Two-Photon FLIM with a Femtosecond Fibre Laser

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We demonstrate two-photon FLIM with a femtosecond fibre laser. Our system consists of a bh DCS-120 scanner attached to a Nikon TE microscope, a 785 nm Toptica Femto Fibre Pro laser, HPM-100 hybrid detectors in a non-descanned beam configuration, and a TCSPC FLIM system with two SPC-150NX TCSPC modules, a GVD-120 scan controller, and a DCC-100 detector controller. We show that the system is perfectly suited to record NADH FLIM data from cells and tissue. It also records autofluorescence images from small organisms. FLIM images are also obtained from stained specimens, such as the Invitrogen BPAE cell and mouse kidney samples.

Motivation

Compared with the classic one-photon excitation, two-photon excitation has a number of advantages. The excitation wavelength is in the NIR, where direct absorption and scattering is lower than in the visible or UV range. Therefore deeper layers in the sample can be reached. Due to the nonlinearity of the two-photon process, excitation occurs preferentially in the focus. Therefore, no pinhole is required to reject out-of-focus fluorescence and scattered light. Consequently, the fluorescence light can be diverted directly behind the microscope lens and sent to the detectors directly. The principle is called 'non-descanned' detection, or NDD. The advantage is not only that losses on the way back through the scanner optics are avoided. More importantly, fluorescence photons which are scattered on the way out of the sample can be collected efficiently and transferred to the detectors. Another advantage is that fluorophores with short excitation wavelengths, such as NADH, can be excited without the need of UV-optimised optics.

The ideal excitation source for two-photon laser scanning microscopes is the titanium-sapphire (Ti:Sa) laser. It delivers high power, short pulse width, and, most importantly, is tuneable in a range from about 750 nm to 1000 nm. On the negative side, Ti:Sa lasers are bulky, expensive, and need frequent maintenance.

In the last decade, more and more femtosecond fibre lasers have become available. Fibre lasers are compact, less expensive than Ti:Sa lasers, and don't need much maintenance. The pulse width is on the orders of 100 fs, which is adequate for two-photon excitation in a microscope. The concern against the fibre lasers is that the power may be insufficient for two-photon excitation, and that they are not tuneable. We will show that both concerns are not generally justified, and that a laser scanning microscope with a fs fibre laser can be used for a wide range of applications.

DCS-120 MP System with Femtosecond Fibre Laser

To demonstrate the capabilities of two-photon FLIM with fibre lasers we used our DCS-120 MP system [1]. The optical part consists of a DCS-120 scan head, a Nikon TE 2000 microscope, a Toptica 785 nm Femto Fibre Pro laser, and two HPM-100 hybrid detectors [2]. The principle is shown in Fig. 1. The laser delivers 120 mW of optical power at a wavelength of 785 nm. The laser beam passes a shutter and a variable attenuator. It is projected into the microscope by a DCS-120 scan head [1]. The fluorescence light is collected back through the microscope lens, separated from the excitation light by a dichroic beamsplitter in the filter carousel of the microscope, and sent out through the back port of the microscope. It passes a laser blocking filter and is split into two spectral components by a standard microscopy beam splitter cube. Each spectral component is fed to a separate detector via a bandpass filter. The signals are recorded by a standard dual-channel bh SPC-150NX TCSPC system [2].

