Metabolic FLIM with Simultaneous pH Imaging

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Abstract: We demonstrate a technique that combines metabolic imaging by NAD(P)H with pH imaging by BCECF (2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein). For excitation we used a femtosecond fibre laser with an emission wavelength of 785 nm. Two-photon excitation with this laser excites both NAD(P)H and BCECF at reasonable efficiency. The fluorescence of the two compounds was split spectrally on the emission side and recorded in two parallel TCSPC FLIM channels. Analysis by bh SPCImage delivers one image for the metabolic state and another for the pH of the tissue.

Technical Background

Metabolic FLIM is based on recording high-resolution lifetime images of the co-enzymes NAD(P)H and/or FAD [1, 3]. In cells and tissues, both compounds exist in a bound and in an unbound form. The fluorescence lifetimes of the bound and unbound forms are different and can be distinguished in high-quality FLIM data: The bound fraction of NAD(P)H has longer lifetime than the unbound one. For FAD it is the opposite. The bound fraction is the fast one, the unbound fraction the slow one [1]. Metabolic imaging is based on the fact that the bound/unbound ratio depends on the metabolic state. Cells running oxidative phosphorylation have lower bound/unbound ratios than cells running glycolysis [6]. Thus, the type of the metabolism is reflected in the apparent fluorescence decay time. The problem of this simple decay-time based approach is that the lifetimes of the decay parameters depend also on other cell parameters, such as mitochondrial pH. A better indicator of the metabolic state is therefore the amplitude ratio of the decay components, a1/a2, (the 'metabolic ratio'), or the amplitude of the fast component, a1, (the 'metabolic indicator). Please see [1] for details. The preferred endogenous coenzyme for this approach is NAD(P)H because the fluorescence decay can be reasonably described by a double-exponential decay model. For FAD this is not exactly the case because the recorded signal contains a contribution from FMN [5].

It has been found that NAD(P)H lifetimes in the mitochondria of cells 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 [7]. 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.

A good candidate for a pH sensitive fluorophore is BCECF (2′,7′-bis-(2-carboxyethyl)-5(6) carboxyfluorescein). We choose it because it has a relatively large pH dependence of its fluorescence lifetime [8], 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.

FLIM System

For the experiments described here we used a Becker & Hickl DCS-120 MP two-photon FLIM system with a femtosecond fibre laser [4]. 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 by the usual Becker & Hickl multi-dimensional TCSPC-FLIM process [1]. With the HPM-100-40 detectors and the SPC-180NX FLIM modules used in our FLIM setup the IRF width is about 85 ps, full width at half maximum.

Results

A typical result is shown in Fig. 1. The figure shows a lifetime image of the NAD(P)H on the left and a lifetime image of the BCECF on the right. Both images were obtained by analysis with a double-exponential model function,

$$f(t) = a_1 e^{-t/\tau 1} + a_2 e^{-t/\tau 2}$$

The amplitude-weighted lifetime, τ_m, and the intensity-weighted lifetime, τ_i, are

$$\tau_m = a_1\tau_1 + a_2\tau_2 \quad \text{with} \quad a_1 + a_2 = 1 \qquad \tau_i = \frac{a_1\tau_1^2 + a_2\tau_2^2}{a_1\tau_1 + a_2\tau_2} \quad \text{with} \quad a_1 + a_2 = 1$$

The NAD(P)H image 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 [8]. The τ-pH curve published in [8] therefore applies also to the BCECF lifetimes in Fig. 1, right.

