithms, parameter constraints, and IRF definitions are defined in the Model-Parameters panel. The panel differs for the least-WLS (weighted least square) fit and for the MLE (maximum-likelihood estimation) fit, see Fig. 537, left and right. Fig. 537 shows the Model Parameter panel as it was in version 9.0 and before. From version 9.1 on the panel has been changed. The new panel is shown in Fig. 538 and Fig. 539.

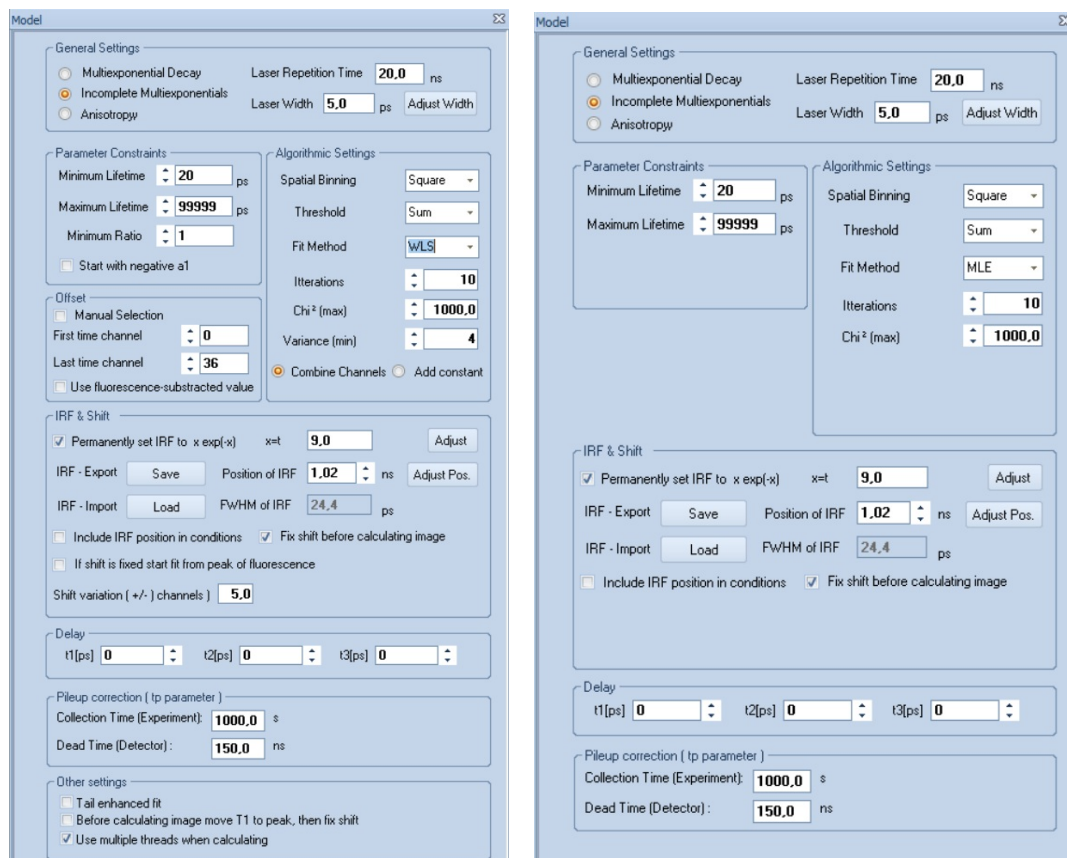


Fig. 537: Model parameters, version 9.0 and before. Left: Weighted-least-square fit. Right: MLE fit.

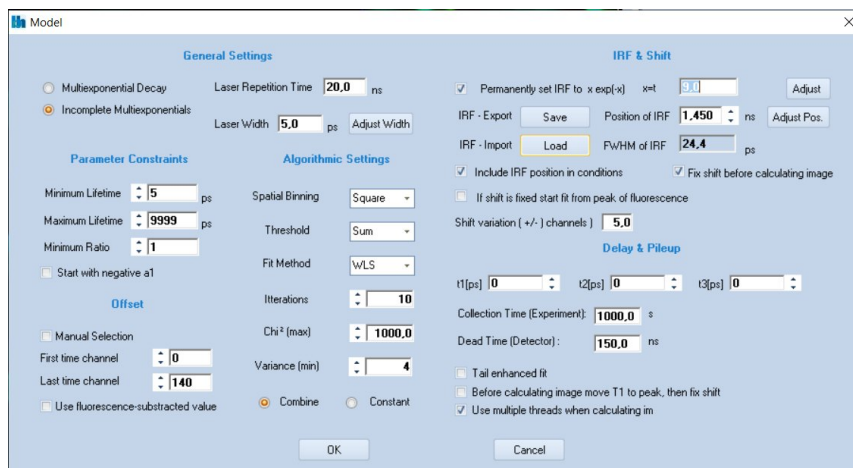


Fig. 538: Model parameters, version 9.1 and later. Weighted-least-square fit.

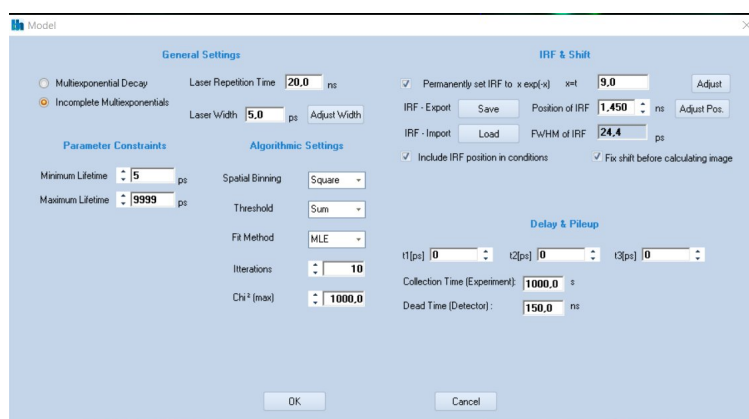


Fig. 539: Model parameters, version 9.1 and later. MLE fit.

Model Options

'Multiexponential Decay' uses the traditional single-, double, and triple-exponential decay models, see Fig. 479. Fluorescence from previous laser pulses is not taken into account. 'Incomplete Multiexponentials' uses the same basic models but takes into account that the fluorescence does not necessarily decay completely within one laser pulse period. It therefore includes residual fluorescence from previous excitation pulse periods. Please see 'Incomplete-Decay Model', page 310.

'Laser Repetition Time' is required for the calculation of incomplete decay and for the phasor plot. 'Laser Width' is the width of the laser pulse. It is used in the determination of the synthetic IRF.

Fit Parameters

'Parameter Constraints' are used to prevent the algorithm from running into irrelevant parameter ranges.

'Offset' is used for WLS only. It allows the user to define a time interval where the counts represent the real counting background. If 'Manual Selection' is not set the offset is determined from the time interval left from the rise of the fluorescence. 'Offset' is irrelevant for MLE because it handles the baseline offset as a real fit parameter.

'Algorithmic Settings' define parameters concerning the fit algorithm. 'Spatial Binning' defines the shape of the area of pixel binning (when used). It can be 'Square' or 'Circle'. 'Threshold' defines whether the 'Threshold' parameter in the decay-curve window is the photon number at the peak of the fluorescence or the total number of photons in the decay function.

'Fit Method' can be 'WLS' (weighted least squares), 'MLE' (maximum likelihood estimation), or MOM (first Moment). Note that only MLE uses GPU processing.

'Iterations' is the maximum number of iterations performed by the fit algorithm. Please note that it refers to the *maximum* number of iterations. In most cases the fit procedure reaches a final fit earlier, and stops when no further improvement is obtained. $\chi^2(\text{max})$ is a maximum of the χ^2 allowed for the WLS algorithm.

'Combine Channels' and 'Add Constant' can be used to tweak the performance of the WLS fit for data with low photon number. Normally 'Combine Channels' is used, i.e. the algorithm uses progressive binning of time channels when the average photon counts per channel approach zero. 'Add Constant' does not bin time channels but adds a 'Minimum Variance' to the least square. The traditional value is '1' but higher values can be used to reduce lifetime bias at low photon number.

IRF Definition

The parameters under 'IRF & Shift' define the IRF and its use in the calculation of the decay functions. We recommend to use 'Fix the shift before calculation'. The IRF is then shifted into an optimum temporal position before the calculation starts, but not shifted any more during the calculation of the entire image. The shift *before the calculation* is limited by 'Shift variation'. In case the fit does not produce reasonable result, please check that 'Shift variation' is large enough.

'Permanently set IRF to $x \exp(-x)$ ' and the parameter right of it define the synthetic IRF. The 'Adjust' button on the right starts an optimisation procedure for the synthetic IRF. Please see 'Instrument Response Function', page 377. The 'FWHM' of the IRF is displayed for information only. It includes the IRF model function $t/t_0 e^{-t/t_0}$ and the width of the laser pulse defined by 'Laser Width'.

Shifted Component Models

The 'Delay' parameters are used for the shifted-component model. t_1 , t_2 , t_3 , are applied as shifts to the corresponding decay components. Negative values shift the component to the left, positive values to the right. For application of the model please see bh TCSPC Handbook [77].

Other Settings

'Other Settings' contain a 'Tail-Enhanced Fit' to improve the fit of weak slow decay components, a function that automatically sets of the left cursor of the decay function to the beginning of the rising edge, and multi-threading. Please note that multi-threading is irrelevant for MLE with GPU processing - the GPU processing is parallel by definition. 'Collection Time' and 'Dead Time' are used only to determine pile-up corrected lifetimes, see 'Parameters of the Decay Functions and their Use in SPCImage', page 384. The parameters are not needed under normal conditions.

Enable / Disable User Access to Parameters

In the default state of SPCImage all model parameters are accessible by the users. It is, however, possible to disable user access to selected model parameters. The function was implemented for clinical applications where certain model parameters must not be changed by unauthorised users. To disable access to parameters, open the 'Preferences' panel and switch to 'Expert Mode'. When back in the model parameters, you now can disable parameters by a double click on the parameter name. The parameter name then turns red, meaning that the parameter access has been disabled. Please see Fig. 540, left and middle. To enable access to disabled parameters double-click again on the name. After switching back to non-expert mode in the Preferences the disabled parameters are greyed out, see Fig. 540, right.

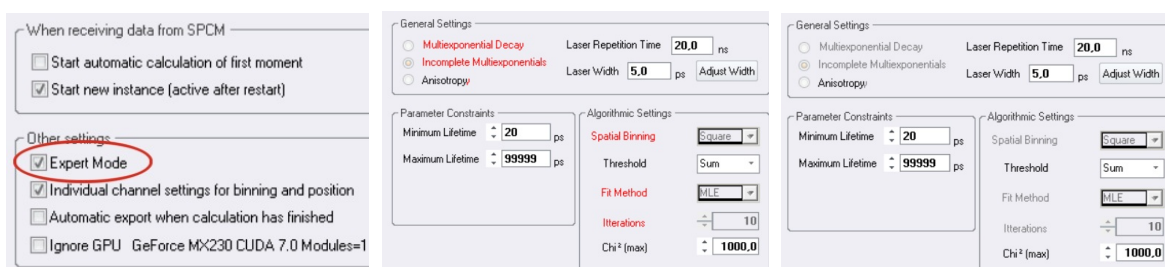


Fig. 540: Enable / disable access to selected model parameters. Left: Expert mode in 'Preferences'. Middle: Disabled parameters are shown in red. Right: In non-expert mode the disabled parameters are greyed out.

Preferences

The 'Preferences' panel defines general options of SPCImage. The panel is shown in Fig. 541.

Layout

This part of the preferences defines the layout of the SPCImage main panel.

Extra Intensity Image: In early SPCImage versions a separate greyscale intensity image was displayed together with the lifetime image. In the past 10 years the display of a separate grey scale image came out of use. Unless you are used to the old style we do not recommend to use this option.

Widescreen adapted: This option uses an aspect ratio which is adapted to modern screens with a wide aspect ratio. Unless you have an old computer screen we recommend to turn on this option.

Toolbar: The toolbar on the left of the main panel can be displayed with small icons (Lean Icon Set) or large icons (Extended Icon set). Unless you are very familiar with SPCImage we recommend 'Extended'.

Lifetime Window

'Zoom in Lifetime Windows only' restricts zoom operations performed in a lifetime window to this window. If the option is not set a zoom in the lifetime window zooms also the image in an intensity window (if one is displayed).

'Center ROI to selected pixel' couples a rectangular ROI (selected by the white image cursors) with the blue image cursor. You can shift the selected area around with the cursor and examine the pixel-parameter histograms for the different areas.

Distribution Window

Pixel parameter histograms can be weighted with the pixel intensity (photon number) or just represent the number of pixels in which a particular parameter value is present. The histograms can be calculated over the full range of the parameter values or only in the parameter range selected in the 'Colour' options panel.

Instrumental Response

The IRF can either be displayed or not displayed in the decay curve window. The calculation of an IRF can be performed at the brightest pixel or at a selected cursor position. To get an impression of whether the IRF reasonable fits to the decay data we recommend to always display the IRF in the decay window. 'Always calculate at brightest pixel' determines the IRF at the brightest spot of the image. However, this can lead to an incorrect result if the brightest spot is a speck of dirt or another artefact. So recommend to turn this option off.

Start Properties

New instance when receiving data from SPCM: When SPCImage contains data and a new data transfer from SPCM is started a new instance of SPCImage can be started or previous data can be overwritten. Starting new instances helps the user compare different data. However, old instances should be closed from time to time.

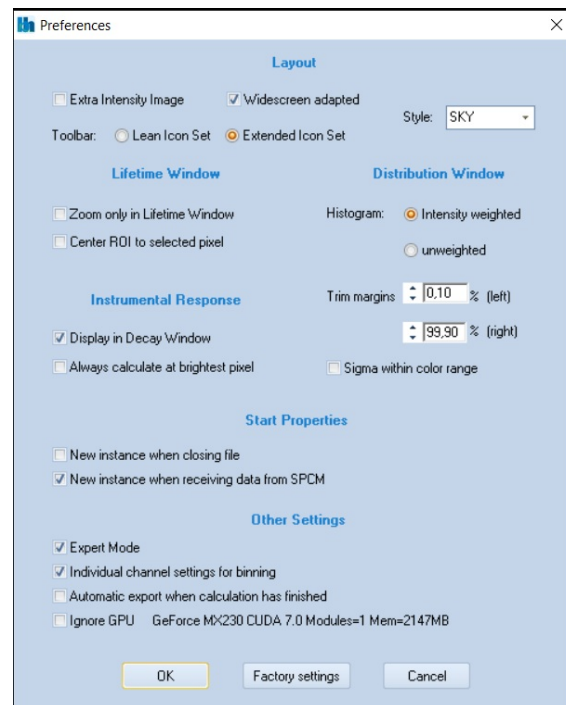


Fig. 541: Preferences panel of SPCImage, showing parameters and recommended settings.

Other Settings

'Expert Mode' puts SPCImage in a mode where the user can disable and enable the access to certain Model Parameters. There is no need to set 'Expert Mode' unless you want restrict the access to certain model parameters or you want enable the access to parameters which have been disabled before. Please see Fig. 540.

'Individual channel settings' means that the binning factor and the cursor position for IRF calculation are individual for several images (from different SPC modules or from different routing channels) loaded into SPCImage. Otherwise the same binning and the same positions for IRF calculations are used.

'Automatic Export' exports data according to the options in the Export panel when the calculation is finished.

'Ignore GPU' prevents SPCImage from using a GPU. (The type of the GPU is shown on the right). There is no need to use this option unless a GPU in the system is causing trouble.

Configurations

'Configurations' allows you to save and load configuration data of SPCImage. A click into Options, Configurations, opens the panel shown in Fig. 542, left. To save the current configuration, left-click into one of the empty fields on the left. The field will then show a small icon of the current image for later identification. To load a configuration, left-click into the field that shows the image of the desired configuration. To delete a configuration, right-click on the field with the configuration to be deleted. Configurations can also be stored into and retrieved from files, please use 'Export' and 'Import' to do so. Important: A configuration to be loaded must have the same timing parameters and number of time channels as the current data.

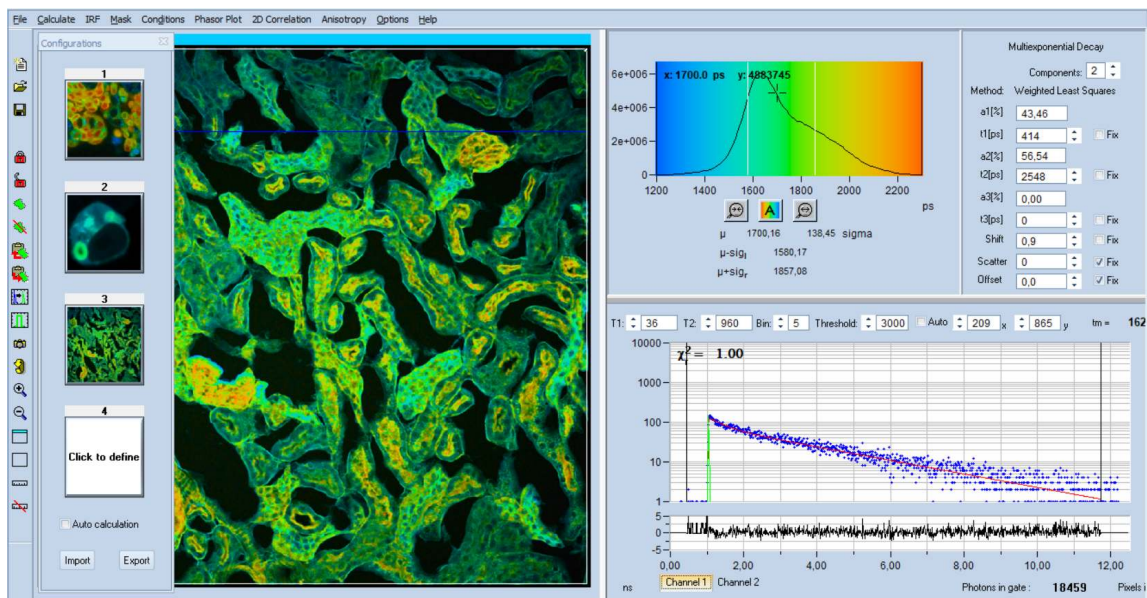


Fig. 542: Loading and saving Configuration data

Analysis of Special Data Types

Single-Curve Data

Data from Cuvette Experiments

Decay data recorded in traditional cuvette-based setups can be imported into SPCImage and analysed with the normal set of models and model options. There are two ways to import the data into SPCImage. Import via the normal import function of SPCImage is shown in Fig. 543.

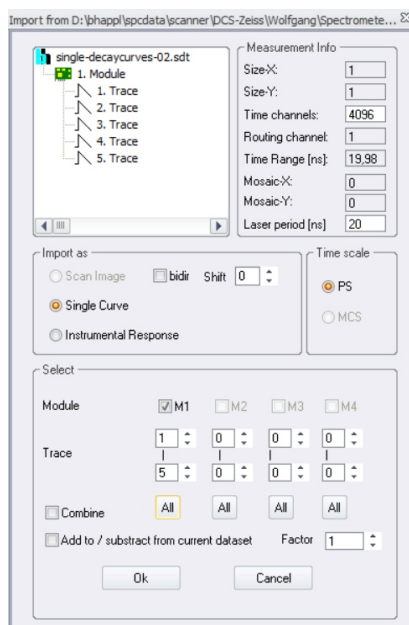


Fig. 543: Import of single-curve data via the import function of SPCImage

Select 'Single Curve', select the SPC module from which you want to import data, select a range of 'Pages' (see SPCM software) and click OK to import the decay curves.

Data can also be transferred to SPCImage by the 'Send Data' of SPCM. Click into 'Main', 'Send Data to SPCImage'. This opens a select panel in the SPCM curve window, see Fig. 544. It contains the numbers of pages (traces) which are active in the '2D Trace Parameters'. Select the curves you want to send, and click on 'OK'.

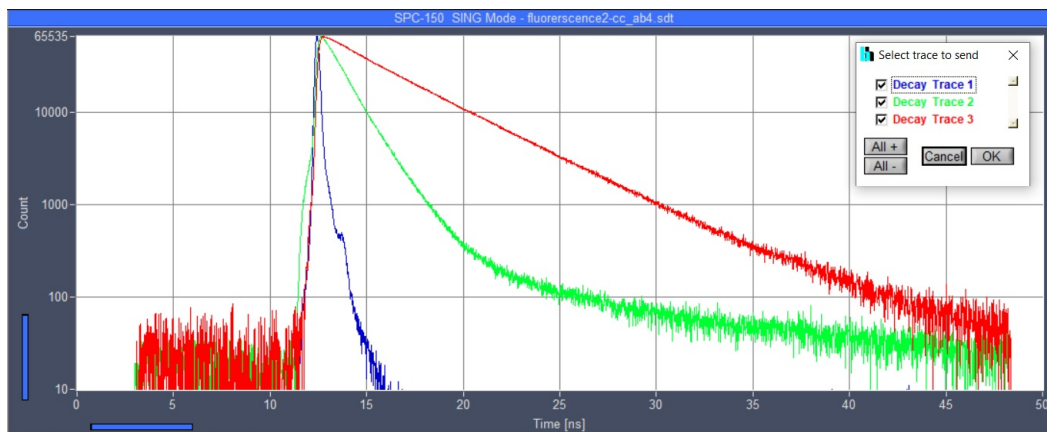


Fig. 544: Sending single curves from SPCM to SPCImage

The result of the data import is a decay curve window as shown in Fig. 545. With the 'Channel' tabs bottom left you can switch between the individual curves (pages in SPCM) which you have imported before. In the example there are three of them. If one of the curves contains an IRF which you want to use for the analysis, select the corresponding 'Channel'. In Fig. 545 it is in 'Channel 1', and 'Channel 1' has been selected.

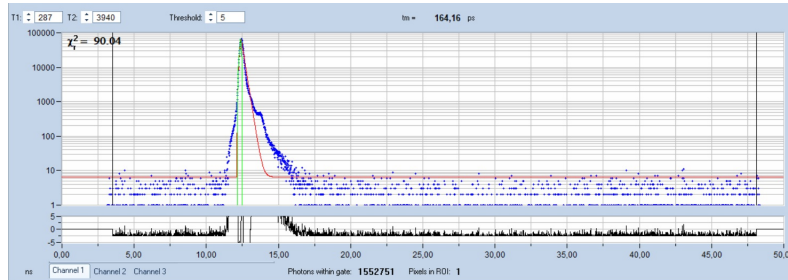


Fig. 545: Decay window after import of data. If there are several curves the desired one can be selected by the 'Channel' tabs. In the example shown Channel 1 is an IRF.

Enclose the signal peak with the cursors, see Fig. 546. Then declare it an IRF. Either click 'Copy from Decay Data' under 'IRF' or  (data to IRF) in vertical bar on the left.

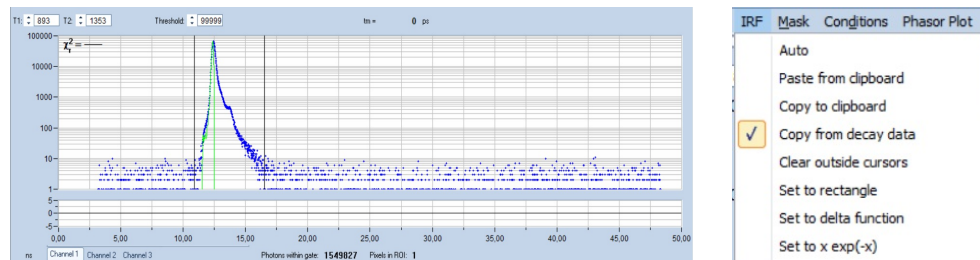


Fig. 546: Signal peak enclosed by cursors for IRF definition. Right: Decay the selected signal part an IRF.