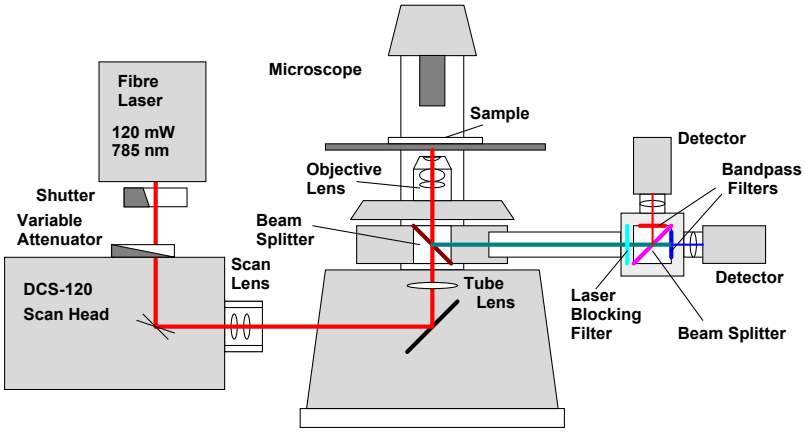

Fig. 1: Optical principle of the 2-photon FLIM system

Results

NADH FLIM

With its 785 nm excitation wavelength the system is perfectly adapted to FLIM of NAD(P)H in live systems. The fluorescence decay function of NAD(P)H bears information on the metabolic state of cells and tissues. The primary information is in the amplitudes of the fast and slow decay component, as we have shown in [2]. These can only be obtained by double-exponential analysis of the decay function. NADH FLIM therefore requires efficient detection of the photons, recording of the entire decay function at high time resolution [3], and efficient data analysis [2, 4]. This is exactly what the bh FLIM systems are designed for [2].

NADH FLIM data of yeast cells are shown in Fig. 2. The data were acquired through a NA = 1.3 oil immersion lens and a 420 to 470 nm bandpass filter. A colour-coded image of the mean (amplitude weighted) lifetime, tm, is shown on the left. The histogram of tm over pixels is shown on the right. The decay curve in 5x5 pixel area around cursor position is shown in the lower right.

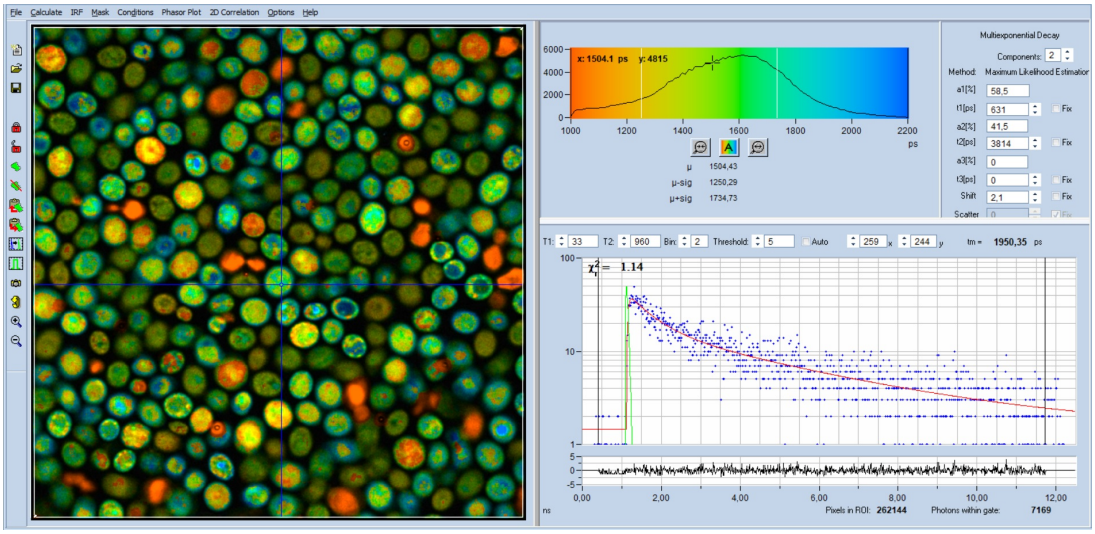

Fig. 2: FLIM data of yeast cells, data format 512 x 512 pixels, 1024 time channels. Analysis with SPCImage NG, double-exponential fit with MLE. Left: Image of mean (amplitude weighted) lifetime, tm. Upper right: Histogram of tm over pixels. Lower right: Decay curve in 5 x 5 pixel area around cursor position.

The decay parameters, shown in the upper right, are typical of NADH. The fast component (t1 = 631 ps) comes from the free NADH, the slow component (T2 = 3914 ps) from the bound NADH. The amplitude (a1 = 58.5 %) indicates a viable cell with normal metabolism [2].

The quality of the decay data is high enough to derive images of the individual decay components from the data. Images of the component lifetimes, t1 and t2, and of the fast-component amplitude, a1, are shown in Fig. 3.