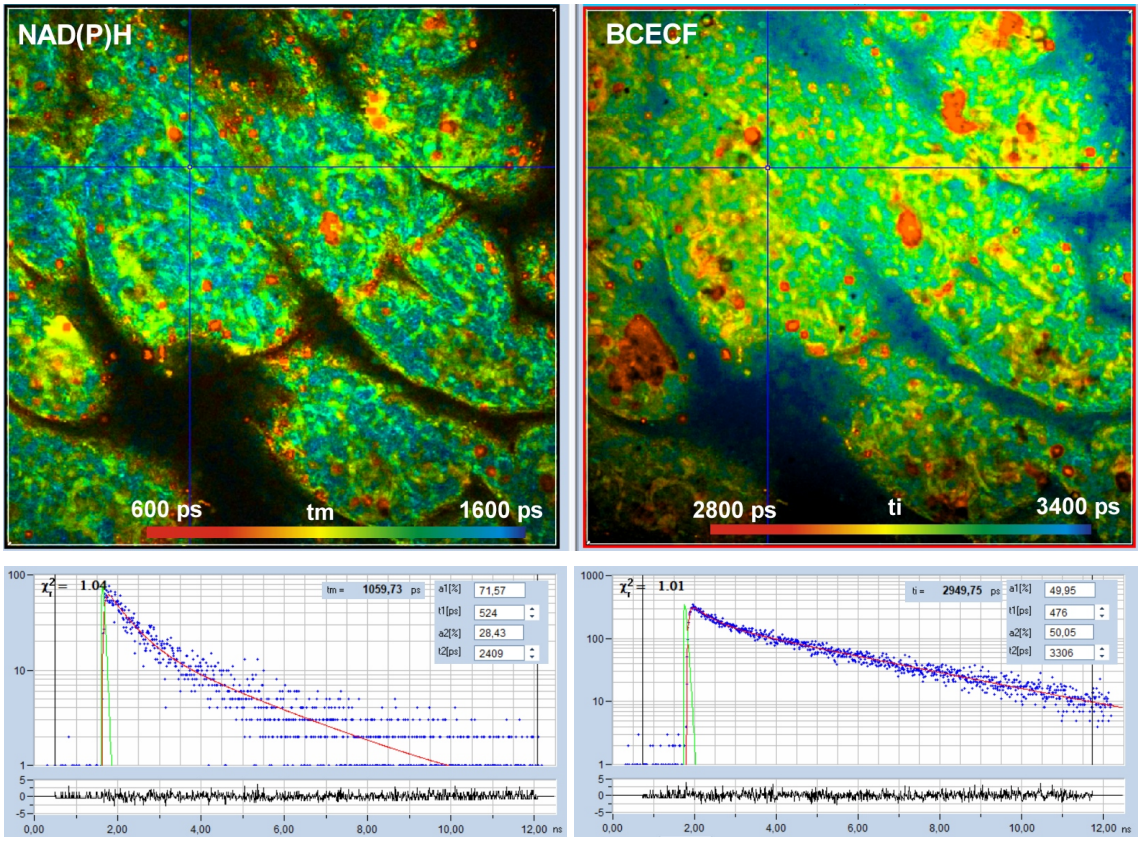

Fig. 1: Upper row: NAD(P)H image of the amplitude-weighted lifetime, t_m, and intensity-weighted lifetime image of BCECF. Lower row: Decay function of NAD(P)H and decay function of BCECF at cursor position. Both images 512 x 512 pixels, 1024 time channels. Data analysis with bh SPCImage NG, MLE fit.

Fig. 1 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 tissue. A more reliable way to characterise the metabolic state and the pH is to use the amplitude, a_1, or the amplitude ratio, a_1/a_2 as an image parameter: For NAD(P)H the primary change is in the fraction of the bound and unbound NAD(P)H, for the BCECF it is in the fraction of protonated and deprotonated BCECF. That means the change in the decay functions is not in the component lifetimes, τ_1 and τ_2, but in the component *amplitudes* a_1 and a_2. When the amplitudes are used as characterisation parameters the result is not influenced by possible changes in the component lifetimes. For many years, amplitude-based approaches in FLIM were considered impossible due to instability in the fit procedures. However, with the MLE fit of Becker & Hickl SPCImage NG software [1, 2] fit instability is no longer a problem. Images of the amplitude, a_1, of the fast decay component for NAD(P)H and BCECF are shown in Fig. 2. As can be seen, there is no indication of excessive noise or fit instability in the images.

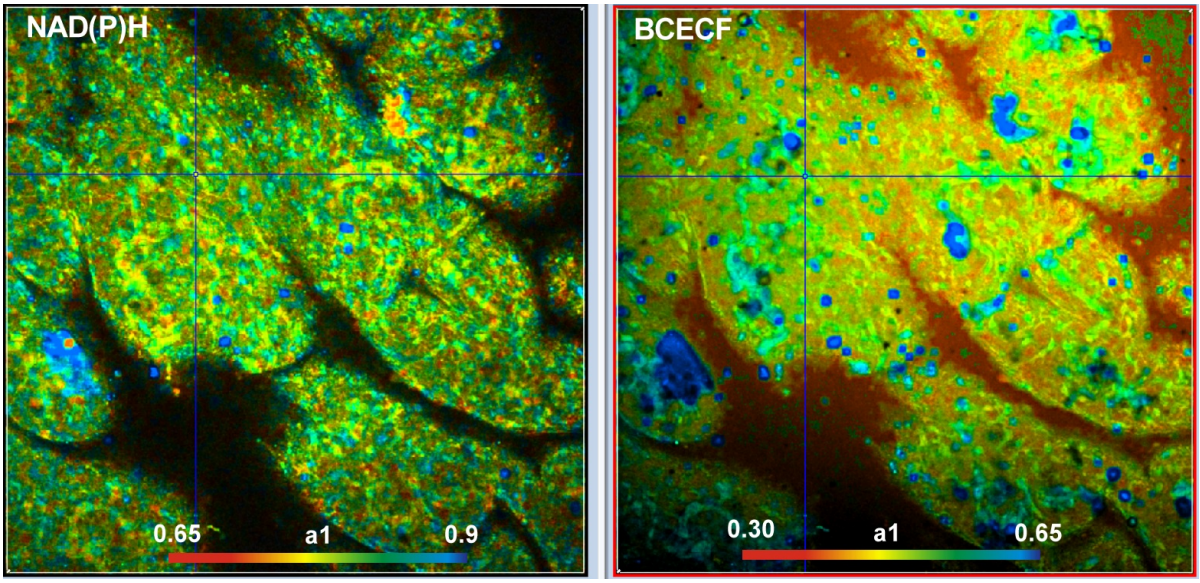

Fig. 2: NAD(P)H image (left) and BCECF image (right) of the amplitude, a_1, of the fast decay component. In the NAD(P)H image a1 represents the fraction of unbound NAD(P)H, in the BCECF image it represents the amount of protonated BCECF.

A Possible Pitfall

Excitation of NAD(P)H inevitably also excites FAD. The emission spectrum of FAD overlaps that of BCECF. Therefore, part of the FAD emission can be mixed into the signal from BCECF. Fortunately, the BCECF signal is (or can easily be made) at least an order of magnitude stronger than the FAD signal. Nevertheless, it is recommended to check whether what is detected in the BCECF channel is really fluorescence from BCECF. This can easily be done by recording images whithout BCECF.

Summary

The approach described in this application note is an extension of metabolic FLIM. By recording a pH image in parallel with an NAD(P)H image additional information on the state of cells and tissues is obtained. Although described for the bh fibre-laser based two photon system the approach can be used for virtually all bh multiphoton FLIM systems. All these systems have two parallel

FLIM channels with independent detectors and TCSPC modules, and all the systems use or can be upgraded to SPCImage NG FLIM data analysis software.

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