The result is shown in Fig. 547, left. The green curve is the IRF. Use 'IRF', 'Copy to clipboard' to have the IRF available for the analysis of decay curves in other channels.

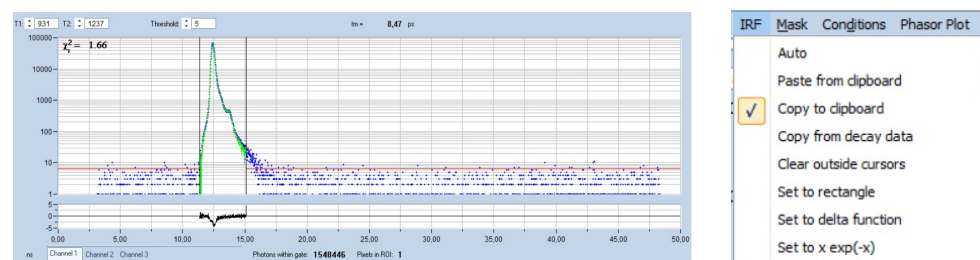


Fig. 547: The signal peak in Channel 1 has been declared an IRF. Right: 'Copy to clipboard' makes the IRF available for analysis of the decay curves in other 'Channels'.

Then click on a 'Channel' which contains a decay curve to be analysed. The curve is still shown with the 'Auto IRF', as shown in Fig. 548. To show it with the measured IRF click 'IRF', 'Paste from Clipboard'. The result is shown in Fig. 549.

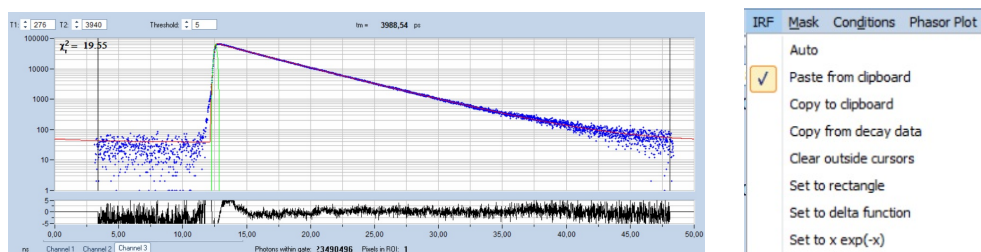


Fig. 548: 'Channel 3' has been selected by the tab bottom left. It contains a decay curve. It is still shown with the 'Auto' IRF.

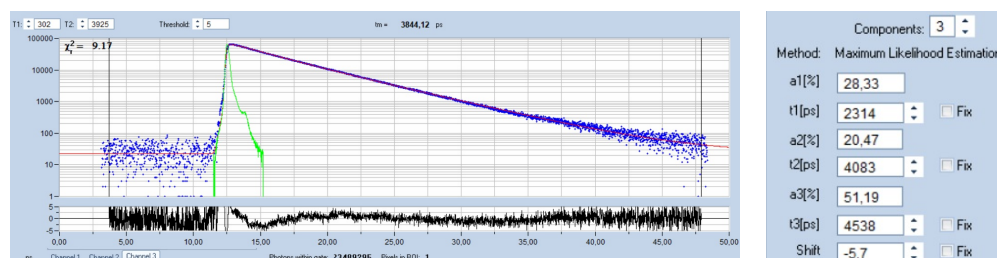


Fig. 549: 'Paste from clipboard' has been executed to analyse the data in channel 3 with the real IRF.

Single-Curve Data from Scanning Experiments

Single-curve data can also be obtained in a FLIM system. A dish with a solution of the dye to be investigated is placed on the microscope stage, and FLIM data of the solution are recorded. To analyse such data, import them into SPCImage by the normal import procedure for FLIM data. Click the  (lock) button to combine the decay data of the entire image area (or of an area selected by the image cursors) into a single decay curve. Select the desired decay model parameters and fit parameters, and choose or create an IRF. The fit process starts instantly (no need to start 'Calculate'), and the decay parameters are shown in the upper right of the SPCImage panel. A result is shown in Fig. 550.

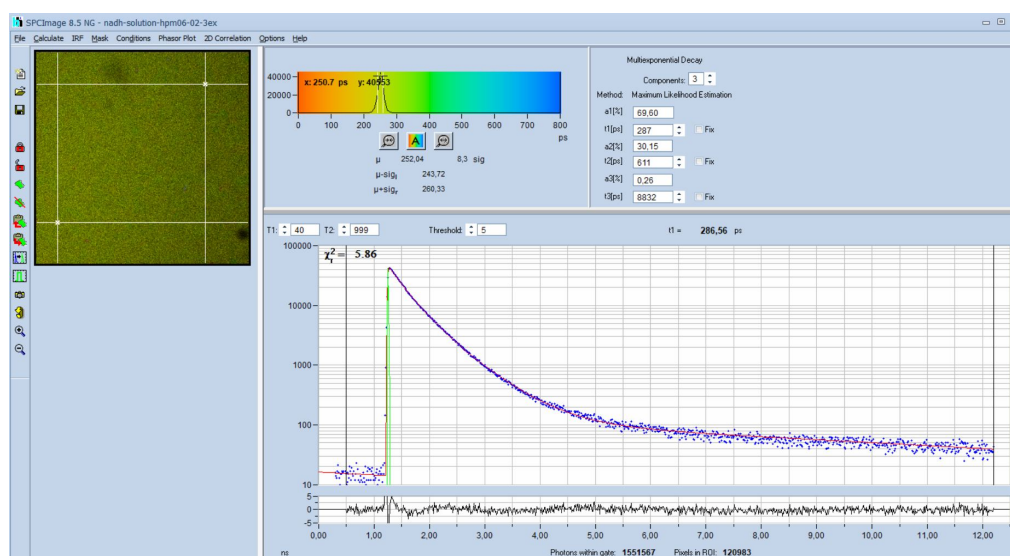


Fig. 550: Combination of decay data from an image area into a single curve, and data analysis with triple-exponential model. NADH solution, DCS-120 MP system with HPM-100-06 detector.

Pseudo-Global Analysis

SPCImage allows you to fix one or several of the component lifetimes. A typical example is the donor fluorescence in FRET experiments. Theoretically, the slow lifetime component comes from non-interacting donor molecules. In first approximation, it should therefore be constant throughout the image. Another example is autofluorescence FLIM. The variation the mean lifetime is essentially (but not entirely!) caused by variation in the amplitudes of the components.

To use fixed lifetimes of the components, first run a double- or triple-exponential analysis with all lifetimes defined as free fit parameters. Take a look at the lifetime histograms of the component lifetimes. Then fix the lifetimes to the most frequent values found in the histograms, and run a new analysis.

An example for autofluorescence data is shown in Fig. 551. Fig. 551, left, shows the result of an analysis with τ_1 , τ_2 , and τ_3 free. Fig. 551, right, shows the result of an analysis with τ_1 , τ_2 , and τ_3 fixed to the maxima of their distributions. The image of the mean lifetime (t_m) is the same. However, the analysis with fixed lifetimes delivers amplitudes at better signal-to-noise ratio.

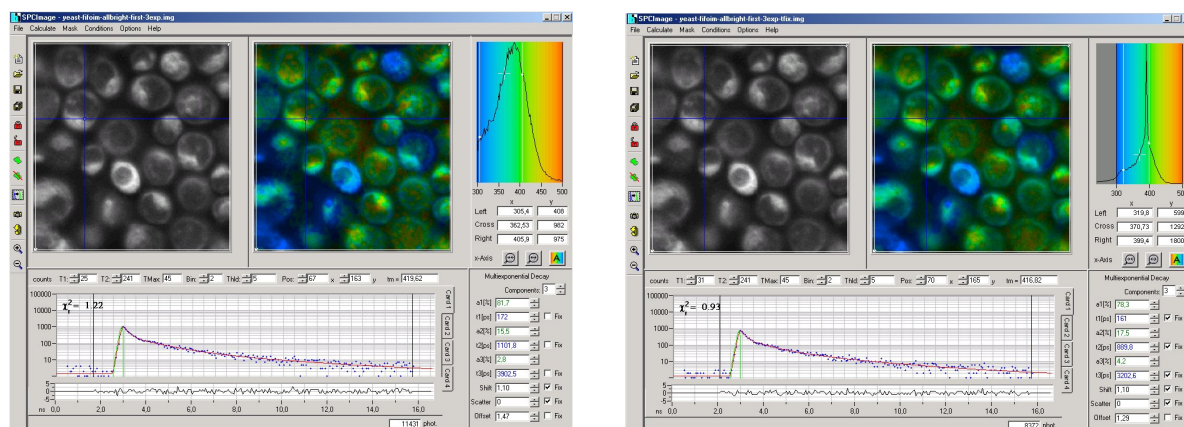


Fig. 551: Left: Analysis with free τ_1 , τ_2 , τ_3 . Right: Analysis with τ_1 , τ_2 , τ_3 fixed to the maxima of their distributions. The result is the same. However, the amplitudes are obtained at higher accuracy.

This does not mean that the systematic errors are similarly low. For all lifetime components there are usually subtle lifetime variations, induced by variation in the local environment or the refractive index [68, 591]. If a lifetime used as fixed parameter is not really constant the fit procedure compensates for the variation by large changes in the amplitude or in the lifetimes of other decay components. The result can be large systematic errors in these parameters.

Global Analysis

Unlike the 'Pseudo-Global' fit described above, a true Global Fit does not use a priori information on the values of any of the component lifetimes. No matter what the component lifetimes are, the global fit only assumes that the lifetimes of one or several decay components *do not vary over the pixels of the image*. That means, the global fit procedure runs iteration cycles each of which is a complete multiexponential fit of all pixels of the entire image. Each cycle uses a different combination of the global parameters until the best possible fit of the entire image is obtained.

Consequently, a global fit is extremely-computation intensive. Assuming that about 20 global iteration cycles are required to obtain the final result the calculation time is 20 times that of a 'normal fit'. In practice, a global fit can therefore only be performed on a GPU. Therefore,

please check whether your system has a GPU and whether SPCImage is using it. You see this from the progress bar during the calculation, see Fig. 552. It should read 'GPU Calculation', and a normal fit should be finished within a few seconds.

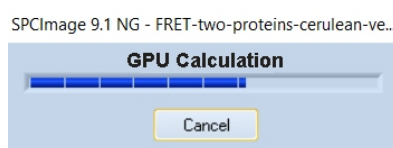


Fig. 552: Progress bar during the calculation of an image

Global analysis is a powerful technique to obtain accurate amplitude images from multi-exponential FLIM data with constant component lifetimes. Different than pseudo-global fitting it does not require that any of the component lifetimes be known. Applications are separation of fluorophores in samples containing mixtures of fluorophores, FLIM-FRET data, and, especially, metabolic-FLIM data. In FRET-FLIM data the lifetime of the non-interacting donor is constant. In metabolic FLIM data the component lifetimes of bound and unbound NADH and FAD are constant, as are the lifetimes of NADPH and FMN. The observed changes in the net lifetime are caused by changes in the amplitudes of the decay components.

For FAD the situation is especially complicated. The data not only contain the decay components of bound and free FAD but also a noticeable contribution from FMN. Therefore triple-exponential decay analysis has to be used. An example is shown in Fig. 553 and Fig. 554. Both images show the amplitudes, a_1 , a_2 , and a_3 of bound FAD, free FAD, and FMN. Fig. 553 shows a 'normal' triple-exponential fit of the data. It is evident that the signal-to-noise ratio is not sufficient to derive the metabolic state from the data. Fig. 554 shows the result of a global fit with the component-lifetimes t_1 (bound FAD), t_2 (free FAD), and t_3 (FMN) defined as global parameters. The improvement in signal-to-noise ratio is amazing. Not only are the amounts of bound and free FAD reproduced clearly, also the amount of FMN is obtained at high accuracy.

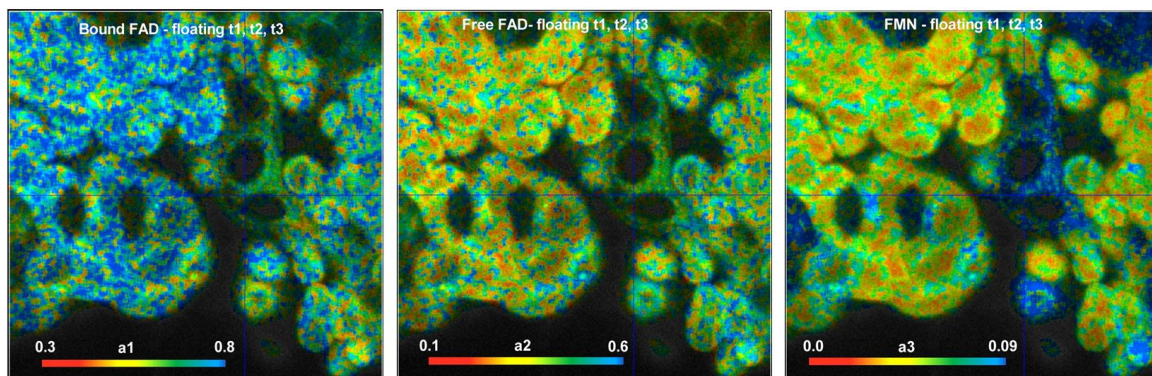


Fig. 553: Human epithelial bladder cells, amplitudes a_1 , a_2 , and a_3 . Standard MLE fit

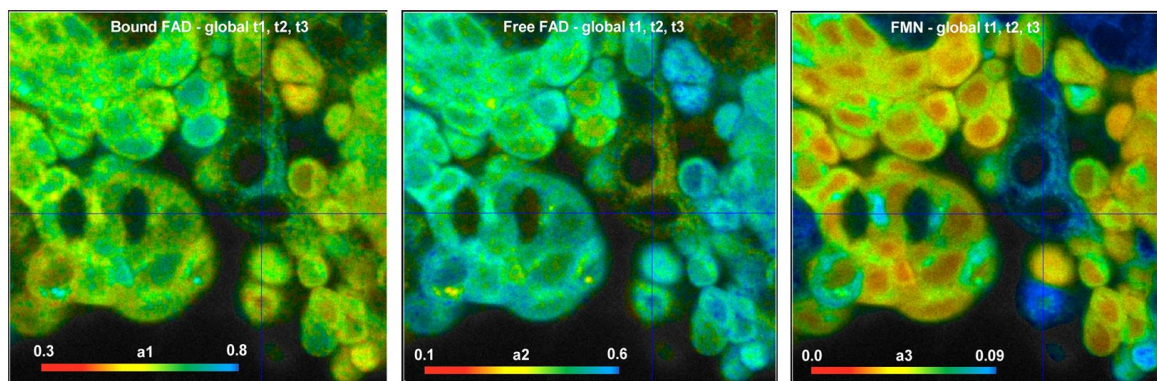


Fig. 554: Same cells as above. Global fit with global component lifetimes t_1 , t_2 , t_3 .

Multi-Wavelength Lifetime Images

Traditional Multi-wavelength FLIM data from SPCImage represent 16 'channels', each of which holds a complete lifetime image for a particular wavelength interval. The data are imported into SPCImage NG as shown in Fig. 555, left. After importing, individual channels can be accessed via 16 individual tabs at the bottom of the decay window, see Fig. 555, bottom right. Individual channels can be selected and analysed individually. However, it is more comfortable to use the 'Analyse all' function as shown in Fig. 555. The function runs the decay fit procedure in all wavelength intervals of the multi-wavelength FLIM data set.

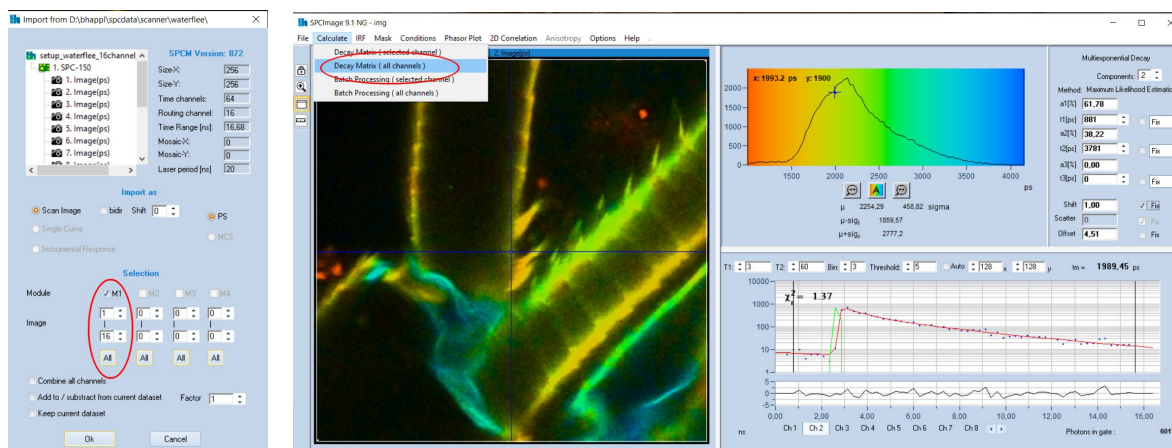


Fig. 555: Left: Importing multi-wavelength data. Right: The data are analysed by 'Calculate', 'Decay Matrix (all channels)'. Individual wavelength channels can be selected via the tabs below the decay-curve window.

The wavelength channels have separate fit parameters. Before starting the analysis, it is recommended to switch through all wavelength channels and set appropriate fit parameters and fit conditions. When this is done, click on 'Calculate', 'Analyse all'.

Recent SPCM versions have a 'Mosaic Imaging' function which can be used to record multi-wavelength FLIM data. Mosaic Imaging records the wavelength channels into individual mosaic elements of a large data array. When such data are imported via the import function of SPCImage the wavelength channels are treated as routing channels, and show up as individual images, as shown in Fig. 556.

However, when the data are sent to SPCImage via the 'Send to SPCImage' function of SPCM the entire array set is sent as a single, large FLIM image, see Fig. 556. Of course, the size of mosaic data is enormous, and the processing effort is high. The analysis can therefore be re-

sonably performed on systems with a GPU only. The result of the analysis is shown in Fig. 556.

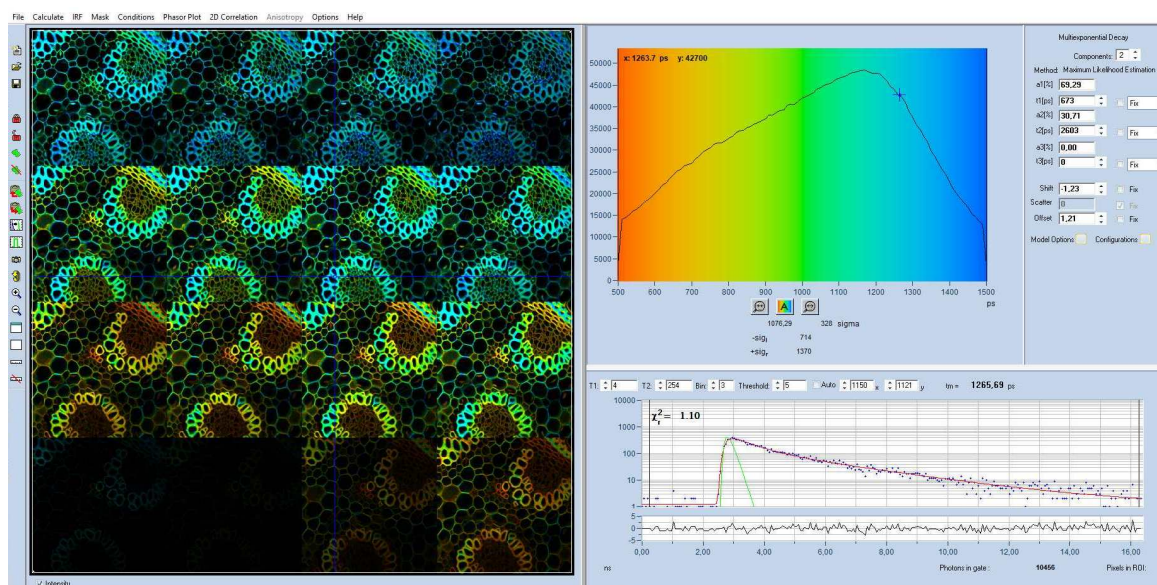


Fig. 556: Multi-wavelength FLIM mosaic, sent to SPCImage by the 'Send to SPCImage' function of SPCM. The data are treated as a single, large lifetime image. Amplitude-weighted lifetime of double-exponential decay.

Analysing the entire multi-wavelength array at once has two advantages over analysis of the individual images. First, the procedure is more convenient to the user and, second, the array can be analysed with global analysis. This is helpful if there are decay components the lifetimes of which do not change with the wavelength. However, there is also caveat. Multichannel detectors usually display a systematic wobble in the transit time over the channels. This can show up as a systematic lifetime variation over the elements of the mosaic. The analysis should therefore be performed with free 'Shift', see Fig. 556 upper right. In the Model Parameters, please turn off 'Fix Shift before calculating Image'.

Spatial and Temporal Mosaic FLIM Data

Mosaic FLIM data contain an array of images in a single FLIM data file. The data can represent a spatial mosaic of images, a Z stack of images, time-series images, or multi-wavelength data. Spatial or temporal Mosaic FLIM data have the same data structure as a single, large FLIM image. The data can either be sent to SPCImage via the 'Send Data to SPCImage' or be loaded into SPCImage via the 'Import' function. Once imported, the data are analysed in a single analysis run. An example is shown in Fig. 557. The mosaic shows 64 images of a leg of a live water flea, recorded with an acquisition time of 0.5 seconds each. Each element of the Mosaic has 252 x 256 pixels, hence the entire mosaic has 2048 x 2048 pixels. The array was analysed by the MLE algorithm, with a double-exponential model.

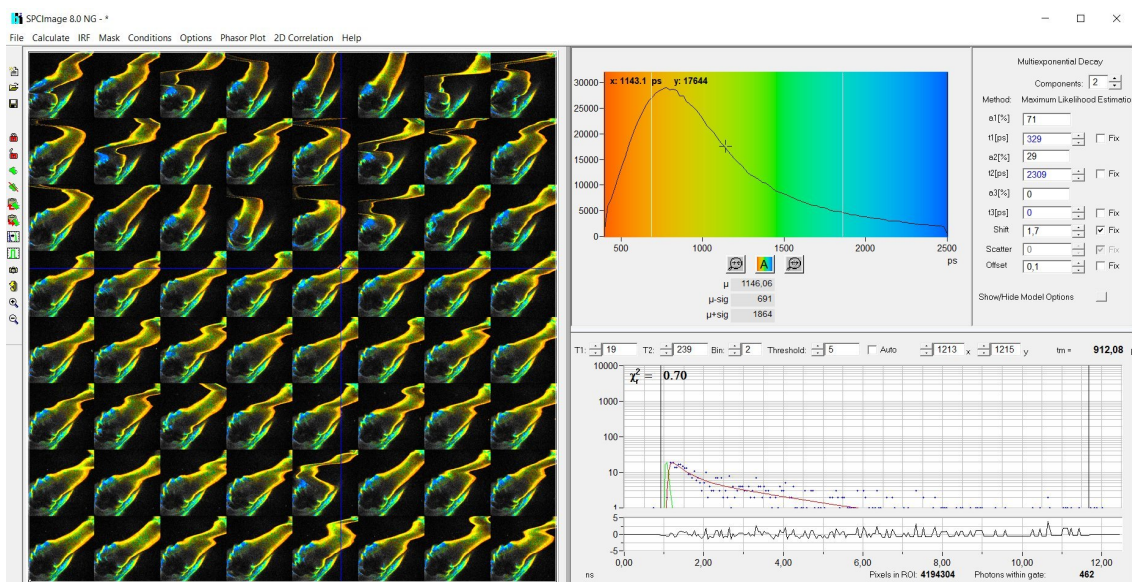


Fig. 557: Mosaic FLIM of the leg of a water flea, 64 images of 0.5 s acquisition time.