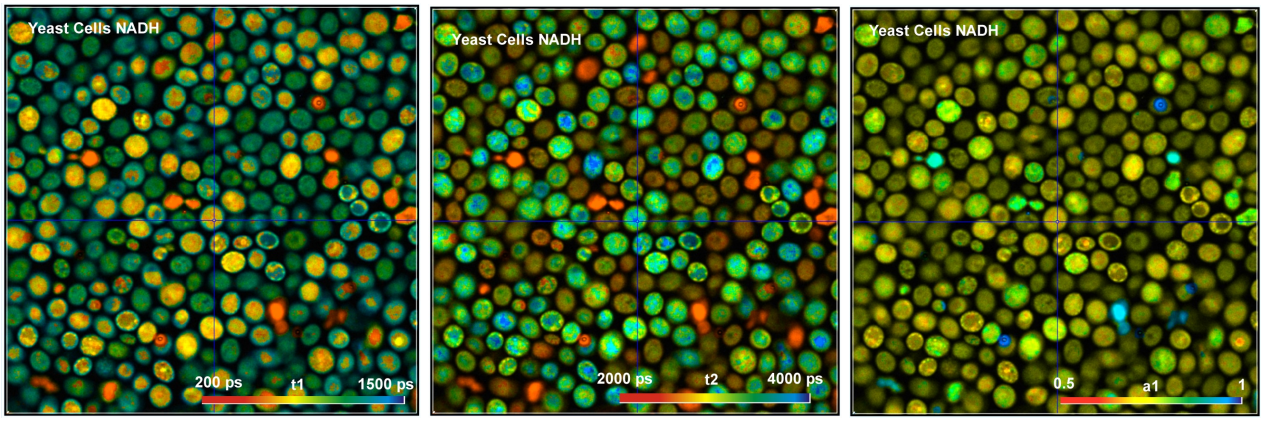

Fig. 3: Yeast cells. Left to right: Images of lifetime of fast decay component, lifetime of slow decay component, and amplitude of fast component.

The data were acquired at a count rate of 300,000 photons per second (average over the entire image). The laser power was about 6 mW. The acquisition time was 2 minutes. No change in the photon rate was observed within this time. In principle, higher count rates (and thus shorter acquisition times for the same number of photons) can easily be obtained by increasing the excitation power. However, power in excess of 6 mW quickly induces changes in the decay behaviour, and, finally, catastrophic destruction of the cells. It should be noted that this is not a special feature of FLIM or of the instrument described here. The limitation of the emission rate is inherent to the sample itself, and a common problem in all autofluorescence experiments.

Fig. 4 shows tm FLIM images of pig skin. The images were acquired through the same filter as the yeast cells. An x20 NA = 0.5 objective lens was used to obtain a large field of view. The FLIM data format is 512 x 512 pixels, 1024 time channels.