When the data are analysed the traditional way the large image size of a mosaic array may result in very long calculation times. We therefore recommend to run the calculation on a GPU and use the MLE algorithm. The calculation time is then reduced to about 10 seconds.

If there is no GPU in the system we recommend to try an analysis of a small part of the Mosaic image first (e.g. of a single mosaic element). Check the results and make sure that the correct model function and the correct model parameters are selected. When you are satisfied with the result start the analysis of the entire mosaic.

INT FLIM Data

INT FLIM ('Intensity-FLIM') is used to obtain a linear intensity scale of FLIM at high count rates. The data are a combination of normal TCSPC FLIM data and pixel intensity data from a fast counter. Images are created by using the fluorescence-decay curves in the pixels from the TCSPC channel and the intensities from the counter channel. Please see Lifetime-Intensity FLIM, page 127 for technical details.

INT FLIM data are transferred from SPCM to SPCImage by the 'Send Data' command. SPCM sends both the FLIM data and the intensity data, and SPCImage loads the intensity data as 'Additional Intensity Data', see Intensity Parameters. The corresponding function is activated automatically, see Fig. 558. Once loaded into SPCImage, INT FLIM data are processed like standard FLIM data, see Fig. 559.

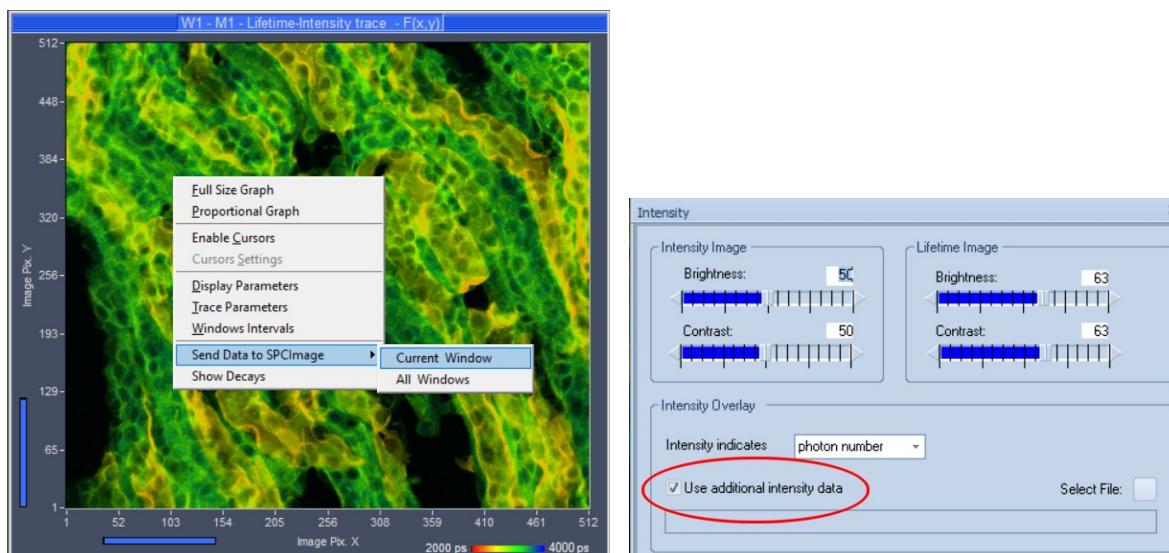


Fig. 558: Transfer of INT FLIM data to SPCImage. Left: Send Data command in SPCM. Right: SPCImage automatically uses the fast-counter data as 'additional intensity data'.

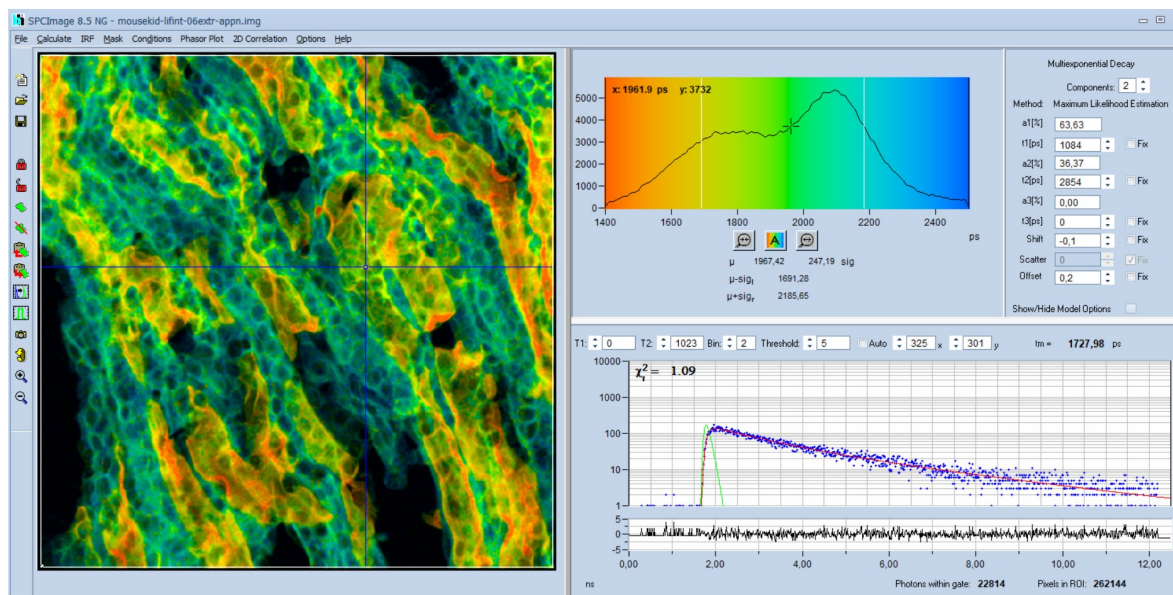


Fig. 559: SPCImage with INT-FLIM data

Phosphorescence Lifetime Images

Analysis of phosphorescence lifetime images, in principle, works the same way as FLIM analysis. A few differences, do, however, exist.

Sending PLIM Data to SPCImage

The SPCM main panel of a typical FLIM/PLIM setup is shown in Fig. 560. From left to right, there is a FLIM window, a PLIM window containing only photons in the ‘laser-off’ periods, and a PLIM window containing the photons both from the laser-on and the laser-off phases, see waveforms above the display windows.

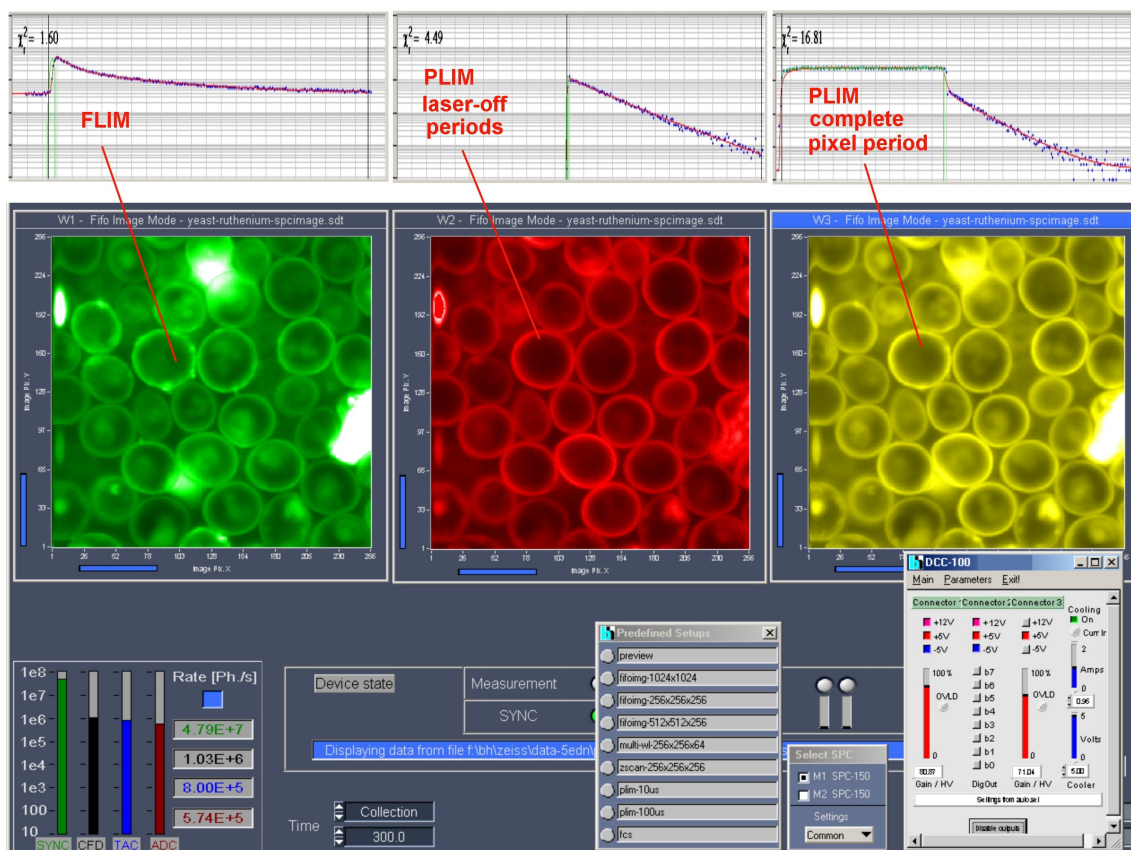


Fig. 560: Main panel of SPCM, configured to show intensity images of FLIM data, of pure PLIM data in the laser-off intervals, and of FLIM and PLIM data in the complete laser-on-off (pixel time) period. Above: Waveforms seen after sending the data to SPCImage

Because the FLIM and the PLIM data have totally different time scales they cannot be simultaneously loaded into SPCImage. To use the ‘Send Data’ function of SPCM, select ‘Selected Window’ in the SPCM Application Options. For sending data, click on the SPCM window you want to analyse, and then transfer it to SPCImage by the ‘Send Data’ command. Note that you can send data from a FLIM window or from a PLIM window, but not both together. The waveforms you see after sending the data of the windows to SPCImage are shown above the SPCM panel in Fig. 560.

IRF of PLIM

For PLIM data acquired from samples with pure phosphorescence usually the automatic IRF generated by SPCImage can be used, see page 377. An example can be seen in the PLIM image of an Autumit crystal shown in Fig. 561.

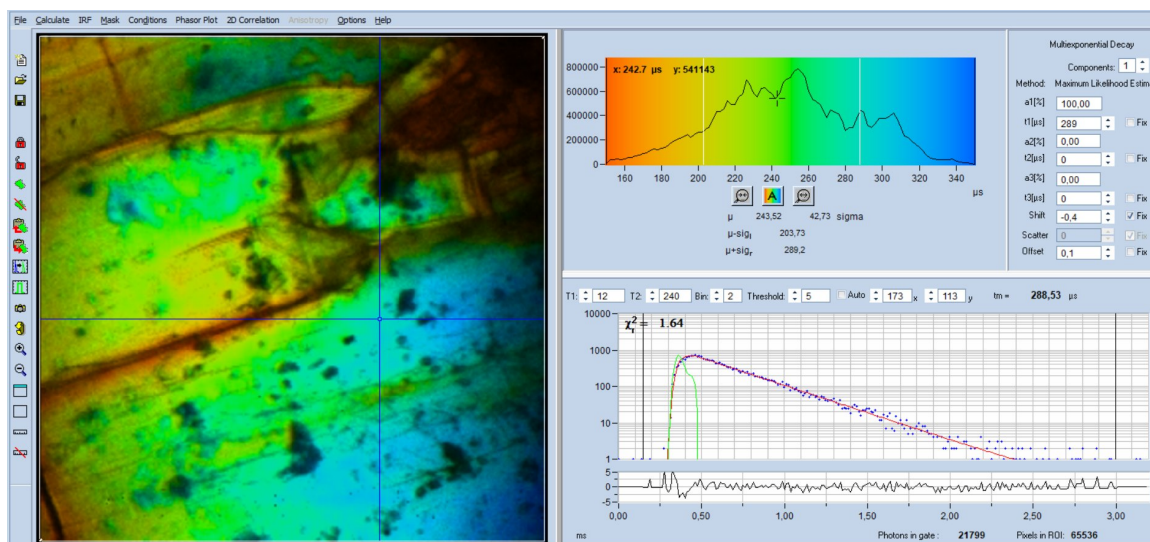


Fig. 561: Analysis of PLIM data from an Autumit crystal. Automatic IRF, single-exponential decay model.

If a sample emits also fluorescence, and FLIM is to be measured simultaneously with PLIM the situation can be different. In that case a much longer laser-on phase (extending over a substantial part of the pixel) is used. The IRF of the phosphorescence signal is then no longer a short pulse, and it may not correctly be reproduced by the automatic IRF generation.

There are several ways to obtain an IRF in these cases. One of them is to use the fluorescence pulse present in the data. Phosphorescence is an emission from the triplet state. The triplet state is populated by intersystem crossing from the first singlet state, S1. This is the state from where fluorescence is emitted. Strictly seen, the IRF of fluorescence is therefore the fluorescence pulse. The fluorescence pulse during the laser-on phases can usually be identified in PLIM data. The IRF is then generated by placing the cursors at the beginning and the end of the fluorescence and clicking the ‘curve to IRF’ button. An example is shown in Fig. 562.

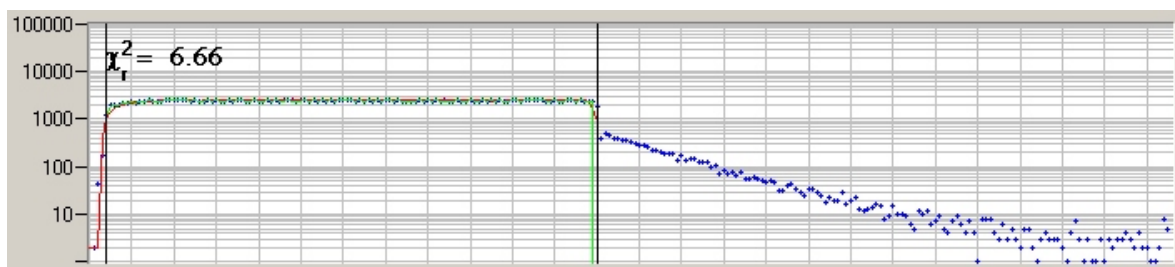


Fig. 562: IRF from the fluorescence pulse during the laser-on phase. The fluorescence pulse has been selected by the cursors in the curve window, and declared an IRF via the ‘Curve to IRF’ button. The green curve is the IRF.

The procedure shown above works well if the laser-on period is dominated by fluorescence. However, if the dominating signals component in the laser-on phase is phosphorescence (this can happen for phosphorescence labels based on rare-earth chelates) the waveform does not represent the effective IRF. In that case, an IRF can be recorded from a sample showing only fluorescence.

A third way is to set the cursors in the curve window to the beginning and the end of the ‘Laser-on’ phase, and define a rectangular IRF by the  (Rectangular IRF) button. Although the generated IRF ignores possible ringing or distortion in the laser-on modulation waveform it usually works reasonably well. A fourth way to deal with the phosphorescence IRF is to ignore it altogether and fit only the phosphorescence data in the laser-off periods, see paragraph below.

Fit Procedure

PLIM data from samples that emit predominately phosphorescence are analysed in analogy to FLIM. The only difference is the time scale, which may require to increase the maximum lifetime in the model parameter panel (see page 342). An example has been shown in Fig. 561. The automatic IRF was used, and the decay profile was fitted by a single-exponential model.

PLIM data containing also fluorescence pose a bigger challenge to data analysis. To fit both the fluorescence and the phosphorescence the waveforms had to be fitted with a model that contains a component with the shape of the IRF for the fluorescence and one or several decay components for the phosphorescence. A model that takes these components into account is, in principle, available in SPCImage: It is a multi-exponential decay model with 'Scatter'. The problem of this model is that it is (because of the 'scatter') extremely sensitive to the IRF shape. The IRF shape is, however, not accurately known. A better way to fit the data is therefore to restrict the fit to the laser-off phase where the signal does not contain fluorescence.

An example is shown in Fig. 563. The data from the complete pixel period were sent to SPCImage. The IRF was taken from the fluorescence pulse during the laser-on phase. The phosphorescence data were fitted only in the decaying part of the curve, selected by the cursors in curve window. The intensity options for the lifetime image were set to 'Gated Intensity', see 'Intensity Parameters', page 324. The intensity data are therefore only from the interval between the cursors, i.e. from the phosphorescence.

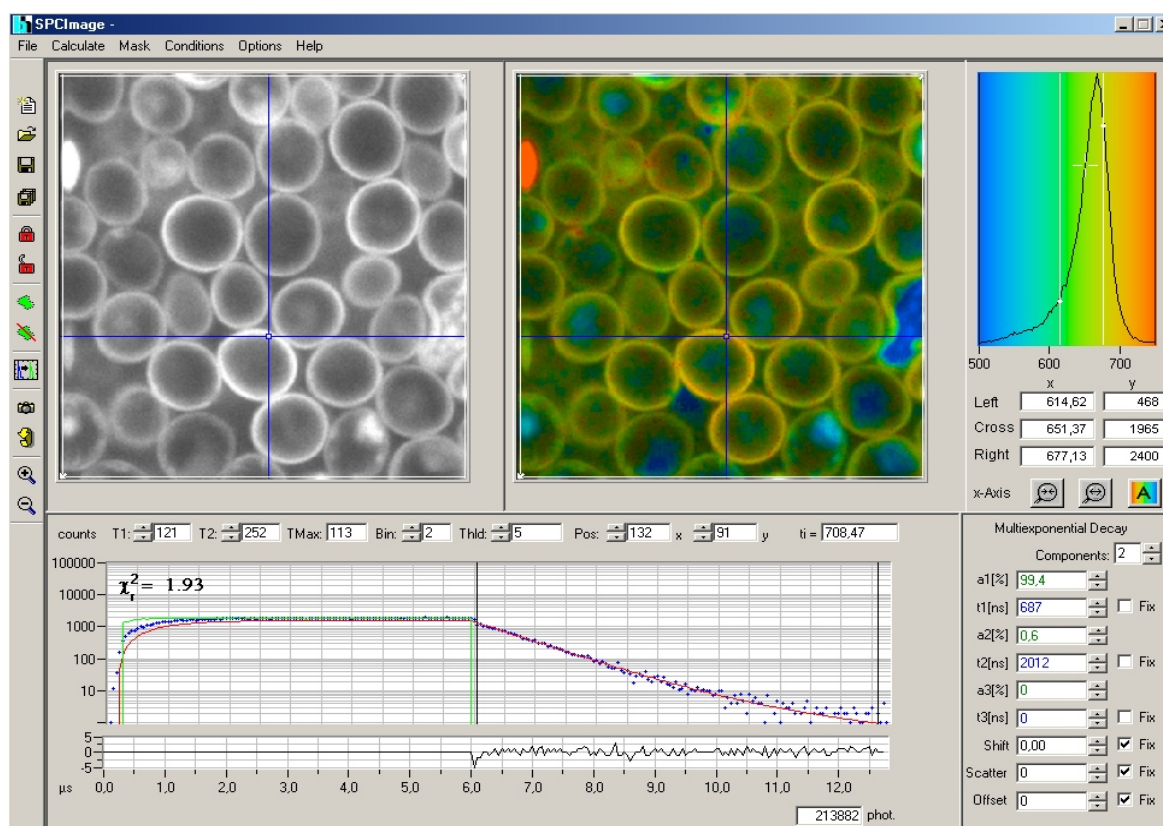


Fig. 563: Analysis of PLIM data from yeast cells stained with ruthenium dye

FLIM Data Acquired Simultaneously with PLIM

FLIM data acquired simultaneously with PLIM are analysed as normal FLIM data. Send the FLIM data from the FLIM windows of SPCM to SPCImage by the 'Send Data' function. In

SPCImage, select the desired model function and model parameters, and start the calculation with 'Calculate Decay Matrix'. It can happen that the FLIM data contain a large counting background. The background can originate from phosphorescence bleedthrough in the fluorescence channel. If fluorescence and phosphorescence were recorded in different TCSPC channels with different detectors the background can often be minimised by using the right filters. However, if the spectral overlap of the signals is large, or if fluorescence and phosphorescence were recorded in the same TCSPC channel some background can be unavoidable. In that case, leave the 'Offset' in the fit conditions floating and use MLE analysis. MLE is less susceptible to high background than WLS. An example is shown in Fig. 564.

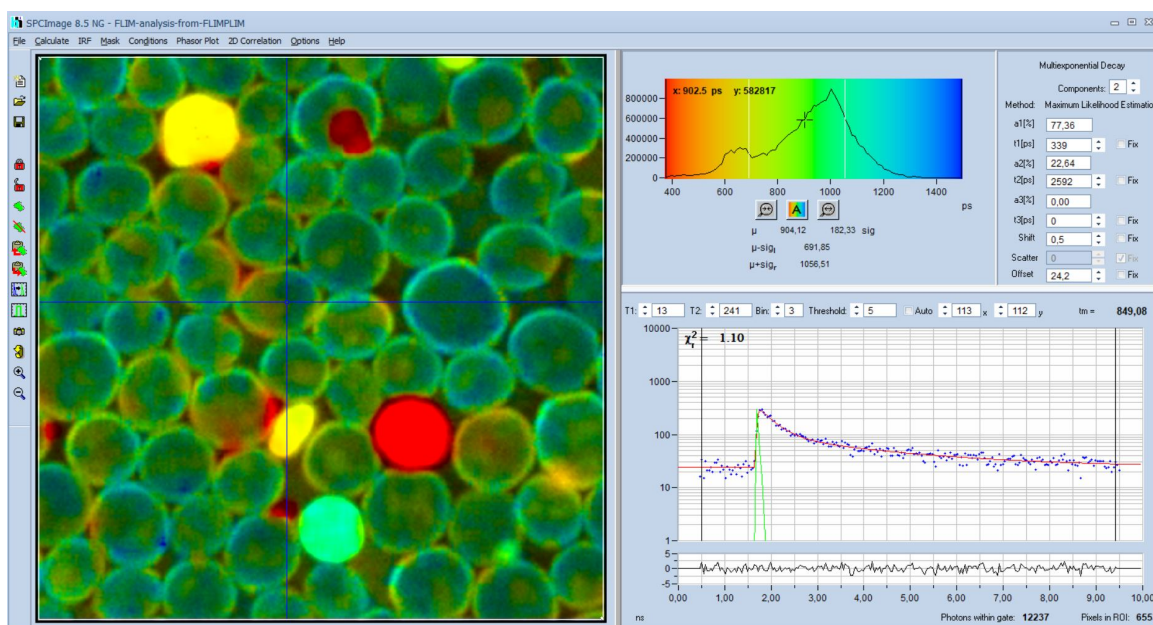


Fig. 564: FLIM data from simultaneous FLIM/PLIM experiment

Batch Processing

The Batch Processing function allows the user to process a large number of similar FLIM data files automatically. The function is reached via 'Calculate' and 'Batch Processing'. Before you start the function import one of the sdt files you want to analyse and calculate a lifetime image from it. Make sure that you have set all model parameters and colour parameters correctly. When you are satisfied by the result proceed by clicking on 'Batch Processing'. This opens a file selection window as shown in Fig. 565, upper right. Select the files to be processed.

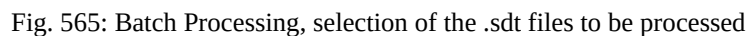


Fig. 566: Batch Processing in progress. Subsequent files are loaded, analysed, and saved.

The analysed data are saved as shown in Fig. 567. The data file extension is ‘.img’. The img files contain not only the decay parameters of the individual pixels but also the raw data. Therefore, an img file can be loaded, and the data can be re-analysed with a different model or with different fit parameters.

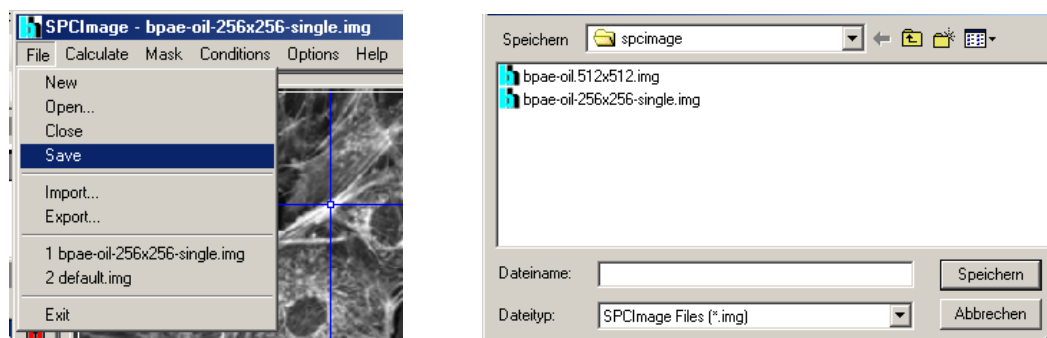


Fig. 567: Saving the data analysed by SPCImage

Export of Data

FLIM data can be exported into other data formats via the 'Export' panel, see Fig. 568. The options under 'Matrix' produce ASCII files of the decay parameters in the pixels of the image. 'Traces' generates ASCII files of the curves displayed in the curve window. 'Image' produces BMP or TIFF files of the intensity or lifetime image. SPCImage versions later than August 2012 have a batch export function. It is used to export data of a series of .img files that have been generated by the Batch Processing function (see page 309). The file selection panel is shown in Fig. 568, right.

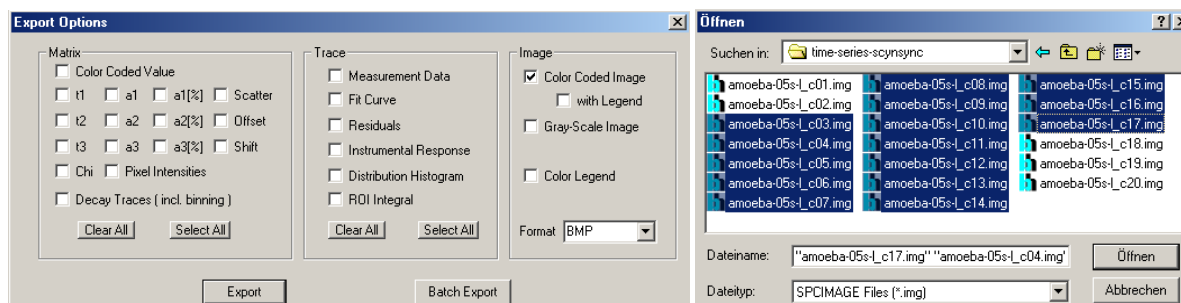


Fig. 568: Left: Export Options of SPCImage. Right: File selection for Batch Export.

Special Commands

Frequently used commands are accessible in a vertical task bar left of the image window. Turn on 'Extended Icon Set' in the 'Preferences' to see all available commands.



Binning of all pixels within a rectangular or polygonal ROI into a single decay curve.



Undo binning of pixels within the ROI.



Defines a polygonal ROI. Decay parameters are calculated only within this ROI.



Discards a previously defined polygonal ROI.



Declares the curve displayed in the decay window an IRF. The parts of the curve outside the cursor range are cut off.



Defines a rectangular IRF. The width and the temporal location are defined by the cursors in the curve window.



The current fit conditions are stored. These include a previously determined IRF, the time-range for the fitting procedure, and the region of interest in the image.



Loads fit the conditions which were stored before.



Zooms into the cursor range of the displayed image or zooms out of the zoomed part of the image.

Supporting Information

Spatial Binning

When an image is recorded by a scanning microscope the point-spread function of the microscope lens is usually ‘oversampled’ to obtain best spatial resolution. As a rule of thumb, the diameter of the central part of the Airy disc should be sampled by 5×5 pixels, see Fig. 569, left. In practice even higher oversampling factors often occur unintentionally. Under these conditions lifetime data should be calculated from several binned pixels. When the binning function of SPCImage is used the lifetime images are built up from the unbinned intensity pixels and the binned lifetime pixels. This yields substantially improved lifetime accuracy without noticeable loss in spatial resolution [91]. Sampling artefacts are largely avoided by overlapping binning, see Fig. 569, right.

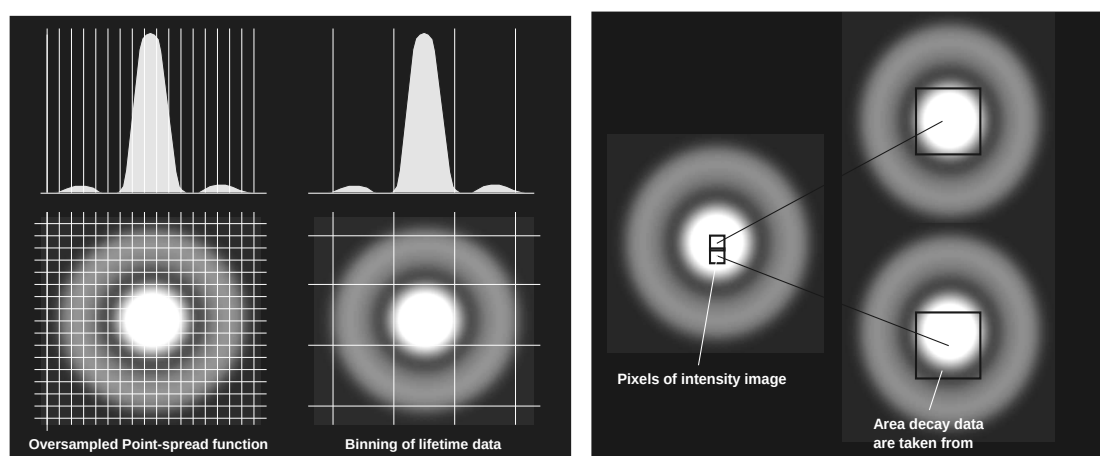


Fig. 569: Left: Oversampling of the Airy disc in the intensity image and binned pixels for lifetime calculation. Right: Overlapping binning of pixels for lifetime calculation.

The binning is controlled by the ‘Bin’ parameter above the decay curve window. The function of the parameter is shown in Fig. 570. ‘Bin’ defines the number of pixels around the current pixel position. Please note that the number of pixels of the lifetime image is not reduced. Only the lifetimes are calculated from the combined pixels, the intensities remain unbinned.

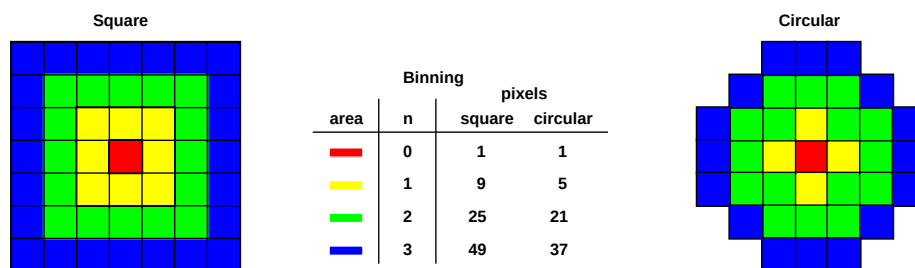


Fig. 570: Function of the binning parameter, n. Binning 'Square' (left) and 'Circular' (right).

Fig. 571 shows lifetime images obtained from 256×256 pixel raw data. The binning parameter is 0 (left) and 2 (right). The upper row was calculated by the WLS fit, the lower row by MLE. It can be seen from these images that the binning causes no loss in image definition and negligible loss in lifetime detail. However, the binning considerably reduces the noise in the lifetime data.

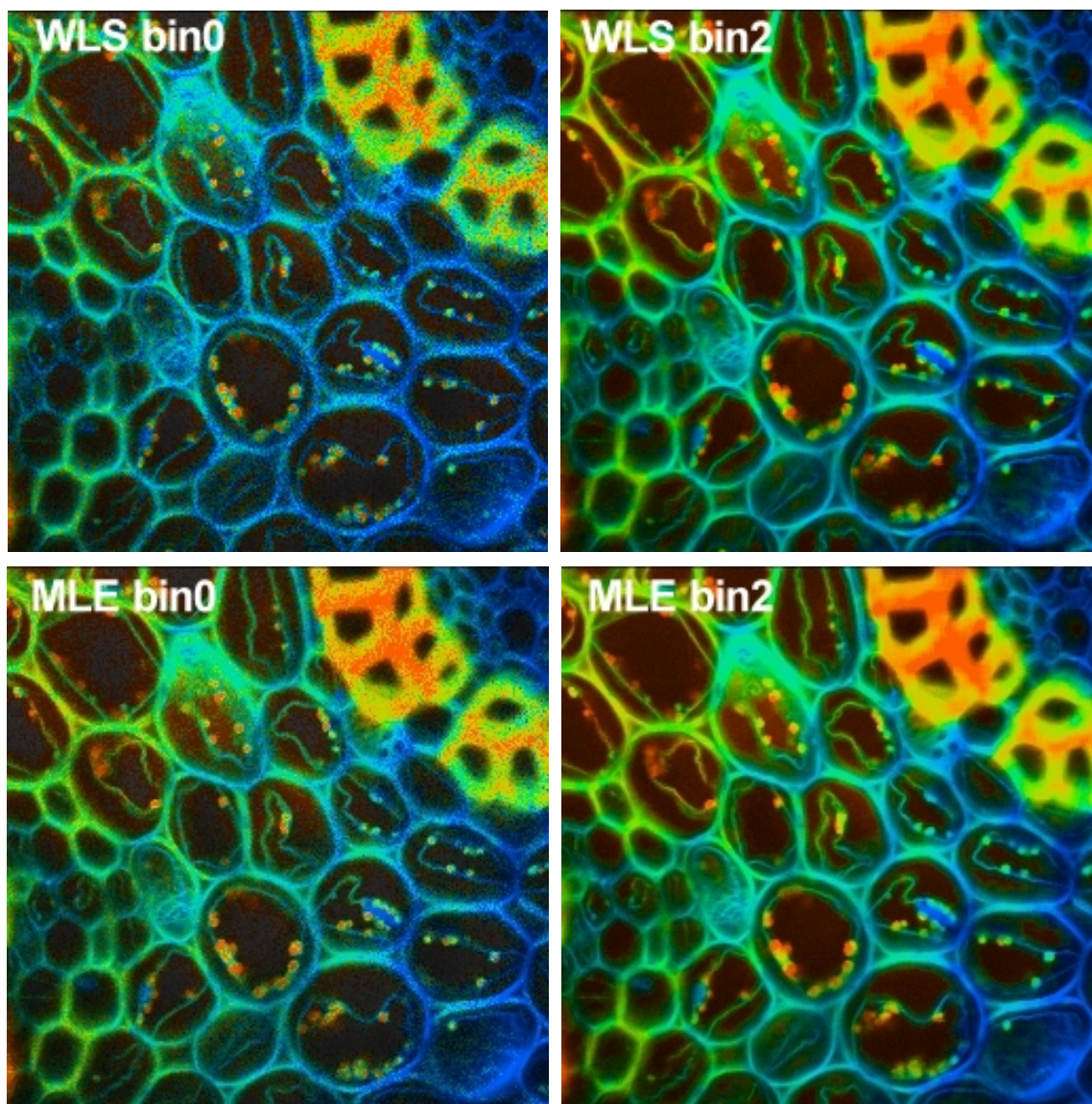


Fig. 571: Lifetime images of a convallaria sample, 256 x 256 pixels, 256 time channels. Left: Binning parameter 0. Right: Binning parameter 2. Upper row: WLS fit. Lower row: MLE fit. Double-exponential fit, amplitude-weighted lifetime, t_m . Lifetime range from 200 ps (blue) to 600 ps (red)

A conclusion from the figure above is that FLIM should *always* be recorded at sufficiently high pixel number to provide the desired spatial resolution. The resulting decrease in the number of photons per pixel can later be compensated by the binning function of SPCImage [91]. Please see Fig. 496, page 318.

Of course, an image with a higher pixel number occupies more data space in the computer and on the hard disc. But this is rarely a problem for state-of-the-art computers and data storage devices. Also the data-processing time for the larger number of pixels is no longer a problem. With a GPU, the processing time is rarely more than a few seconds even for the largest images.

Temporal Binning

SPCImage NG has no function for binning of TCSPC time channels. Temporal binning leads to sampling artefacts, such as uncertainty in the position of the IRF and the rising edge of the fluorescence. With the advanced fit algorithms used in SPCImage NG temporal binning is detrimental to the fit accuracy. Please see [91].

The Convolution Integral

In a real FLIM system the fluorescence is excited by laser pulses of non-zero width, and detected by a detector that has a temporal response of non-zero width. The effects on the temporal shape of the recorded signal are shown graphically in Fig. 572. The laser pulse can be thought to be broken down into a sequence of (infinitely) narrow pulses of different amplitude (Fig. 572, left). Each of these sub-pulses produces a fluorescence decay of an amplitude proportional to the amplitude of the sub-pulse, and starting at the time of the sub-pulse. The sum of all these decay functions is the real optical waveform of the fluorescence signal, see bottom of Fig. 572, left.

The real fluorescence signal is measured by a detector the temporal response of which has non-zero width, see Fig. 572, middle. Again, the detector response can be thought to consist of a sequence of infinitely short pulses. Also here, the measured waveform is the sum of shifted signal components of different amplitude.

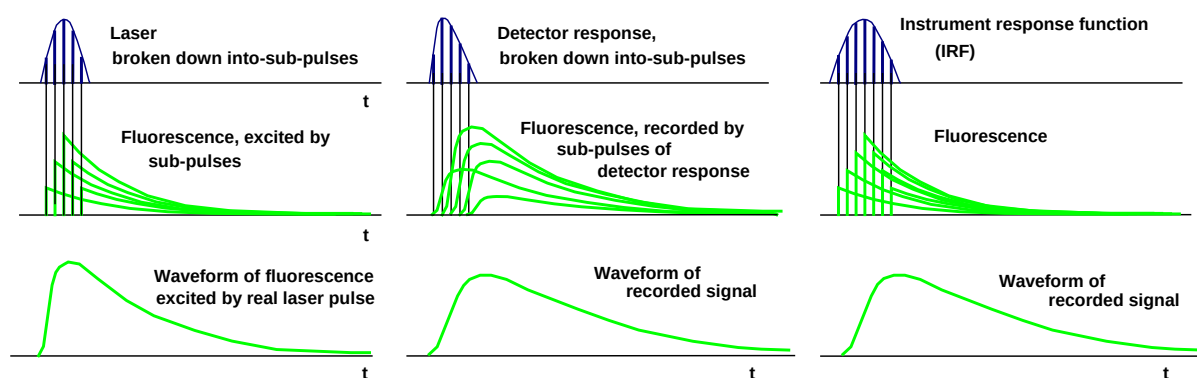


Fig. 572: Left: Convolution of the laser pulse with the fluorescence decay. Middle: Convolution of the real fluorescence waveform with the detector response. Right: Laser pulse and detector response combined into IRF pulse, convolution of fluorescence decay with IRF.

The transformation of the signal waveforms shown above is called ‘convolution’. In a linear system, the convolution of signal waveforms is a commutative operation. The laser pulse shape and the detector response can therefore be combined in a single ‘instrument response function’, or IRF, see Fig. 572, right. The IRF is the convolution of the laser pulse shape with the detector response, or the pulse shape the system would record if it directly detected the laser. The convolution of the fluorescence decay function with this IRF delivers the same result as the two subsequent convolution steps shown in Fig. 572, left and middle.

Mathematically, the convolution of the fluorescence decay with the IRF can be expressed by the convolution integral

$$f_m(t) = \int_{\tau=0}^t f(\tau) IRF(t-\tau) d\tau,$$

with $f_m(t)$ = measured fluorescence function, $f(t)$ = true fluorescence decay function.

The convolution integral cannot be reversed, i.e. there is no analytical expression of $f(t)$ for a given $f_m(t)$ and $IRF(t)$. There is also another implication: The measured data contain noise from the statistics of the photons, i.e. $f_m(t)$ itself is not accurately known. Any attempt to directly *calculate* $f(t)$ from the recorded data is therefore in vain. The standard approach to solve the de-convolution problem is to use a fit procedure: A model function of the fluorescence decay function is defined, the convolution integral of the model function and the IRF is calculated, and the result is compared with the measured data. Then the parameters of the model

function are varied until the best fit with the measured data is obtained [427]. This operation is repeated for all pixels of the image.

Decay Models

Basic Multi-Exponential Decay Model

The basic model functions used in SPCImage are sums of exponential terms:

Single-exponential model: $f(t) = e^{-t/\tau}$

Double-exponential model: $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$ with $a_1 + a_2 = 1$

Triple-exponential model: $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$ with $a_1 + a_2 + a_3 = 1$

The models are characterised by the lifetimes of the exponential components, τ , and the amplitudes of the exponential components, a . In principle, models with any number of exponential components can be defined. However, higher-order models become so similar in curve shape that the amplitudes and lifetimes of the components cannot be obtained at any reasonable certainty. Therefore, FLIM analysis does not use model functions with more than three components.

To account for possible detector background or daylight pickup the models used in SPCImage includes an Offset parameter in the models. The composition of the three basic model functions is illustrated in Fig. 573.

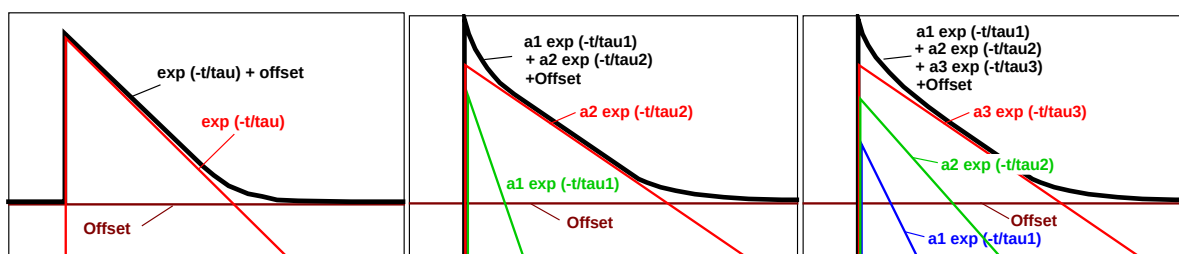


Fig. 573: Single, double, and triple-exponential decay models

The basic decay models can be combined with a number of options, see below.

Incomplete-Decay Model

The incomplete-decay model includes the fluorescence remaining from the previous laser pulses in the model function, see Fig. 574.

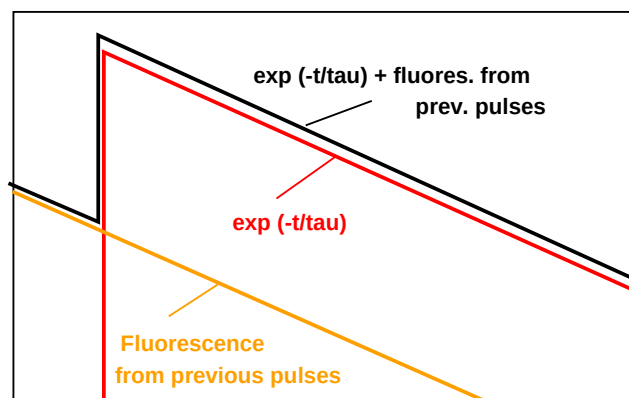


Fig. 574: Incomplete decay model. Red: Decay function. Orange: Residual fluorescence from previous laser pulses. Black: Sum of decay function and fluorescence from previous laser pulses. Shown for single-exponential decay. Similar models exist for double- and triple-exponential decay functions.

A comparison of the standard decay model and the incomplete decay model is given in Fig. 575. A single-exponential decay was recorded at 80 MHz repetition rate. The fluorescence lifetime was 4 ns. Consequently, there is a noticeable contribution from fluorescence excited by the previous laser pulses. It can be seen left of the rising edge of the fluorescence pulse. The standard decay model interprets this signal as an offset, see Fig. 575, top. As a result, an imperfect fit of the fluorescence is obtained. The fluorescence lifetime is determined too short because a fraction of the fluorescence photons in the tail of the decay is interpreted as background signal.

The Incomplete-Decay model interprets the signal correctly. It obtains a near-perfect fit of the decay curve, and delivers the correct lifetime, see Fig. 575, bottom. The incomplete model also has advantages when a multi-exponential decay has slow lifetime components. According to our own experience, it not only delivers these components with their correct amplitudes and lifetimes but also delivers a more pronounced χ^2 minimum. This is especially the case if the part left of the rising edge of the fluorescence pulses is included in the fit.

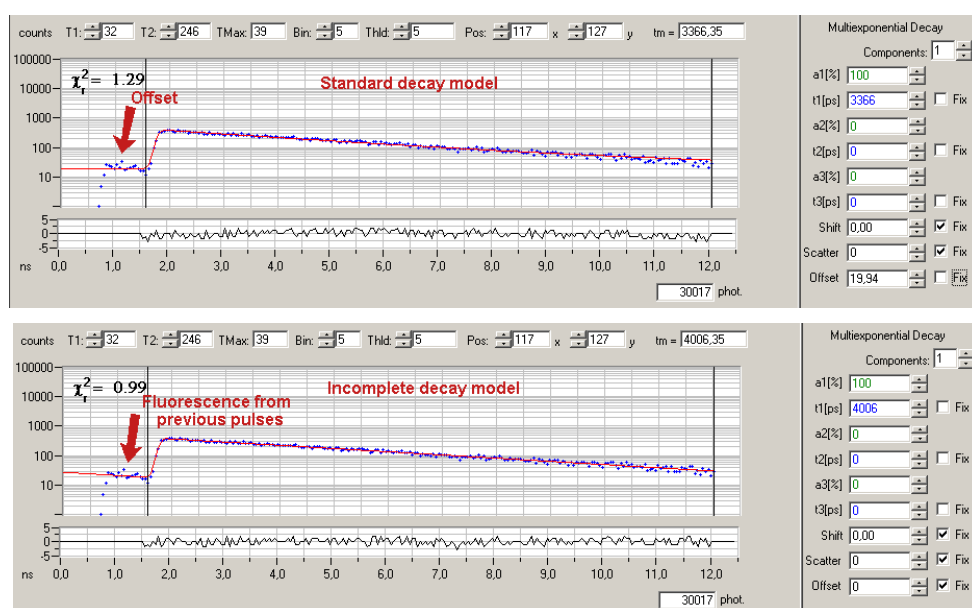


Fig. 575: Fit of a 4-ns-decay excited at 80 MHz by a standard decay model (top) and an incomplete-decay model (bottom). The incomplete decay model interprets the signal left of the rising edge correctly, and thus obtains a better fit.