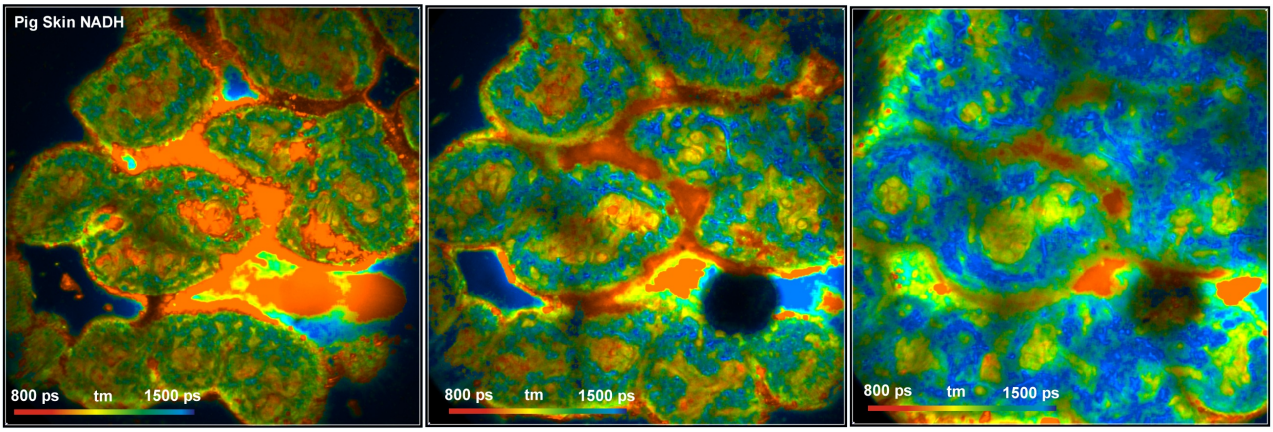

Fig. 4: Pig Skin, through 420 to 470 nm filter. Different depth in tissue. Amplitude-weighted lifetime of double-exponential decay

The images do not only contain fluorescence from NADH but also from other tissue constituents, such as collagen. The laser power was adjusted to obtain an average count rate of 1 MHz to 2 MHz over the entire image. The laser power under these conditions was 4 mW to 6 mW, depending on the depth in the tissue. Higher excitation power induces changes in the sample, such as warp in spatial dimensions, a gradual decrease in the emission intensity and a decrease in the average lifetime.

Autofluorescence FLIM of Small Organisms

Fig. 5 shows a lifetime image of a brine shrimp (*artemia salinas*). The image was recorded through an x20 NA = 0.5 microscope lens without a bandpass filter. The FLIM data format was 512 x 512 pixels, 1024 time channels. Fluorescence decay functions of different areas are shown on the right. Because no bandpass filter was used the data contain not only fluorescence but also SHG. SHG delivers ultra-fast decay signals, as can be seen in the upper decay curve in Fig. 5.

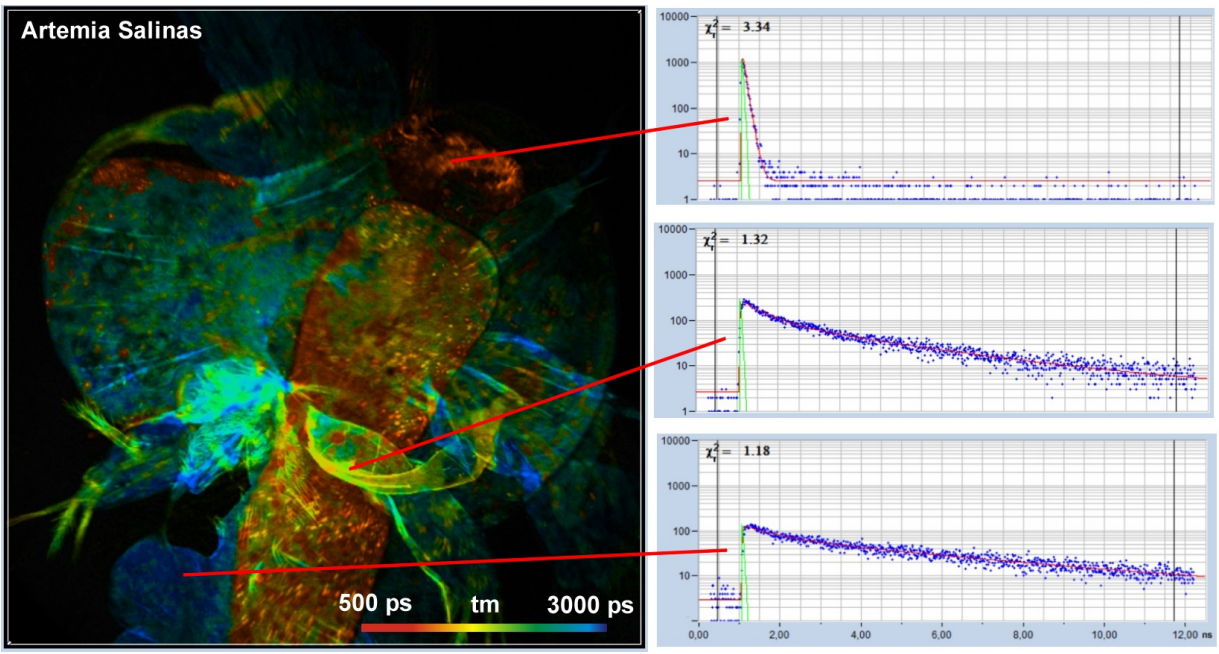

Fig. 5: Autofluorescence FLIM image of a brine shrimp, *artemia salinas*. Mean (amplitude-weighted) lifetime of double-exponential decay. Decay functions of selected areas shown on the right.