Difficulties can arise if the data contain both an offset and a contribution from incomplete decay. The influence on the shape of the model functions is very similar, especially if the incomplete decay is caused by very slow decay components. The advantages of the incomplete decay model can therefore be fully exploited only if there is negligible offset (from daylight, detector dark counts or afterpulses) in the decay signals. The 'Offset' should then be set to zero and fixed. The incomplete decay option is available in SPCImage since 2003. A theoretical evaluation has been published recently by Leung et al. [368].

Shifted-Component Model

The shifted-component model has been developed for analysis of fluorescence-lifetime-ophthalmoscopy (FLIO) data. In FLIO data, the fluorescence of the fundus of the eye is overlaid by fluorescence from the front parts of the eye, especially from the crystalline lens. The lens fluorescence causes unpredictable changes in the detected fundus lifetimes. Moreover, the lens fluorescence causes a distortion in the rising edge of the fluorescence signal. This

makes it difficult to shift the IRF in the correct position. SPCImage NG therefore has a shifted-component model of the form

$$f(t) = a_1 e^{(-t+td1)/\tau1} + a_2 e^{(-t+td2)/\tau2} + a_3 e^{(-t+td3)/\tau3}$$

where $td1$, $td2$, $td3$ are temporal shifts of the corresponding decay components. The td parameters are fixed parameters which are given by the geometry of the measurement object, in this case the eye.

It turned out that the lens fluorescence in the FLIO data is almost entirely represented by the slow decay component, $e^{(-t+td3)/\tau3}$. For geometric reasons the lens component arrives about 150 ps before the fundus fluorescence. The data are thus analysed with $td1 = td2 = 0$ and $td3 = -150$ ps. The shifted-component model massively improved the reliability of FLIO analysis. It even made it possible to separate the signals from the fundus from that of the crystalline lens [77, 85, 86].

Multi-Exponential Models with Fixed Decay Times

Multi-exponential decay analysis, both with the basic models and with the incomplete-decay and shifted-component options, can be performed with fixed decay times of the components. The idea behind this is that the fluorescence lifetimes are often known, or can be determined by independent measurements. Fixing one or several decay times does, of course, result in a substantial increase in accuracy for other decay parameters. Conceivable applications are FRET, where the fluorescence lifetime of the non-interacting donor is supposed to be constant, and NADH and FAD FLIM, where the lifetimes of the unbound components are mostly constant.

Unfortunately the idea has a flaw. Except for a few fluorophores with extremely rigid molecular structure fluorescence lifetimes depend on the molecular environment. They can therefore not a priori be considered to be constant. However, if the value to which the decay time has been fixed is not correct or not constant throughout the sample large systematic errors in the other decay parameters can result. Fixed decay times should therefore be used consciously and carefully, and the results be checked versus analysis with free parameters. Please see 'Pseudo-Global Analysis', page 351.

Fixing Shift, Scatter and Offset

Shift, scatter and offset can be fixed in the basic model parameters, see 'Model Functions', page 309. There is no objection against fixing scatter and offset to zero if a look at the decay functions show that their contribution is negligible.

Fixing the 'Shift' can be more problematic. 'Shift' is a temporal offset of the data referred to the IRF. On the one hand, the analysis runs faster and more accurate with fixed shift. On the other hand, a wrong shift directly offsets the obtained decay times. We therefore recommend to leave the shift unfixed but set the option 'Fix shift before calculating image' in the model parameters, see Fig. 537, page 342. The analysis procedure then determines the optimum shift first, then fixes it to the determined value, and after that performs the decay analysis with the fixed shift.

Fit Procedures

Weighted Least Squares

The principle of the traditional least-square fit is shown in Fig. 576. The differences, $\delta(t)$, between the points of the model function, $f_{\text{mod}}(t)$, and the data points, $n(t)$, are calculated. In principle, the squares of the differences, $\delta(t)^2$, could be calculated, summed up, and this sum be used as an optimisation parameter.

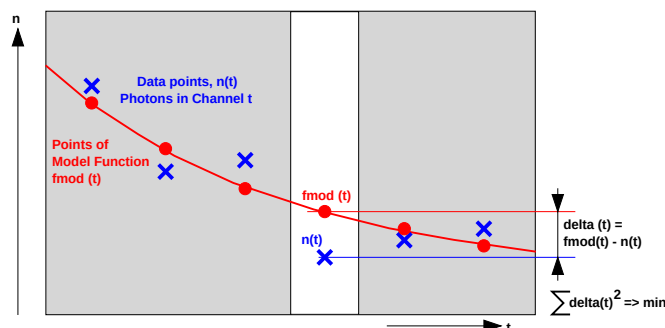


Fig. 576: Least-square fit

For fluorescence decay curves, this procedure has a flaw. The photon numbers, $n(t)$, are Poisson-distributed. That means the noise is larger in channels with higher photon number: The noise in $n(t)$ is $n(t)^{1/2}$. Therefore, the squares of the differences must be weighted with the square of the reciprocal expectation value of the noise, i.e. $1/n(t)$.

The correct weighting of the delta-squares is the problem of the least-square fit. The correct weight according to the Poisson distribution would be $1/n(t)$. This is, of course, not possible because there are time channels with $n(t)=0$. The weight of these channels would be infinite, which is a practical impossibility. The commonly used solution is to use a weight of $1/(n(t)+1)$:

$$S = \sum \frac{\Delta(t)^2}{n(t)+1}$$

The weighting with $1/n(t)+1$ avoids the singularity problem for $n(t)=0$, but, of course, does not weight the deltas in channels with low $n(t)$ or $n(t)=0$ correctly. The result is a bias towards shorter lifetimes for decay data of low photon number.

Maximum-Likelihood Estimation

MLE is based on calculating the *probability* that the values of the model function correctly represent the data points of the decay function. The principle is illustrated in Fig. 577. To each point of the model function, $f_{\text{mod}}(t)$, a Poissonian distribution,

$$p(n) = \frac{E^n e^{-E}}{n!}$$

with an expectation value equal to $E=f_{\text{mod}}(t)$, is associated, see Fig. 577, right. From this distribution the probability, $p(n(t))$, is calculated. The probability $p(n(t))$ tells how likely it is that the point of the model function is a correct representation of the data point. $p(n(t))$ is calculated for all time channels, i.e. for all pairs of data points and model-function points. The product of these probabilities is the probability that the model function represents the data. The parameters of the model functions are then optimised until the maximum probability, $\prod p(n(t))$, is obtained.

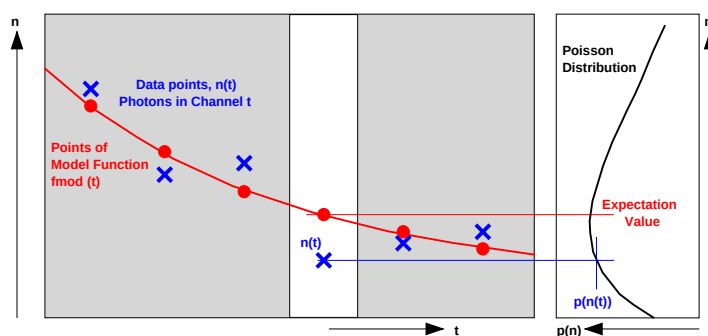


Fig. 577: Principle of MLE fit. For each point of the model function, $f_{\text{mod}}(t)$, a Poissonian distribution is associated. The function delivers a probability $p(n(t))$, that a given data point, $n(t)$ fits to the corresponding point of the model function. The product of $p(n(t))$ over all time channels is used for optimising the parameters of the model function.

The MLE fit has no problem with data points with low photon number or even with a photon number of zero. The Poissonian distribution associates correct probabilities to all these situations, and the product of these probabilities correctly describes the quality of the fit. Consequently, there is no bias toward shorter lifetime, as it occurs in the weighted least square fit.

Lifetime Calculation by First-Moment

The first moment of a photon distribution is the average arrival time of the photons. There is a simple relation between the first moment and the fluorescence lifetime: The lifetime is the difference of the first moments of the decay curve and the IRF, see Fig. 578. The advantage of first-moment calculation is that the lifetime is obtained at *ideal accuracy*. There is no error contribution from numerical effects or fit uncertainty.

The disadvantage is that the first moment is correct only if the decay data are free of background and if the entire decay curve is included in the calculation. Moreover, first-moment calculation also does not resolve multi-exponential decay functions into their components. When first-moment calculation is applied to multi-exponential data the result is an ‘apparent lifetime’ which is close to the lifetime obtained by a single-exponential fit.

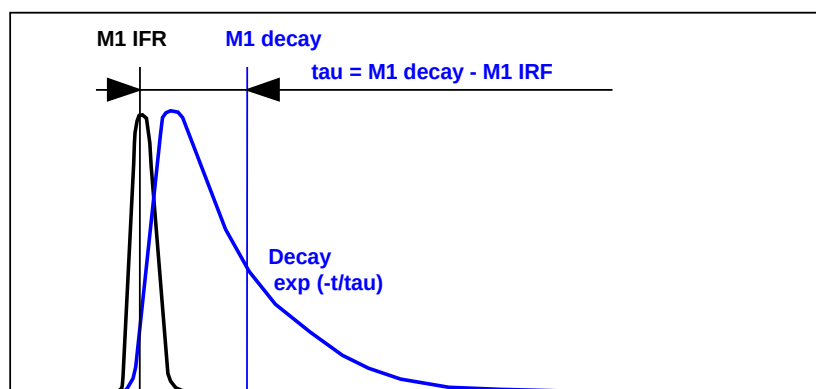


Fig. 578: First-moment calculation of fluorescence lifetime. The lifetime is the different of the first moments of the decay curve and the IRF.

Two examples of lifetime calculation via the first moment are shown in Fig. 579. The calculation interval (cursor range) in Fig. 579, top, includes all photons of the decay data. The lifetime obtained from first-moment calculation is correct. In Fig. 579, bottom a calculation interval was selected that does not include the later part of the decay function. Consequently, the first moment calculated in the range selected is too small, and the lifetime is determined too short.

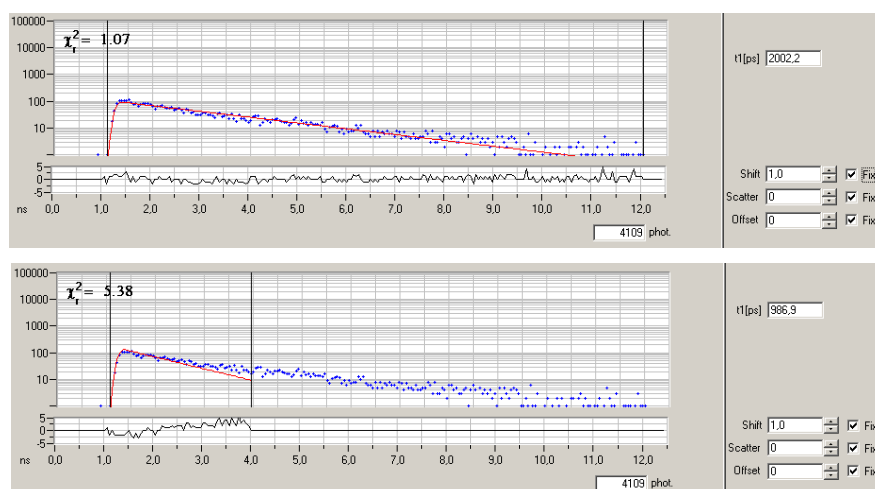


Fig. 579: Lifetime calculation via the first moment. Top: All photons of the decay function have been included in the moment calculation. The obtained (single-exponential) lifetime is correct. Bottom: If the late photons are not included in the calculation the lifetime is determined too short.

How Many Exponential Components Are Needed?

Users are often uncertain which model, in particular which number of exponential components, they should use to fit the data. In most cases the answer is simple: Use a number of decay components equal to the number of fluorophores or fluorophore fractions you expect to contribute to the fluorescence of the sample.

If there is no a priori knowledge about the fluorescence mechanisms in the sample the model can be found by trial and error. Select a characteristic spot of the sample. Increase the binning factor until you see a clean fluorescence decay function. Then change the number of components and check the displayed χ^2 and the curve of the residuals. A good fit is characterised by a χ^2 close to one, and residuals showing no noticeable systematic variations. Often you see a poor fit already by comparing the fitted curve (red) with the photon data (blue) in the decay window.

In most cases your decay curves will be fitted adequately by a single- or double-exponential model. If you define more exponential components than needed you normally obtain two components of almost identical lifetime, or an additional lifetime component of very long lifetime and low amplitude.

An example is shown in Fig. 580. Fitting the data with only one component (Fig. 580, top) delivers a large χ^2 and clearly visible systematic variation in the residuals. With some experience, you may also spot systematic deviations between the decay data and the red curve calculated by the fitting procedure.

Fitting the data with two components delivers a perfect χ^2 and removes any systematic deviations in the residuals. This is an indication that the fit cannot be improved by adding more exponential components.

An attempt to fit the data with three components (Fig. 580, bottom) indeed does not deliver any improvement. Instead, it delivers a third lifetime component almost identical with the second one. This is a clear indication that the double-exponential model is the right one.

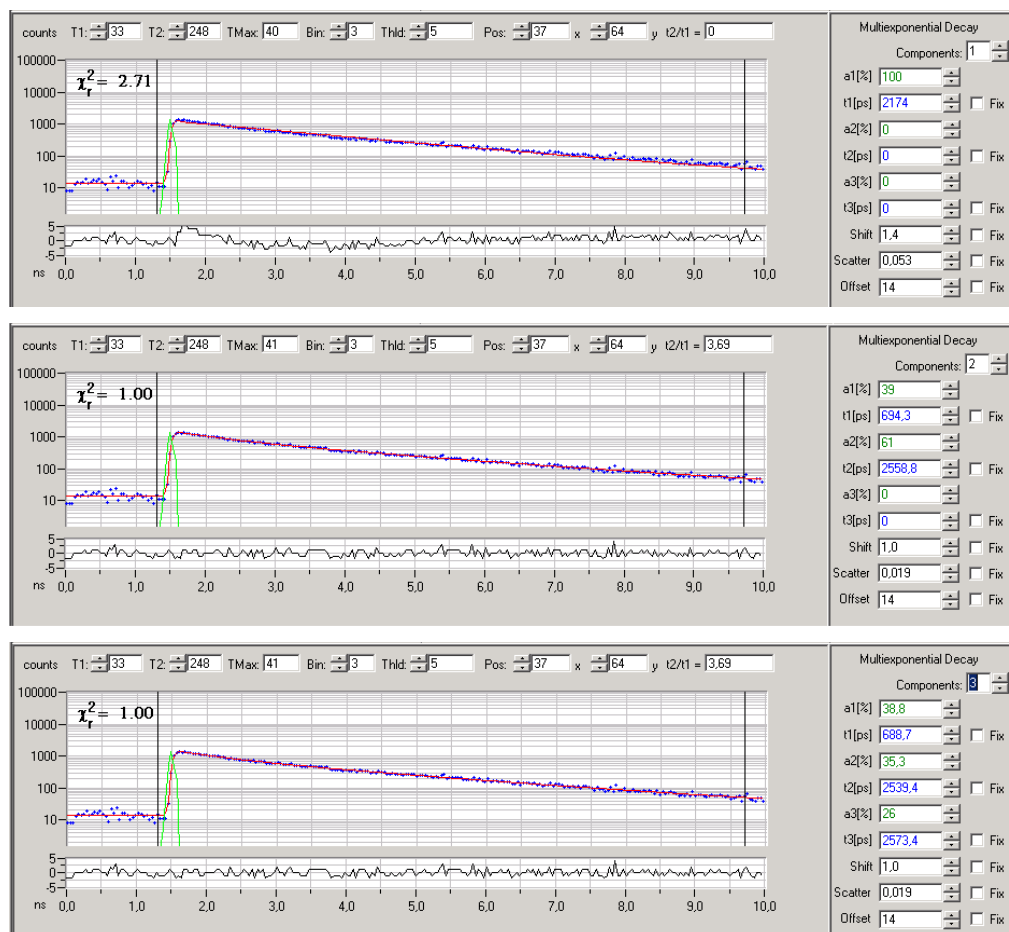


Fig. 580: Top to bottom: Fitting a decay profile with one, two, and three exponential components

Over-Determined Models

The statistical errors of the fluorescence parameters are, of course, smallest if the correct model function is used. However, the parameters used in the model should be restricted to those actually needed to describe the measured fluorescence waveform. If more parameters are used the fit may achieve a slightly better χ^2 but the individual parameters may vary wildly. This is especially the case if parameters are used that have an almost identical influence on the model function. A few typical cases of over-determined models are described below.

Single-Exponential Decay Fitted with Double-Exponential Model

If a single-exponential decay function is fitted with a double-exponential model the fit procedure delivers two decay components of nearly identical lifetimes. The lifetimes are correct, and an amplitude or intensity-weighted lifetime (t_m or t_i) derived from the components will be correct as well. However, the amplitudes of the two components have no influence on the model function. The amplitudes will thus remain undetermined, and may fluctuate strongly. An example is shown in Fig. 581. FLIM data of a rhodamine 110 solution were recorded and analysed by a single- and a double-exponential model. The average photon number per pixel is 2700. Rhodamine 110 in water delivers a near-perfect single-exponential decay. Fig. 581, left shows the result of a single-exponential fit. The lifetime image is nicely homogenous. The lifetime distribution shows a relative variance of about 0.019. This is the theoretical value: The expected relative variance for a photon number N is $1/\sqrt{N}$, i.e. 0.019 for 2700 photons.

The lifetime image and the distribution obtained by a double-exponential model are shown in Fig. 581, middle. The lifetime distribution has a relative variance of 0.026. This is more than the theoretical value but still reasonably good. However, the amplitudes, a_1 and a_2 , are fluctuating wildly because they have no influence on the shape of a double-exponential model with two almost equal lifetimes. See Fig. 581, right.

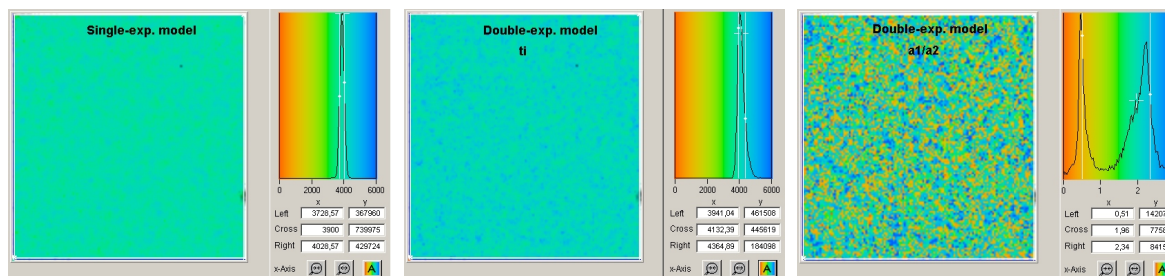


Fig. 581: Comparison of single-exponential and double exponential model applied to single-exponential decay. Rhodamine 110 solution. Left: Fit with single-exponential model, lifetime image and lifetime distribution. Middle: Double-exponential model, intensity-weighted lifetime. Right: Double-exponential model, amplitude ratio, a_1/a_2 .

The situation is similarly difficult for real double-exponential decay profiles with component lifetimes that very close to one another [320]. Also here, reasonably accurate values are obtained for the mean and average lifetime, t_m and t_i , but not necessarily for the lifetimes and amplitudes of the individual components.

Shift Parameter and Extremely Short Lifetime Component

The convolution integral of a lifetime shorter than the width of the IRF is very similar to a shift of the curve, see Fig. 582. The shift parameter may thus conflict with a short decay component: A slightly shorter lifetime and a slightly larger shift and vice versa deliver almost similar shapes of the model function. The effect is variation of the lifetime and the shift in opposite directions. Therefore, the shift parameter *must* be fixed when lifetimes on the order of the IRF width or shorter are to be determined.

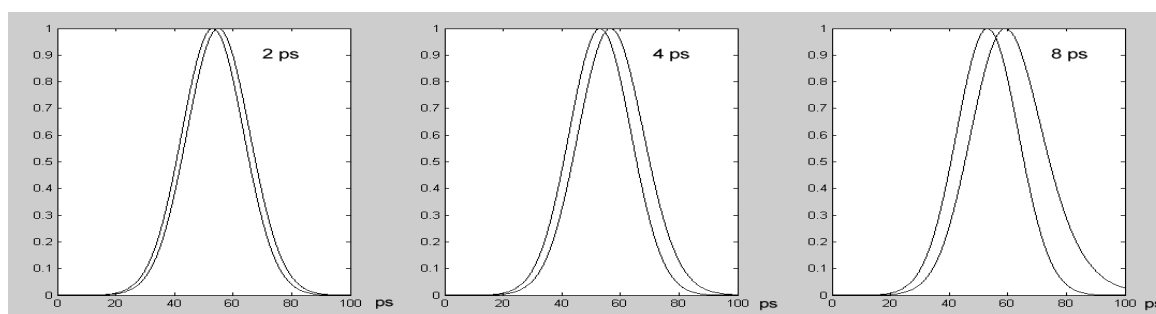


Fig. 582: The result of a convolution of the IRF with a fast decay function is very similar to a shift. Simulated data, IRF width 25 ps FWHM

Scatter Parameter and an Extremely Short Lifetime

Similar problems can occur if the signal contains SHG components and extremely fast lifetime components. Also here, a change in the amplitude of the fast decay component can be compensated by an opposite change in the scatter. The best advice is record the fluorescence and the SHG signal independently through different filters.

Offset Parameter and Long Lifetime

Long lifetimes are difficult to distinguish from an offset in the signal. This is especially the case when the fluorescence does not fully decay within the recorded time interval or within the excitation pulse period. The situation is further complicated by the fact that an apparent

offset can have different reasons: It can be a real offset caused by daylight pickup or after-pulsing of the detector, or it can be residual fluorescence from the previous pulses. An example is shown in Fig. 583.

In Fig. 583, left, the apparent offset left of the rising edge of the fluorescence pulse was fitted by the 'Offset' parameter of SPCImage. The fit quality looks good, although subtle deviations can be seen in the tail of the decay curve. In Fig. 583, middle, the offset was fixed to zero. The signal part left of the rising edge is not fitted. Nevertheless, the fit of the tail looks better than Fig. 583, left. The lifetime is determined more than 12% longer than in Fig. 583, left.

The reason becomes clear when the data are fitted by the 'Incomplete Decay' model, see Fig. 583, right. The 'Offset' parameter was fixed to zero. The fit is good, and also the signal left of the rising edge fits correctly. The result shows that the apparent offset is fluorescence from the previous pulses that has not completely decayed. Interpreting it as an offset due to background signals (Fig. 583, left) is wrong and leads to the wrong lifetime.

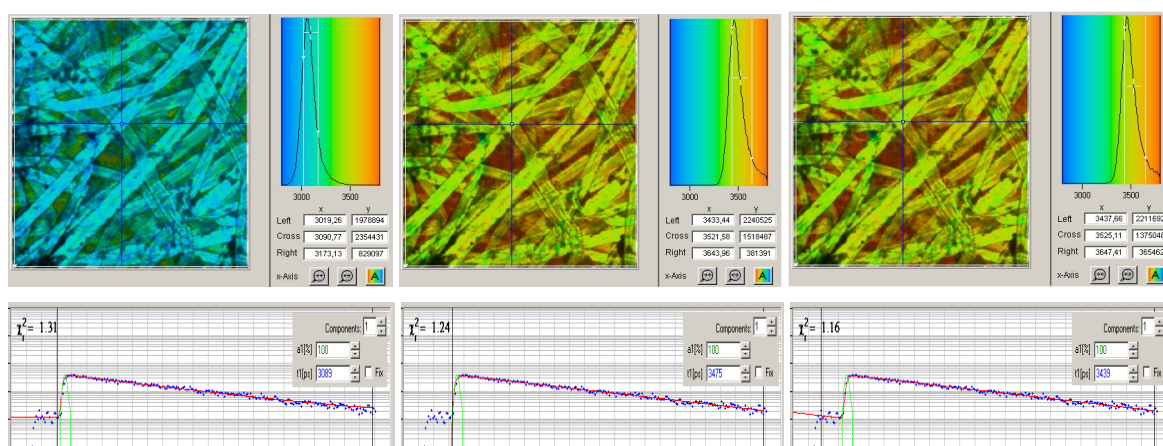


Fig. 583: Left: 'Offset' parameter used to fit the signal left of the rising edge of the fluorescence pulse. Middle: Offset parameter fixed to zero, signal left of rising edge not fitted. Right: Offset parameter fixed to zero, signal left of rising edge fitted by 'Incomplete Decay' model.

Wrong interpretation of the offset is a frequent source of obtaining 'wrong' lifetimes in two-photon microscopes. 'Lifetime standards', such as fluorescein, have lifetimes on the order of 3 to 5 ns. At 80 MHz repetition rate, the fluorescence does not completely decay between the excitation pulses, causing the problems described above.

To avoid the offset problem we recommend to strictly avoid daylight pickup. If there is still an offset in the signal, check whether it is fitted by the incomplete decay model. In that case, do not forget to set the correct repetition rate in the Model Parameter Options, see Fig. 537, page 342, and fix the 'Offset' to zero. If the signal part left of the rising edge is fitted correctly by the incomplete decay model the apparent offset is residual fluorescence.

Instrument Response Function

Automatic IRF: Calculating an IRF from Fluorescence Decay Data

SPCImage NG has functions to derive the IRF from recorded FLIM data. The calculation of the 'Auto IRF' is based on the assumption that the fluorescence lifetime is long compared to the width of the IRF. In that case, the rising edge of the fluorescence signal is (almost) the integral of the IRF. The IRF can therefore be obtained from the fluorescence signal: The calculation procedure fits the rising edge of the fluorescence signal with a suitable function, $\text{rise}(t)$. The differentiated function, $d \text{ Rise}(t) / dt$, is the IRF. Fig. 584, left, shows how this works. A typical result is shown on the right.

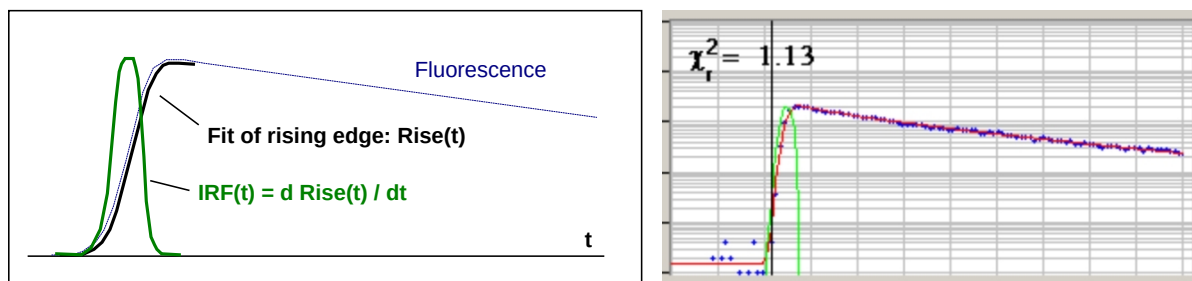


Fig. 584: Calculation of IRF from fluorescence data. Principle shown left, typical result shown right.

The calculation of the Auto IRF is performed automatically every time raw data are loaded. It is run on combined decay curves from pixels of an area centred around the 'Hot Spot', i.e. from the brightest part of the image. Occasionally, it happens that the hot spot does not contain valid decay curves, e.g. if it is a speck of dust or another contamination. In that case, disable 'Always calculate at brightest pixel' in the preferences panel (see Fig. 541, page 346), select a better position by the blue image cursor, and click on 'IRF', 'Auto'.

Analysis with the automatic IRF delivers a reasonably good fit and a colour-coded τ_m image virtually identical with an image obtained with the real IRF, compare Fig. 591, page 381. The τ_m in the selected spot is nearly the same. Even the lifetimes and amplitudes of the decay components do not differ by more than 10%. This is the more surprising as the fastest decay component has a lifetime of only 400 ps, which is not far from the width of the instrument response function.

Nevertheless, there may be cases when the IRF calculation does not deliver the correct result. This can happen when the decay curves contain lifetime components with decay times close to or shorter than the IRF width, or with a contribution from SHG. The rising edge is then faster than the integral of the true IRF. Consequently, the IRF is calculated too short. This may bias fast lifetimes towards longer values, and make it difficult to extract an SHG contribution from the FLIM data.

Fully Synthetic IRF of the Type $x \exp(-x)$

The idea behind the fully synthetic IRF is to model the IRF by a function of the type

$$\text{irf}(t) = t/t_0 e^{-t/t_0}$$

The function has only one parameter, t_0 , which determines the width. The function has a steep rise, and a slow, almost single-exponential tail. It closely resembles the IRF of a GaAsP hybrid detector.

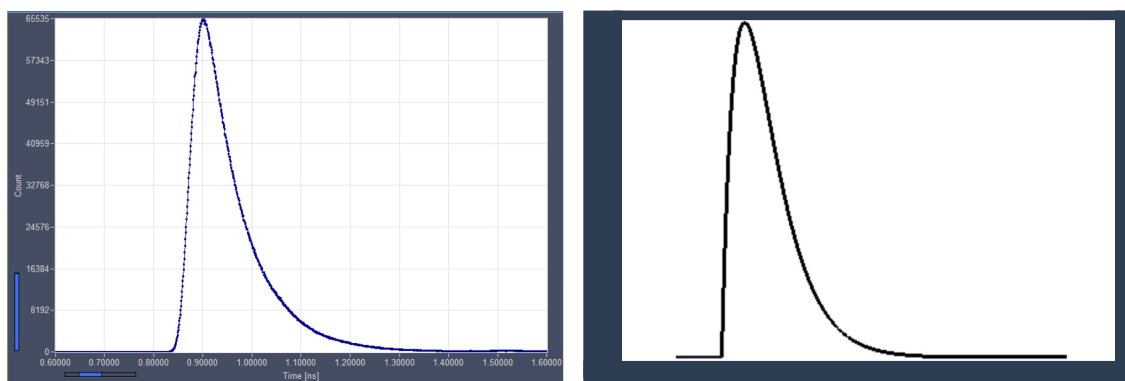


Fig. 585: IRF of a TCSPC system with HPM-100-40 hybrid detector (left) and shape of the function $\text{irf}(t) = t/t_0 e^{-t/t_0}$ (right)

The function is also a good approximation to the IRFs of normal PMTs and most single-photon avalanche photodiodes. To account for possible influence of the laser pulse shape the function is convoluted with a Gaussian curve of the (known) laser pulse width, t_l .

$$f(t) = e^{-(t/t_l)^2}$$

The advantage of the fully synthetic IRF is that it works also for short decay times, in presence of ultra-fast components, and in combination with the shifted-component model.

The parameter t_0 can further be refined by an automatic optimisation procedure. The principle is illustrated in Fig. 586, left. The procedure runs a fit of the fluorescence model parameters together with the parameter t_0 to the decay data. The IRF in subsequent steps of the procedure is shown in Fig. 586, right. Provided a correct model function is used the result is an optimised IRF function, with the correct width parameter, t_0 . The optimisation procedure is accessible via the 'Model' parameters panel, see page 342. Make sure that 'Permanent IRF' is set and that the left decay-window cursor is left of the rising edge of the decay curve.

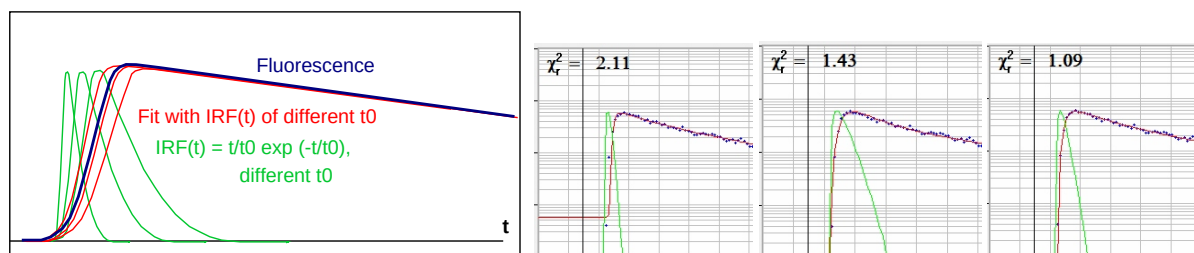


Fig. 586: Left: IRF optimisation procedure. Right: IRF shape in subsequent steps of the procedure.

For successful IRF optimisation it is important that a reasonable model of the fluorescence decay function is used. If the decay model does not have enough components the IRF optimisation may compensate the deficiency of the decay model with a wrong IRF. On the other hand, if the model is too flexible, the combination of the decay model and the IRF model can become 'over-determined'. The procedure can then fit the data with a small IRF width, t_0 , and a large lifetime of the fast decay component, t_1 , or vice versa. The situation occurs sometimes with triple-exponential decay models when amplitude of the third component is low. If this happens, go back to a double-exponential model for IRF optimisation, and then switch to a triple-exponential model for decay analysis.

The IRFs from the procedure above yield surprisingly precise fit results. An example of FLIM analysis with a synthetic IRF is shown in Fig. 587.

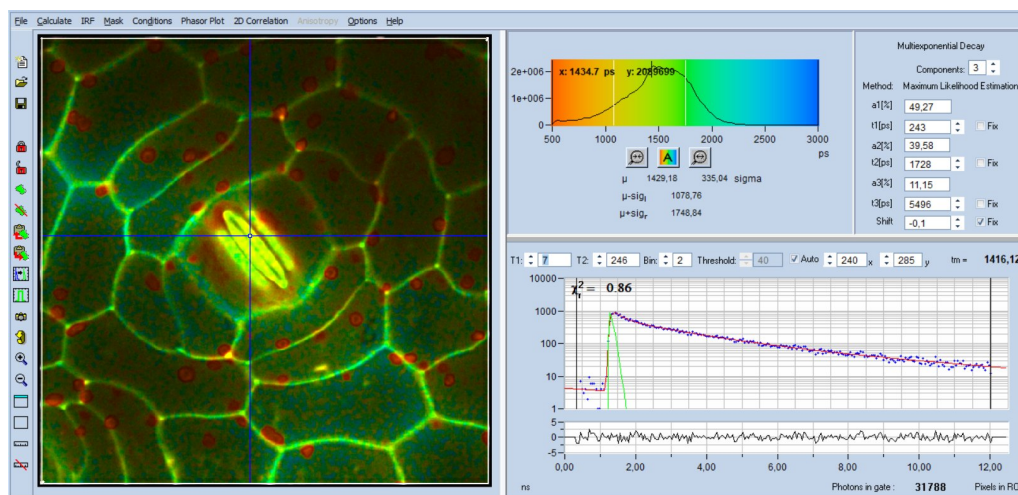


Fig. 587: Plant tissue FLIM data analysed with synthetic IRF. Amplitude-weighted lifetime of triple-exponential decay model. Autofluorescence, emission wavelength 500 to 550 nm. The result is virtually identical with the result of an analysis with the real IRF, see Fig. 591.

Measured IRF

Principle

To say it plainly: Most of the measured IRFs are wrong. We therefore do not recommend to use a measured IRF unless this is absolutely unavoidable. IRF measurement and the associated pitfalls are described in [77]. If you think you need a measured IRF or you can't withstand the temptation to measure one, please follow the instructions given below.

The IRF is obtained from scattering or SHG data. The data must be reasonably free of fluorescence, reflections, and background. It is important that the IRF data be recorded under the same conditions as the FLIM data to be analysed. In particular, TCSPC system parameters affecting the timing and the time scale, such as TAC, CFD, SYNC parameters and ADC Resolution, must be the same. Cable lengths, optical path length, delay box settings, DCC detector gain, and electrical laser power (in the scan control panel) must be the same. Needless to say, IRF data must be from the same detector channel as the data to be analysed.

A frequent source of errors in IRF acquisition is baseline shift due to counting background. Surprisingly, the problem is rarely mentioned in the TCSPC literature. If the IRF data contain background - either from room light pickup or from afterpulsing of detectors - the fit of the convoluted model to the fluorescence data delivers wrong lifetimes.

The effect of IRF background is illustrated in Fig. 588. The convolution of the model function with the true (background-free) IRF yields a waveform that represents the measured fluorescence decay function. Convolution of the model function with a continuous background yields an integral term, see Fig. 588 middle right. Convolution with an IRF containing background delivers the measured fluorescence decay function plus an integral term, see Fig. 588, lower right. It does not fit the waveform of the fluorescence data. Even a small IRF background can affect the fit results noticeably: The background extends over far more time channels than the true IRF and thus has a large influence on the convolution integral.

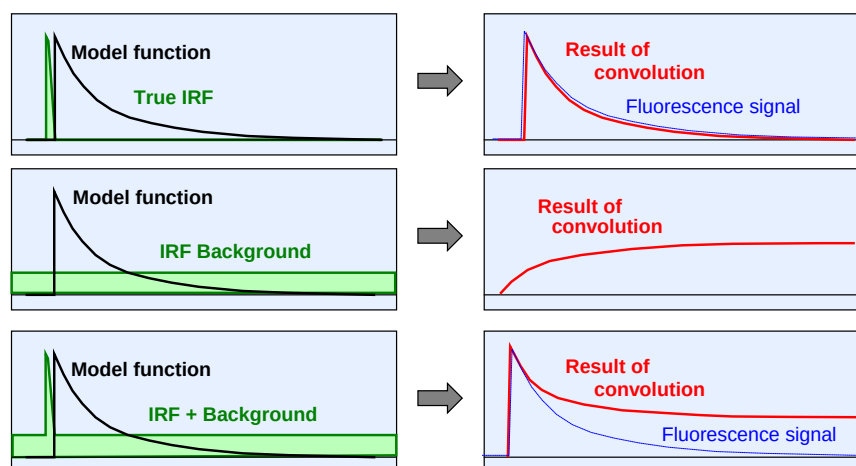


Fig. 588: Effect of background in a recorded IRF on the result of the convolution with the decay model

Background correction of recorded IRF data is therefore essential. In principle, background could be subtracted from the data. However, the background contains noise. Subtracting the average background from all time channels does not fully clean up the data. Therefore SPCImage allows you to *cut off* the signal portions outside the valid part of the IRF.

Loading a Measured IRF

To generate an IRF from measured data proceed as follows:

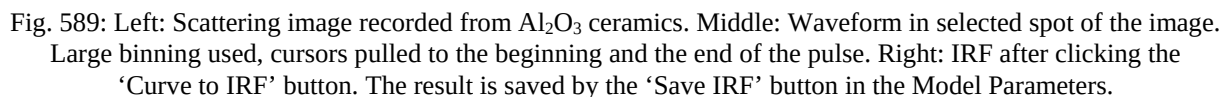
- Import or load an .sdt or .img data FLIM file that contains IRF data.
- Use the blue image cursor to select a region that contains good data. Keep away from fluorescent inclusions or suspicious contaminations. Increase the binning until you see a clean waveform in the curve window.
- Place the cursors in the decay-curve window at the beginning and the end of the valid part of the IRF.
- Click on the  (Curve to IRF) button. This declares the selected part of the curve an IRF.
- Click on the  (Store Conditions) button. This saves the IRF you generated. The IRF will stay in the 'Fit Conditions' until you store a new one.
- To retrieve the IRF after loading new FLIM data click on  or 'Load fit conditions'.
- Alternatively, you can use 'IRF', 'Copy to clipboard', and 'Paste from clipboard'. This saves/retrieves the IRF for the current session.

One-Photon IRF

Fig. 589 through Fig. 591 give an example of analysing one-photon FLIM data with an IRF obtained from scattering data.

Fig. 589, left, is a scattering image obtained from aluminium oxide ceramics. The image was recorded following the instructions given in [77]. No laser blocking filter was used. To reduce possible fluorescence from contaminations or laser background at longer wavelengths a 460 ± 20 nm bandpass filter was inserted in the detection beam path. The image therefore contains mostly scattered laser light.

A suitable spot was selected in the image (Fig. 589, left) and the binning factor increased until about 10,000 photons were in the curve. The waveform obtained this way is shown in (Fig. 589, middle). A click on the  (Curve to IRF) button declares the data points within the cursor interval an IRF. See green curve in Fig. 589, right. The generated IRF can be saved and recalled by 'Save IRF' and 'Load IRF' in the model parameter panel. Within the same SPCImage session, the IRF can be temporarily saved and recalled by 'Copy to Clipboard' and 'Paste from Clipboard' under 'IRF' in the top bar.



Please notice that there is virtually no difference between the synthetic IRF (left) and the measured IRF (right). Also the decay parameters are closely the same. That means the entire effort to measure the IRF and prepare it for use in SPCImage was unnecessary. The synthetic IRF works equally well.

The screenshot displays the WinAQC software interface, which is used for analyzing fluorescence decay data. The interface is divided into several panels:

- Top Panel:** Contains a menu bar with options: File, Calculate, IRF, Mask, Conditions, Phasor Plot, 2D Correlation, Anisotropy, Options, and Help.
- Left Panel:** A vertical toolbar with icons for various functions such as opening files, saving, zooming, and fitting.
- Main Image Panel:** Displays a fluorescence image of cells. A region of interest (ROI) is highlighted in yellow, and a multiexponential decay fit is overlaid on the image. The fit parameters are shown in the top right corner:
 - $\chi^2 = 1434.7$ ps
 - $\chi^2 = 1954.086$ ps
 - $\mu = 1440.87$ ps
 - $\mu + \sigma = 1083.28$ ps
 - $\mu + \sigma = 1762.71$ ps
- Right Panel:** Contains a table of fit parameters and their values, along with checkboxes for fixing certain parameters.

Multiexponential Decay	
Components: 3	
Method:	Maximum Likelihood Estimation
a[%]	49.31
t1[ps]	251
a2[%]	39.74
t2[ps]	1751
a3[%]	10.95
t3[ps]	5540
Shift	0.9
- Bottom Panel:** Displays a histogram of the decay data. The x-axis is labeled "ns" and ranges from 0.00 to 12.00. The y-axis is labeled "ns" and ranges from -5 to 10000. The histogram shows a sharp peak at approximately 1.5 ns, with a fitted curve overlaid. The fit parameters are shown in the top right corner:
 - $\chi^2 = 0.86$
 - $\chi^2 = 0.86$
 - $\chi^2 = 0.86$

Fig. 591: Data analysed with measured IRF. Plant tissue, autofluorescence, emission wavelength 500 to 550 nm.
Compare with result of analysis with synthetic IRF, Fig. 587

Two-Photon IRF from SHG Data

In two-photon microscopes a measured IRF is usually obtained from SHG data. Good SHG signals are obtained from crystalline urea or sugar [77]. The procedure to generate an IRF from the FLIM data is the same as described for one-photon excitation. Load the data into SPCImage, select a time interval around the signal peak, and declare the data within the cursor interval an IRF. An IRF from SHG of sugar is shown in Fig. 592.

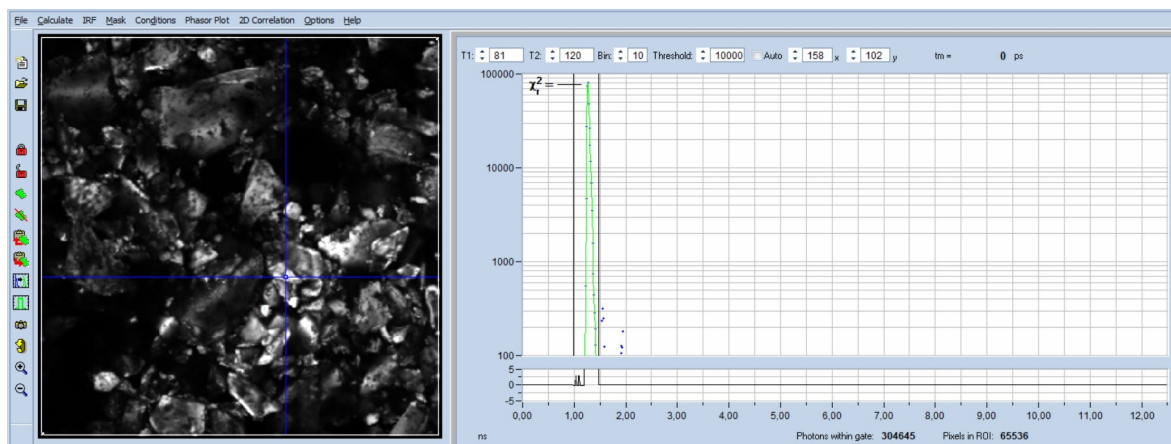


Fig. 592: IRF from SHG of sugar. DCS-120 MP multiphoton FLIM system, HPM-100-06 detector

Often an IRF can be derived directly from the sample investigated, without data from an external IRF measurement. This is the case when the sample contains collagen, starch or other SHG-active compounds. A spot with a dominating SHG signal can then be selected in the FLIM image and the corresponding waveform declared an IRF.

An example is shown in Fig. 593. A collagen structure was selected in the intensity image, see Fig. 593, left. The data shown in the curve window confirm that the selected spot is dominated by an ultra-fast signal, see Fig. 593, middle. However, the signal is not entirely free of fluorescence, see the tail in the recorded waveform. However, the amplitude of the tail is three orders of magnitude below the peak of the SHG pulse. Consequently, a reasonable IRF is obtained by just deleting the signal outside the SHG pulse. To do so, enclose the SHG pulse by the decay cursors and declare the selected part an IRF, see green curve in Fig. 593, right.

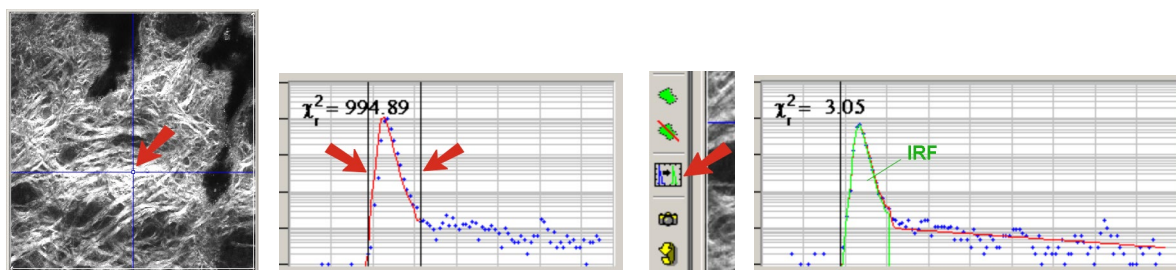


Fig. 593: Generating an IRF from the SHG signal from collagen in a tissue sample. Left to right: Select a collagen structure in the intensity image, set the cursors in the curve window to the beginning and the end of the SHG pulse, and declare the selected part of the curve an IRF. The IRF is the green curve shown right.

The IRF is then used to analyse the image from which it was extracted. A result is shown in Fig. 594.

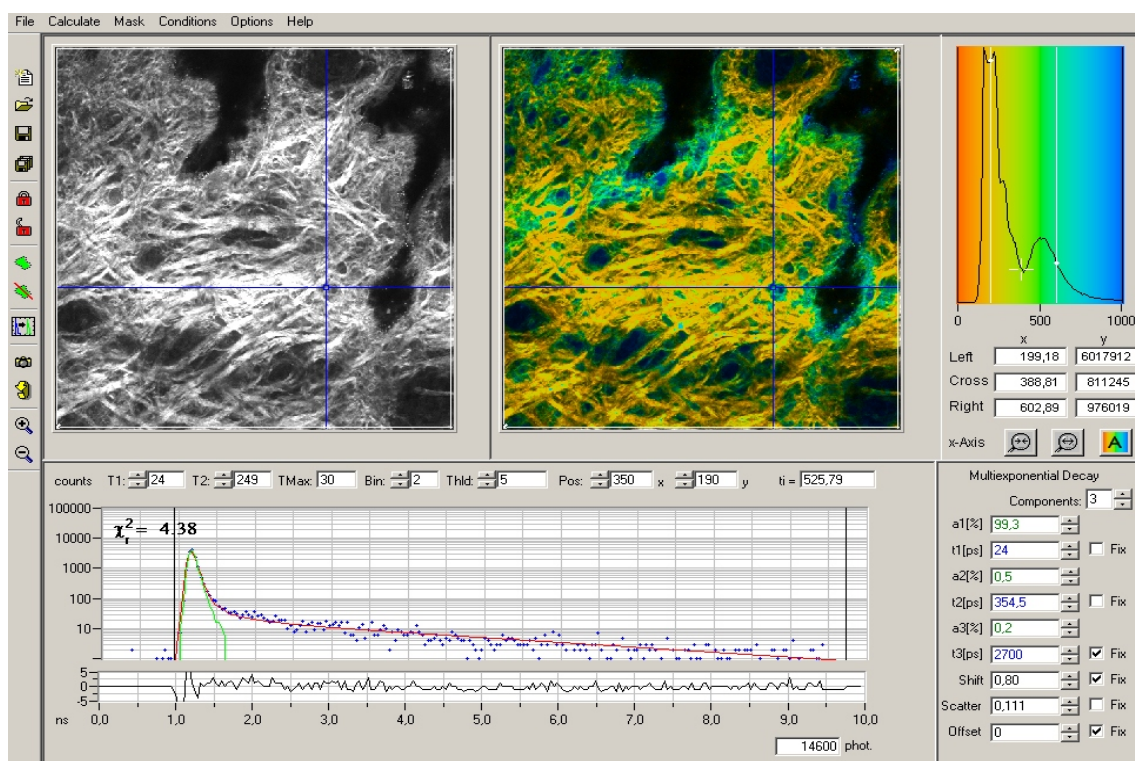


Fig. 594: Analysis with the IRF derived from SHG in the same image

Parameters of the Decay Functions and their Use in SPCImage

Amplitude-Weighted Mean Lifetime, t_m

The mean lifetime, τ_m , is an average of the lifetimes of the components of a multi-exponential decay weighted by their amplitude coefficients. It is

for a single-exponential decay: $\tau_m = \tau$

for a double-exponential decay: $\tau_m = a_1\tau_1 + a_2\tau_2$ with $a_1 + a_2 = 1$

for a triple-exponential decay: $\tau_m = a_1\tau_1 + a_2\tau_2 + a_3\tau_3$ with $a_1 + a_2 + a_3 = 1$

If a decay function deviates from a single exponential function the mean lifetime τ_m , is *not* the same as the ‘apparent’ lifetime obtained from a single-exponential fit or from first-moment analysis. To obtain an equivalent of the apparent lifetime the lifetimes of the decay components had to be weighted by their integral intensities, see ‘average lifetime’. Calculating a mean lifetime from a multi-exponential decay is therefore often considered incorrect. However, the mean lifetime has a real physical meaning:

The terms $a_n\tau_n$ in the calculation of the mean lifetime are proportional to the intensities contained in the individual decay components. Thus, the mean lifetime is proportional to the total fluorescence quantum efficiency of the emitting species [360]. The mean lifetime is therefore the correct value to calculate classic FRET efficiencies from a multi-exponential decay. Thus, if you use TCSPC FLIM to verify steady-state FLIM results you have to use the mean lifetime. Please note, however, that the FRET efficiency obtained this way averages the emission of all donor molecules, no matter whether they are linked to an acceptor or not. The classic FRET efficiency therefore does not necessarily deliver the correct distance, see ‘Fluorescence Resonance Energy Transfer (FRET)’, page 233.

Mean Lifetime of the First Two Components, t_{m12}

t_{m12} is the amplitude-weighted lifetime of the first two decay components of a triple-exponential decay:

$$\tau_{m12} = (a_1\tau_1 + a_2\tau_2)/(a_1 + a_2)$$

It is used to display fundus images in FLIO analysis [77, 85].

Intensity-Weighted Average Lifetime, t_i

The average lifetime, t_i , is the average of the lifetimes of the decay components weighted by their integral intensities. The integral intensity of a lifetime component is the product of its lifetime and its amplitude. The intensity-weighted lifetime, t_i , is thus

for a single-exponential decay $\tau_i = \tau$

for a double-exponential decay $\tau_i = \frac{a_1\tau_1^2 + a_2\tau_2^2}{a_1\tau_1 + a_2\tau_2}$

for a triple-exponential decay $\tau_i = \frac{a_1\tau_1^2 + a_2\tau_2^2 + a_3\tau_3^2}{a_1\tau_1 + a_2\tau_2 + a_3\tau_3}$

For a mono-exponential decay, the average lifetime is, of course, identical with the lifetime of the decay. For multi-exponential decay functions the average lifetime comes close to lifetimes

obtained by single-exponential analysis or by modulation techniques. t_i is therefore also called ‘apparent lifetime’. The average lifetime should therefore be used if a decay profile is not truly single exponential but its lifetime has to be compared with a single ‘decay time’ given in the literature. Please note also that the average lifetime, t_i , is more sensitive to changes in the slow lifetime component while the mean lifetime, t_m , is more sensitive to changes in the fast component.

A practical example is shown in Fig. 595. The sample has a pronounced double-exponential decay. From left to right, the figure shows the amplitude-weighted lifetime, t_m , the intensity weighted lifetime, t_i , (both from a double exponential fit), and the lifetime of a single-exponential fit. Differences are clearly visible.

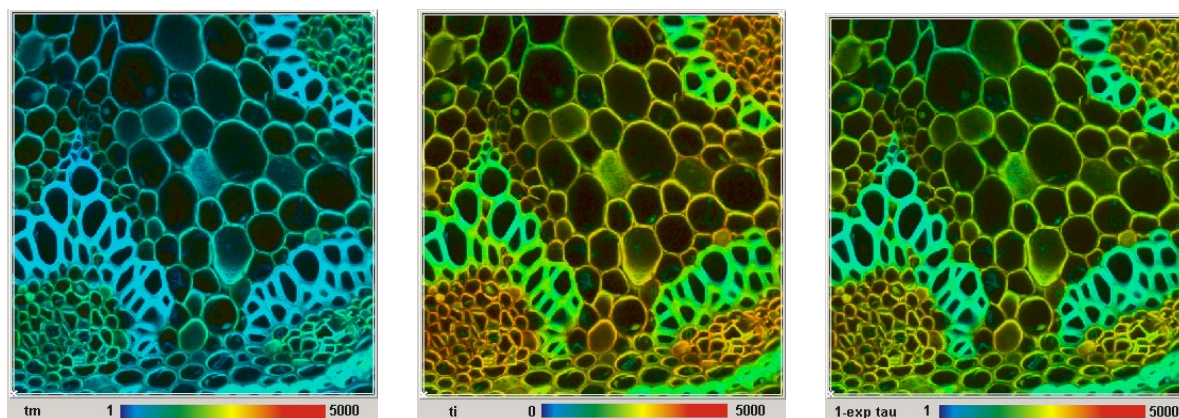


Fig. 595: Left to right: Lifetime images of the mean (amplitude-weighted) lifetime, the average (intensity-weighted) and the lifetime of a single-exponential fit. Convallaria sample, the decay profiles deviate strongly from single-exponential functions.

Lifetimes of the Decay Components, t_1 , t_2 , t_3

For a multi-exponential decay model the lifetimes of the individual components, t_1 , t_2 , or t_3 , can be selected and used as colour of the lifetime image. The lifetimes are often used to distinguish several fluorophores or different binding states of a single fluorophore present in the same pixel. For example, in FRET measurements there is usually an interacting and a non-interacting donor fraction. In this case t_1 is the lifetime of the interacting fraction, t_2 is the lifetime of the non-interacting one. In autofluorescence measurements t_1 and t_2 are the lifetimes of unbound and bound NADH fractions [548, 159].

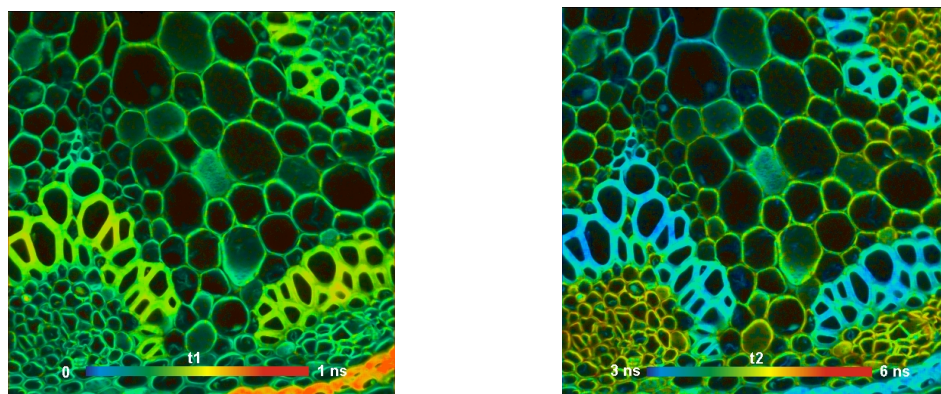


Fig. 596: Lifetimes of the components of a double-exponential decay. Same data as in Fig. 595.

Amplitudes of the Decay Components, a_1 , a_2 , a_3

The colour of the image can be assigned to one of the amplitudes, a_1 , a_2 , or a_3 , of a multi-exponential decay. Amplitude images are used to show the relative concentration of similar fluorophore molecules in different local environment, different binding states, or different states of FRET. Amplitudes of the decay components are often surprisingly stable, with noticeably higher signal-to-noise ratio than the lifetimes.

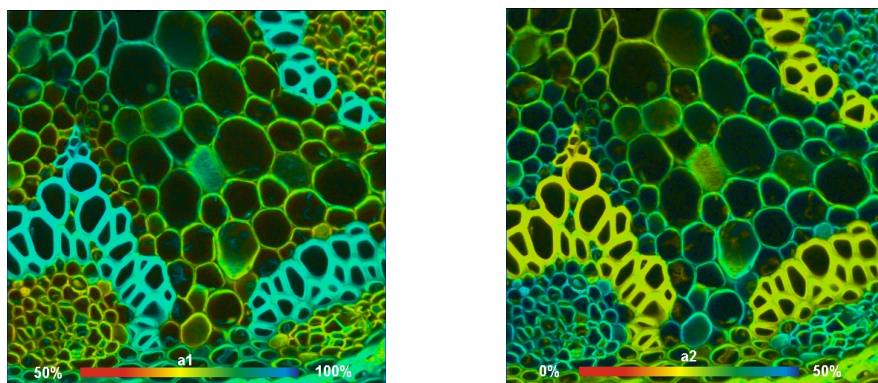


Fig. 597: Amplitudes of the components of a double-exponential decay. Same data as in Fig. 595.

Ratios of Lifetimes of Decay Components

SPCImage can display ratios of the lifetimes of different decay components. The lifetime ratio of two decay components is especially useful for FRET. The fast component, t_1 , is the lifetime of the interacting donor fraction. The lifetime of the slow component, t_2 , is the lifetime of the non-interacting donor. The ratio of both is directly related to the distance of donor and acceptor. The charm of this approach is that it is calibration-free: The reference lifetime is the non-interacting donor lifetime, t_2 . t_2 is derived from the same specimen, the same cell, and the same pixels as the interacting-donor lifetime t_1 . Variations in t_2 , e.g. by variation in the refractive index or in the pH, have no influence on the distance calculation.

Ratios of Amplitudes

Ratios of amplitudes can be used the same way as the amplitudes themselves. For example, the ratio a_1/a_2 of a FRET decay is the concentration ratio of the interacting and non-interacting donor fraction. Amplitude ratio images often have a surprisingly high signal-to-noise ratio. In metabolic FLIM the a_1/a_2 ratios of NADH and FAD are indicators of the metabolic state, see chapter 'Autofluorescence FLIM of Cells and Tissue', page 248.

Classic Redox Ratio

The ratio of the intensities of an NADH image and an FAD image is the 'Redox Ratio', an important indicator of the metabolic state of a cell. In NADH/FAD FLIM data recorded by multiplexed TCSPC the intensities are available via the photon numbers, N_{nadh} and N_{fad} of the corresponding recording channels. The ratio, $N_{\text{fad}} / N_{\text{nadh}}$ is the redox ratio.

Classic FRET Efficiency, E_{class}

An image of the FLIM-based classic FRET efficiency is obtained by selecting E_{class} from the list of decay parameters. The classic FRET efficiency is

$$E_{\text{class}} = 1 - \frac{\tau_m}{\tau_2}$$

τ_m and τ_2 come from an analysis of the donor image with a double-exponential model. E_{class} is the lifetime-based equivalent of the intensity-based classic FRET efficiency. However, please keep in mind that it does not necessarily represent the real FRET efficiency. E_{class} is an

average of the real FRET efficiencies of the interacting and the non-interacting donor fraction. This is a general problem of the classic FRET approach, not a problem of SPCImage [103]. Please see 'Fluorescence Resonance Energy Transfer (FRET)', page 233.

Classic FRET Efficiency, Single-Exponential Equivalent

SPCImage offers also a classic FRET efficiency

$$E_{\text{1exp}} = 1 - \frac{\tau_i}{\tau_2}$$

It approximates the FRET efficiency that is obtained if the donor signal is treated as a single-exponential decay or measured with a FLIM system that only delivers single-exponential 'Lifetimes'. The result neither resembles the intensity-based FRET Efficiency nor the real FRET efficiency of the interacting donor fraction. It is simply wrong [103], please see 'Fluorescence Resonance Energy Transfer (FRET)', page 233. SPCImage makes it available to allow the user to compare FRET results with published data based on wrong FRET calculations.

FRET Efficiency of Interacting Donor Fraction, E_{int}

The decay components of the interacting and non-interacting donor fraction can be used to calculate the real FRET efficiency of the interacting donor fraction. The FRET efficiency is

$$E_{\text{int}} = 1 - \frac{\tau_1}{\tau_2}$$

τ_1 and τ_2 are the lifetime components from the interacting and non interacting donor, respectively. Please note that E_{int} is significantly higher than the classic FRET efficiency because it does not include a contribution from the non-interacting donor fraction. Please see 'Fluorescence Resonance Energy Transfer (FRET)', page 233. Of course, E_{int} can only be used if the decay has been analysed by a double-exponential model and the components of the double-exponential decay are clearly resolved.

FRET Distance, r/r_0

SPCImage is able to calculate the ratio of the donor-acceptor distance, r , to the Förster radius, r_0 . Please see 'Fluorescence Resonance Energy Transfer (FRET)', page 233.

Relative Intensity Contribution, q_1, q_2, q_3

q_1, q_2 , and q_3 are the relative intensity contributions of the decay components. The q values are the products of the lifetimes with their amplitude factors. The q values can be useful to visualise the distribution of different fluorophores in autofluorescence images. The q values are especially sensitive to slow lifetime components.

Scatter

The scatter is the amount of light emitted as a prompt response to the laser pulse. Except for directly scattered laser light leaking through improperly selected filters, the source may be Raman scattering, or, in two-photon excitation systems, second-harmonic generation (SHG). An image with the scatter parameter used as colour shows SHG or other prompt effects clearly, see Fig. 598. A possible fluorescence background can be reduced by using time-gating of the intensity. Please note that exact scatter determination requires the use of the real instrument-response function.

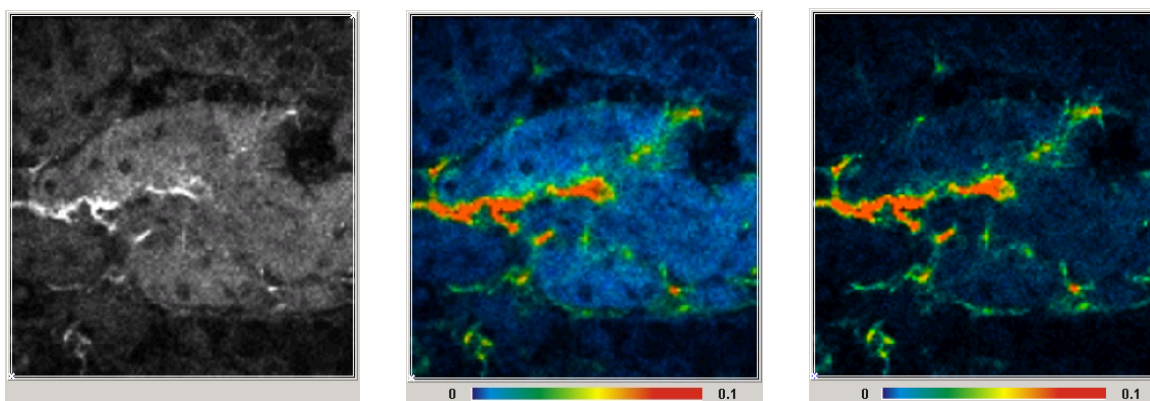


Fig. 598: Extraction of SHG signals from FLIM data. Left: Intensity image. Middle: Image with colour representing the amount of prompt response. Right: Further suppression of fluorescence background by time-gating the intensity.

Accuracy of the Fit: χ^2

The χ^2 parameter indicates the quality of the fit. A χ^2 image may be used to check whether the used model is appropriate in all areas of the image. An interesting application of the χ^2 image has been described in [301]: The decay data in a lifetime image are expected to be mono-exponential or close to mono-exponential. The data are therefore analysed by a single-exponential model. An increased χ^2 indicates that the corresponding pixels are contaminated by another fluorophore of different lifetime, usually autofluorescence. Autofluorescence by itself deviates so strongly from a single-exponential decay that it shows up clearly in a χ^2 image.

An example of a χ^2 image from a single-exponential analysis is shown in Fig. 599, left. An a_1/a_2 image of a double-exponential analysis is shown right. It can clearly be seen that large χ^2 correlates with large a_1/a_2 , i.e. with strong deviation from the single-exponential decay.

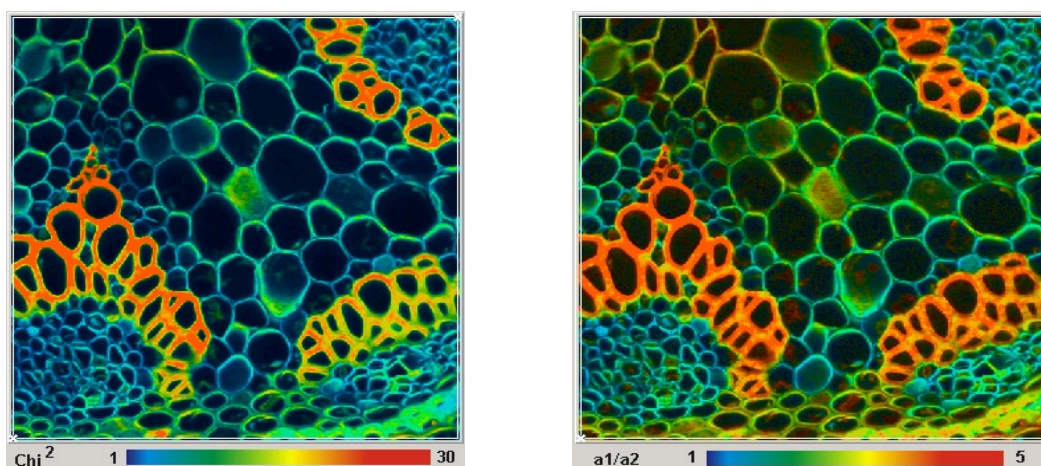


Fig. 599: χ^2 image (left) of a single-exponential analysis and a_1/a_2 image (right) of a double-exponential analysis. χ^2 is largest where a_1/a_2 is large, i.e. where the decay profile has the strongest deviations from a single-exponential function.

Offset

The offset in the decay data can be used as a colour parameter. This may be considered useless because an offset usually originates from daylight pickup or from detector background. However, an offset may also be phosphorescence. Phosphorescence lifetimes are much longer than the laser pulse period normally used for FLIM. The phosphorescence signal then piles up

over many signal periods and causes an baseline offset in the decay data. This can be used to identify phosphorescence, i.e. from Lanthanide dyes or from various nanoparticles. An example is shown in Fig. 600.

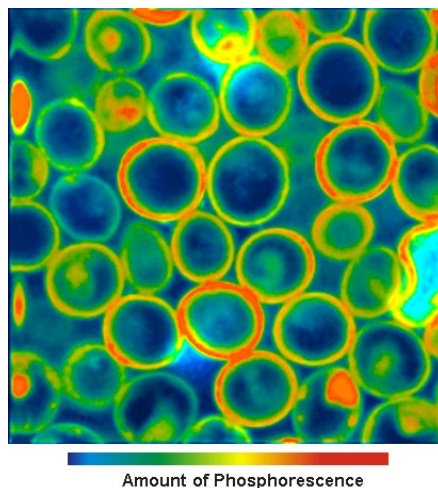


Fig. 600: Amount of phosphorescence derived from offset in decay data. Yeast cells stained with ruthenium dye.

Phasor Plot

Principle

The phasor plot is based on the analysis of the decay data in the frequency domain. A transformation of the decay data, $n(t)$, into the frequency domain delivers a complex function of frequency, $G(\omega) + iS(\omega)$ with

$$G(\omega) = \frac{1}{N} \sum \cos(\omega t) n(t) \quad \text{and} \quad S(\omega) = \frac{1}{N} \sum \sin(\omega t) n(t)$$

As it turns out, a good representation of the decay function is obtained already if only G and S at the fundamental repetition frequency (the first Fourier component) are used. The decay function is then described by just two numbers, G and S , for $\omega = 2\pi/T$, where T is signal period of the fluorescence signal. Because G and S are no longer functions of ω in this case they can also be considered weighted moments of the decay functions, $n(t)$, where the weight functions are cosine and sine functions.

$$G = \frac{1}{N} \sum \cos(2\pi t / T) n(t) \quad \text{and} \quad S = \frac{1}{N} \sum \sin(2\pi t / T) n(t)$$

The data points $G + iS$, can also be expressed by the magnitude, M , and the phase, φ , in the plane of complex numbers:

$$M = \sqrt{G^2 + S^2} \quad \varphi = \arctan S / G$$

IRF

For analysis of real measurement data still the IRF has to be included in the calculation. In the complex Fourier data plane the convolution integral transforms into a multiplication i.e.

$$G + iS = (G_{fl} + S_{fl}) \cdot (G_{irf} + iS_{irf}) \quad \text{or} \\ (G_{fl} + iS_{fl}) = (G + iS) / (G_{irf} + iS_{irf})$$

which can also be written as

$$G_{fl} = G \frac{\cos \varphi_{irf}}{M_{irf}} + S \frac{\sin \varphi_{irf}}{M_{irf}} \quad S_{fl} = -G \frac{\sin \varphi_{irf}}{M_{irf}} + S \frac{\cos \varphi_{irf}}{M_{irf}}$$

In this form, the representation of a decay curve is called a 'phasor', and a density plot of the phasors of all pixels of an image or an image area is called a 'phasor plot' [183, 184].

Relationship between Decay Curves and Phasors

The relationship between different decay curves and their phasors is illustrated in Fig. 601. The phasors of single-exponential decays are located on a semi circle in the G - S plane. The phasor of a fast decay (a) is located on the right, at low phase and large amplitude. The phasor of a slow decay (b) is located farther left, at larger phase and lower amplitude. The phasor of a double-exponential decay (c) is a linear combination of the phasors of the decay components. It is located inside the semicircle.

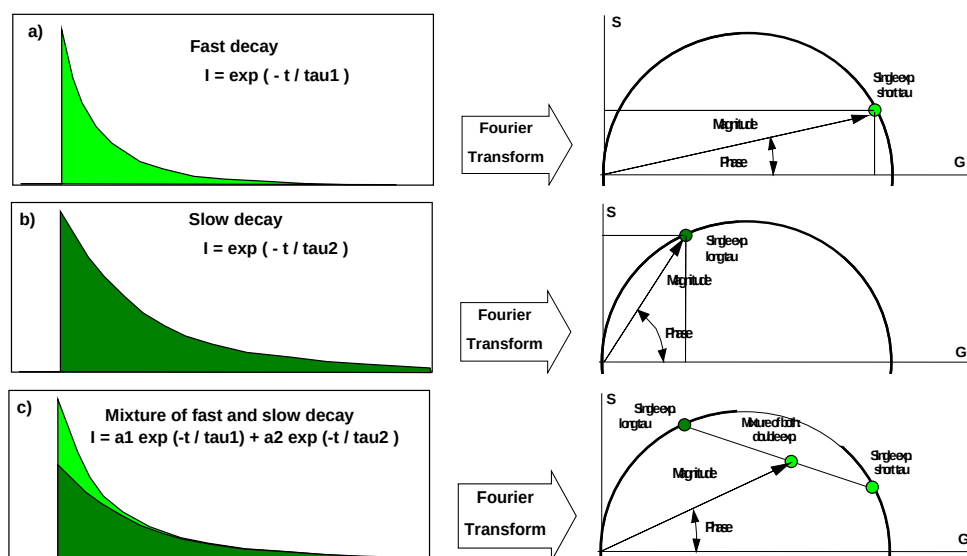


Fig. 601: Relationship between decay functions in the time domain (left) and phasors in the frequency domain (right)

A fluorescence-lifetime image and the corresponding phasor plot are shown in Fig. 602. The pixels of objects with different decay functions in the FLIM image (left) form clusters in the phasor plot (right).

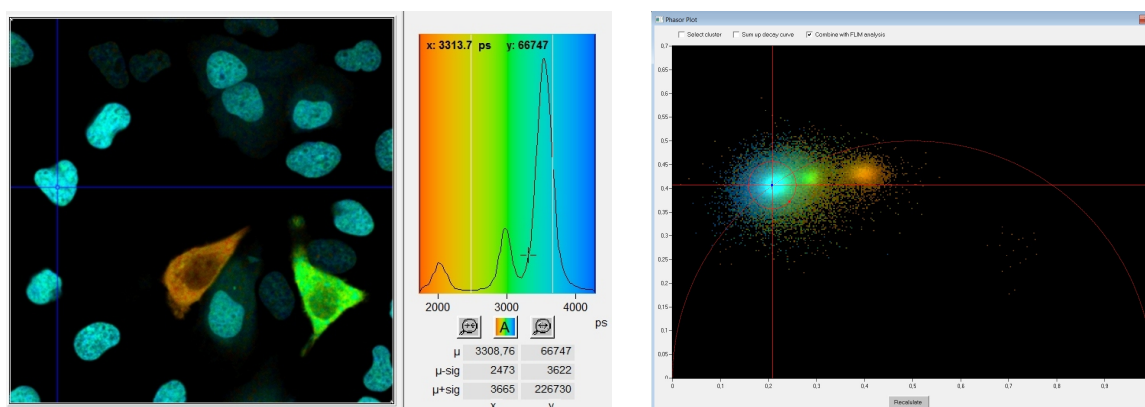


Fig. 602: Lifetime image (left) and phasor plot (right). Pixels with similar decay profiles form clusters in the phasor plot.

Different phasor clusters can be selected see Fig. 603 and Fig. 604, left, and the corresponding pixels back-annotated in the time-domain FLIM images, see Fig. 603 and Fig. 604, right. SPCImage is able to calculate combined decay curves over the selected areas (Fig. 603 and Fig. 604, middle), or create decay parameter histograms over the selected areas.

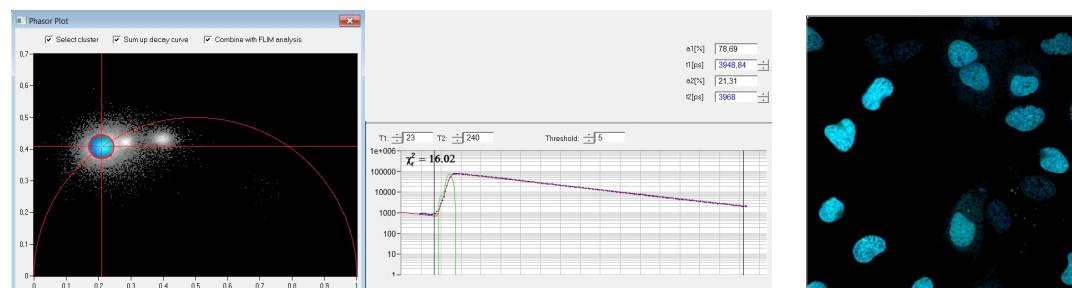


Fig. 603: Left: Selecting a cluster of phasors in the phasor plot. Middle: Combination of the decay data of the corresponding pixels in a single decay curve. Right: Display of the pixels corresponding to the selected cluster in the phasor plot.

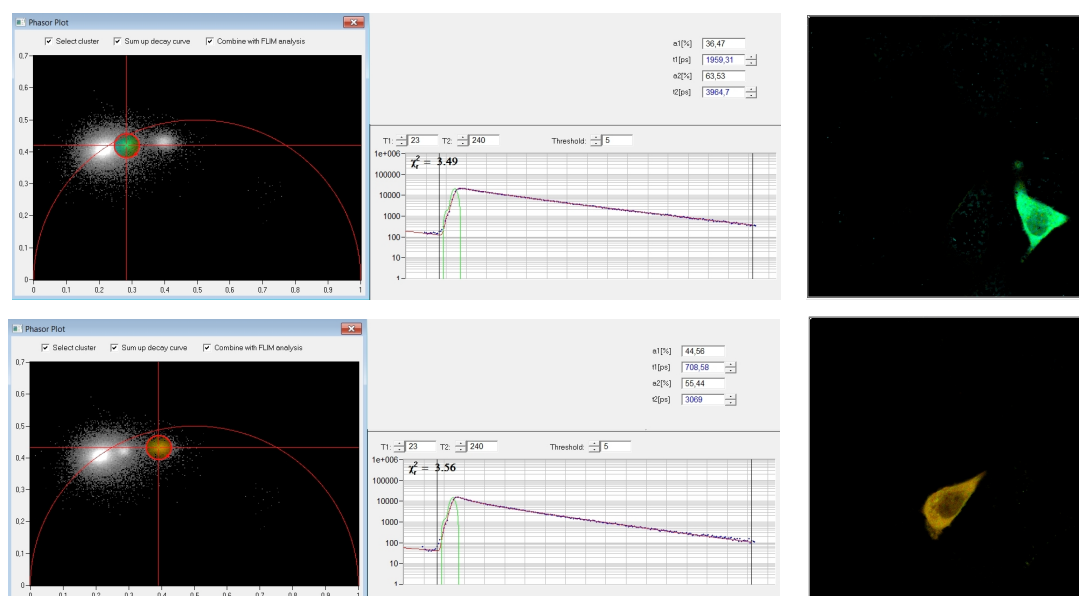


Fig. 604: As figure above but other phasor clusters selected

Truncation Effects

As all moment-based lifetime techniques, phasor analysis is sensitive to truncation effects. If the signal is not recorded over the full signal period, photons at the end of the period may be not included in the phasor calculation, with the result that the phasor is not correct. Recording intervals shorter than the signal period is a common situation in TCSPC FLIM. The time measurement principle covers just a bit less than one signal period, and recording two signal periods and extracting just one period from the data would dramatically decrease the photon efficiency. Moreover, TCSPC decay functions are usually analysed by fit procedures in the time domain. Recording the full signal period is not necessary in this case. It is more important that the photons are recorded into sufficiently small time channels, especially when fast decay components are present.

The effect of recording an incomplete signal period is illustrated in Fig. 605. As long as the fluorescence decays completely in the observation time interval (Fig. 605, left) there are no photons in the last (un-recorded) part of the signal period. Phasors calculated from the data are correct. If the fluorescence does not fully decay within the observation time photons in the last part of the signal period are not included in the calculation of the phasor (Fig. 605, right). Both G and S become larger (note that the weight function for S is negative in the truncated part), therefore the phasor shifts to left and (to a lesser extend) up in the phasor diagram.

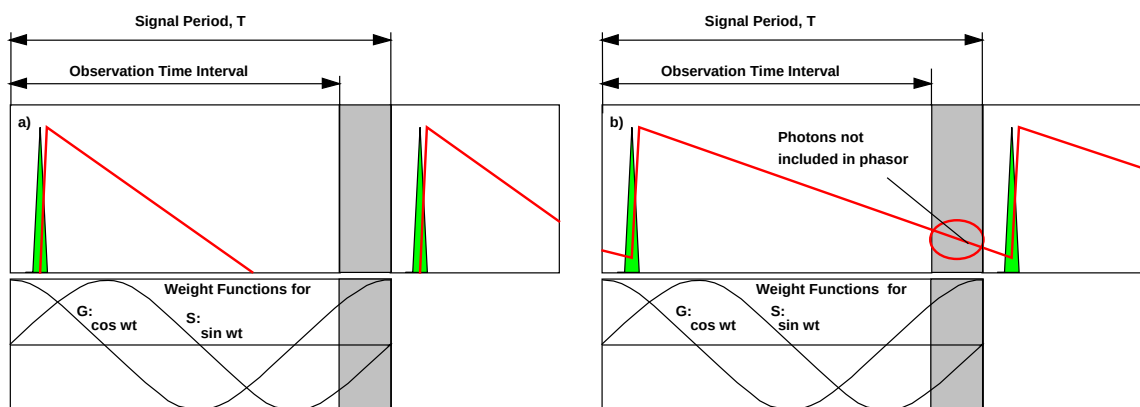


Fig. 605: Systematic errors can occur if the fluorescence does not entirely decay in the observation time interval

In practice, truncation effects have little influence on the performance of the phasor plot in SPCImage. SPCImage uses the phasor plot mainly for image segmentation. Decay parameters are derived from MLE processing in the time domain. Lifetimes and amplitudes are therefore correct even in presence of truncation effects.

When the phasor plot is used in combination with the incomplete-decay model SPCImage detects possible truncation situations. It then fills the missing part of the decay data by data points from the fitted model function, see Fig. 606. Of course, this substitution works precisely only if the correct model function is used and a time-domain analysis has been performed before the phasor plot is calculated.

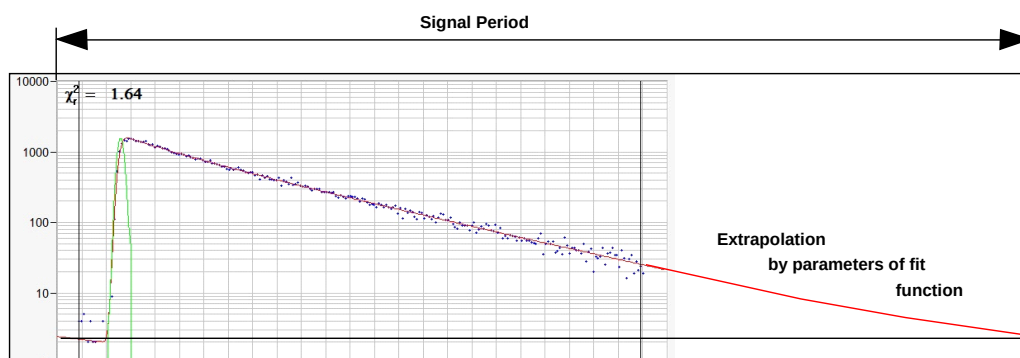


Fig. 606: Filling of the missing part of the decay data by data points from the fitted model function.

Ambiguity of the Phasor Representation

Another rarely described feature is the ambiguity of the phasor representation. Unless a phasor is located directly on the semicircle (i.e. the decay function is single-exponential) it does not unambiguously represent a particular decay profile. As shown schematically in Fig. 607, a given phasor can be obtained for different combinations of component lifetimes and amplitudes.

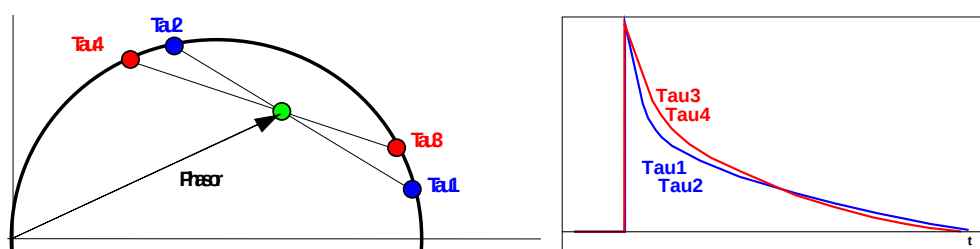


Fig. 607: Ambiguity of the phasor representation. A given phasor within the semicircle can represent different decay profiles.

Possible ambiguity should be taken into consideration if pixels of similar phasor signature are combined into a single decay curve. The combined curve can, in principle, contain decay components from different decay curves with the same phasor. It need not strictly represent the decay profile of every individual pixel within the selected image area.

Specifications

General

Principle

FLIM by Multi-Dimensional TCSPC
 One-photon excitation with confocal detection
 Multiphoton excitation with non-descanned detection
 Classic FLIM in two parallel channels
 FLIM with laser multiplexing
 Multi-wavelength FLIM
 Simultaneous FLIM / PLIM
 Time-series FLIM
 Z stack FLIM
 Spatial Mosaic FLIM
 Temporal Mosaic FLIM

Basic imaging modes

Scan head

Optical principle

Laser inputs

Laser power regulation, optical

Outputs to detectors

Main beamsplitter versions

Secondary beamsplitter wheel

Pinholes

Pinhole size

Emission filters

Connection to microscope

Coupling of lasers into scan head (visible)

Coupling of laser into scan head (Ti:Sa)

bh DCS-120 scan head
 confocal, beam scanning by fast galvanometer mirrors
 two independent inputs, fibre coupled or free beam
 continuously variable via neutral-density filter wheels
 two outputs, detectors are directly attached
 multi-band dichroic, wideband, multiphoton
 3 dichroic beamsplitters, polarising beamsplitter
 100% to channel1, 100% to channel2
 independent pinhole wheel for each channel
 11 pinholes, from about 0.5 to 10 AU
 2 filter sliders per channel
 adapter to left side port or port on top of microscope
 single-mode fibres, Point-Source type, separate for each laser
 free beam, 1 to 2 mm diameter

Scan Controller

Principle

Scan waveform

Scan format

Frame size, frame scan

Line size, line scan

X scan

Y scan

Number of lasers

Laser power control, electrical

Laser multiplexing

Beam blanking

Scan rate

minimum pixel time for frame size

Zoom=1

Zoom=8

minimum frame time for frame size

Zoom=1

Zoom=8

Scan area definition

Fast preview function

Beam park function

Laser control

bh GVD-120 or GVD-140
 Digital waveform generation, scan waveforms generated by hardware
 linear ramp with cycloid flyback
 line, frame, or single point
 16x16 to 2048x2048 pixels
 16 to 2048 pixels
 continuous or pixel-by-pixel
 line by line
 2 4
 via electrical signal to lasers
 frame by frame, line by line, or within one pixel
 during flyback and when scan is stopped
 automatic selection of fastest rate or manual selection

	64x64	128x128	256x256	512x512	1024x1024	2048x2048
15.6µs	12.8µs	6.4µs	3.2µs	1.6µs	1.2µs	
6.4µs	3.2µs	1.6µs	0.8µs	0.6µs	0.5µs	
64x64	128x128	256x256	512x512	1024x1024	2048x2048	
0.19s	0.37s	0.64s	1.24s	2.6s	6.5s	
0.037s	0.074s	0.173s	0.320s	1.0s	2.7s	

 via zoom and offset or interactive via cursors during preview
 1 second per frame, 128 x 128 pixels
 via cursor in preview image or cursor in FLIM image
 2 Lasers, on/off, frame, line, pxl multiplexing

Diode lasers

Number of lasers simultaneously operated

Wavelengths, standard

Wavelengths, optional

Mode of operation

Pulse width, typical

Pulse frequency

Power in picosecond mode

Power in CW mode

bh BDS-SM lasers
 2
 405nm and 473nm or 405nm and 488nm
 375nm, 445nm, 510nm, 640nm, 685nm, 785nm
 picosecond pulses or CW
 40 to 70 ps
 selectable, 20MHZ, 50MHZ, 80MHZ
 0.25mW to 1mW injected into fibre. Depends on wavelength version.
 10 to 40mW injected into fibre. Depends on wavelength version.

Femtosecond Lasers for Multiphoton Excitation**Femtosecond fibre lasers**

Coupling	free beam, diameter 1 to 2 mm
Wavelengths	780 nm, 920 nm

Ti:Sa Laser

Coupling	free beam, diameter 1 to 2 mm
Wavelength	740 to 1200 nm

Detectors (standard)

No. of detectors	2
Spectral Range	300 to 710nm
Cathode type	GaAsP
Peak quantum efficiency	40 to 50%
IRF width with bh diode laser	120 to 130 ps
Detector area	3mm
Background count rate, thermal	300 to 2000 counts per second
Background from afterpulsing	not detectable
Power supply and overload shutdown	via DCC-100 controller of TCSPC system

bh HPM-100-40 hybrid detector**Ultra-fast hybrid detectors (optional)**

Spectral Range	bh HPM-100-06 and -07 hybrid detector -06: 300 to 600nm -07: 300 to 750nm
Peak quantum efficiency	20 %
IRF width (fwhm, with Ti:Sa laser)	19 ps
Detector area	3mm
Background count rate, thermal	100 to 500 counts per second
Background from afterpulsing	not detectable
Power supply and overload shutdown	via DCC-100 controller of TCSPC system

Detectors (optional)

Spectral Range	400 to 900nm
Cathode type	GaAs
Peak quantum efficiency	15 to 20%
IRF width with bh diode laser	130 to 180 ps
Detector area	3mm
Background count rate, thermal	1000 to 8000 counts per second
Background from afterpulsing	not detectable
Power supply and overload shutdown	via DCC-100 controller of TCSPC system

bh HPM-100-50 hybrid detector**Detectors (optional)**

Spectral range	380 to 700nm
Cathode type	GaAsP
Number of wavelength channels	16
Spectral width of wavelength channels	12.5 nm
IRF width with bh diode laser	250 ps
Power supply and overload shutdown	via DCC-100 controller of TCSPC system

bh Multi-Wavelength GaAsP FLIM detector**TCSPC System**

Number of parallel modules (recording channels)	bh SPC-180NX	or	SPC-QC-104
Number of recording channels per module	2		1
Number of detector (routing) channels	1		3
Principle	16		
Electrical time resolution	TAC/ADC principle		TDC Principle
Minimum time channel width	2 ps rms		15 ps rms
Dead time	405 fs		4 ps
Saturated count rate, per channel	100 ns		20 ns
Dual-time-base operation	10 MHz		100 MHz
Source of macro time clock	via micro times from TAC and		via macro time clock
Input from detector	internal 40MHz or clock or from laser		400 MHz
Reference (SYNC) input	constant-fraction discriminator		constant-fraction discriminator
Synchronisation with scanning	via frame clock, line clock and pixel clock pulses		
Scan rate	any scan rate		
Synchronisation with laser multiplexing	via routing function		
Recording of multi-wavelength data	simultaneous, via routing function		
Basic acquisition principles	on-board-buildup of photon distributions		buildup of photon distributions in computer memory

Operation modes	generation of parameter-tagged single-photon data		
	online auto or cross correlation and PCH		
	f(t), oscilloscope, f(txy), f(t,T), f(t) continuous flow		
	FIFO (correlation / FCS / MCS) mode		
	Scan Sync In imaging, Scan Sync In with continuous flow		
	FIFO imaging, with MCS imaging, mosaic imaging, time-series imaging		
	Multi-detector operation, laser multiplexing operation		
	cycle and repeat function, autosave function		
Max. Image size, pixels (SPCM 64 bit software)	2048x2048	1024x1024	512x512
No of time channels, see [77]	256	1024	4096

Data Acquisition Software, please see [77] for details

Operating system	Windows 10 or Windows 11, 64 bit
Loading of system configuration	single click in predefined setup panel
Start / stop of measurement	by operator or by timer, starts with start of scan, stops with end of frame
Online calculation and display, FLIM, PLIM	in intervals of Display Time, min. 1 second
Online calculation and display, FCS, PCH	in intervals of Display Time, min. 1 second
Number of images displayed simultaneously	max 8
Number of curves (Decay, FCS, PCH, Multiscaler)	8 in one curve window
Cycle, repeat, autosave functions	user-defined, used for for time-series recording, Z stack FLIM, microscope-controlled time series
Saving of measurement data	User command or autosave function
Link to SPCImage data analysis	Optional saving of parameter-tagged single-photon data automatically after end of measurement or by user command

Data Analysis: bh SPCImage, integrated in bh TCSPC software package

Data types processed	FLIM, PLIM, MW FLIM, time-series, Z stacks, single curves
Procedure	MLE fit with iterative convolution or first-moment calculation
	Global fit
Processing	GPU based
IRF	synthetic IRF or measured IRF
Model functions	single, double, triple exponential decay single, double, triple exponential incomplete decay models
Parameters displayed	amplitude- or intensity-weighted average of component lifetimes ratios of lifetimes or amplitudes, fractional intensities of components or ratios of fractional intensities parameter distributions FRET efficiency, FRET distance, Interacting FRET fraction Metabolic state, FLIRR
Parameter histograms, one-dimensional	Pixel frequency over any decay parameter or ratio of decay parameters
Parameter histograms, two-dimensional	Pixel frequency over two decay parameters
Phasor plot	Pixel frequency in amplitude / phase plane Simultaneous with display of lifetime images and decay curves Selection of clusters and decay analysis of combined pixels

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