The excitation power for the acquisition of the image shown in Fig. 5 was less than 4 mW. Despite the relatively low NA of the objective lens and the correspondingly low collection efficiency, the average count rate was more than 1 MHz.

FLIM of Samples with Exogenous Fluorophores

To show that the objections against non-tuneable lasers are not generally justified we recorded a few FLIM images of samples with exogenous fluorophores. Fig. 6, Fig. 7, and Fig. 8 show tm images of the commonly used convallaria sample (stained with acridine orange), of an Invitrogen BPEA Cell sample (Alexa 488), and of an Invitrogen mouse kidney sample. All images have a FLIM format of 1024 x 1024 pixels, 1024 time channels. An objective lens of NA = 0.5 and x20 magnification was used to obtain a wide field of view. Despite the relatively low NA strong signals

were obtained at a laser power of no more than 5 mW. This indicates that a 785 nm fibre laser can be used for two-photon excitation of a wide range of fluorophores.

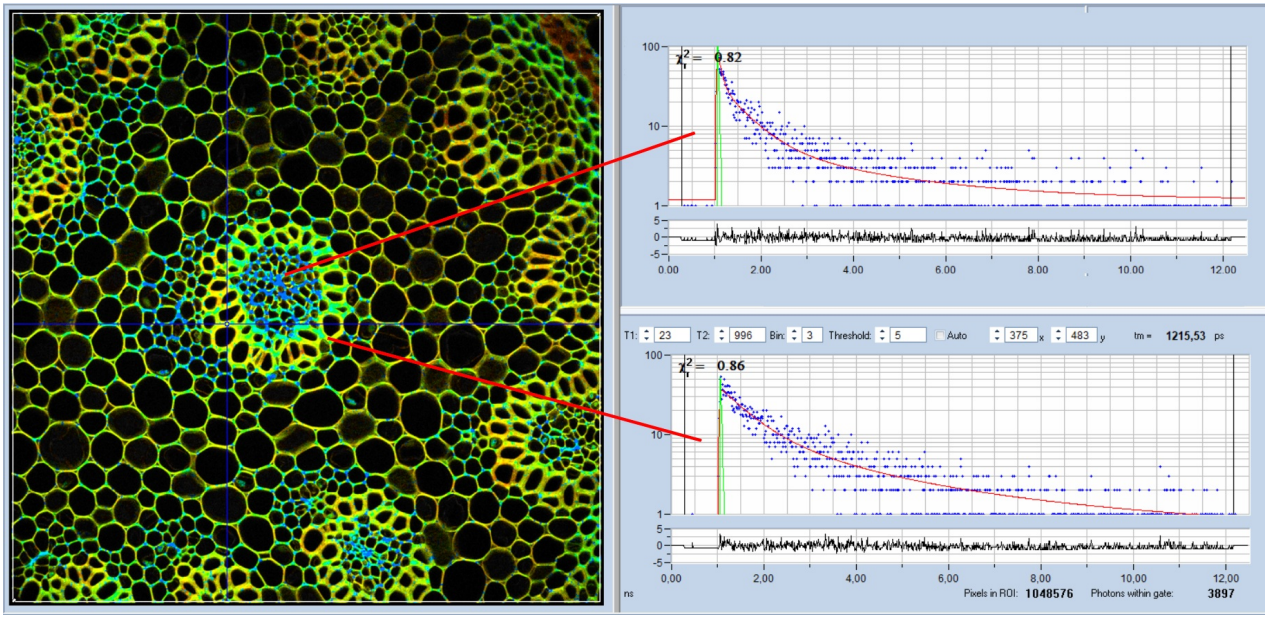

Fig. 6: Convallaria FLIM Image. 1024 x 1024 pixels, 1024 time channels. Decay functions in two selected 5 x 5 pixel spots shown on the right. Note the fast IRF (green curve), which is the result of the use of an ultra-fast HPM-100-06 detector.

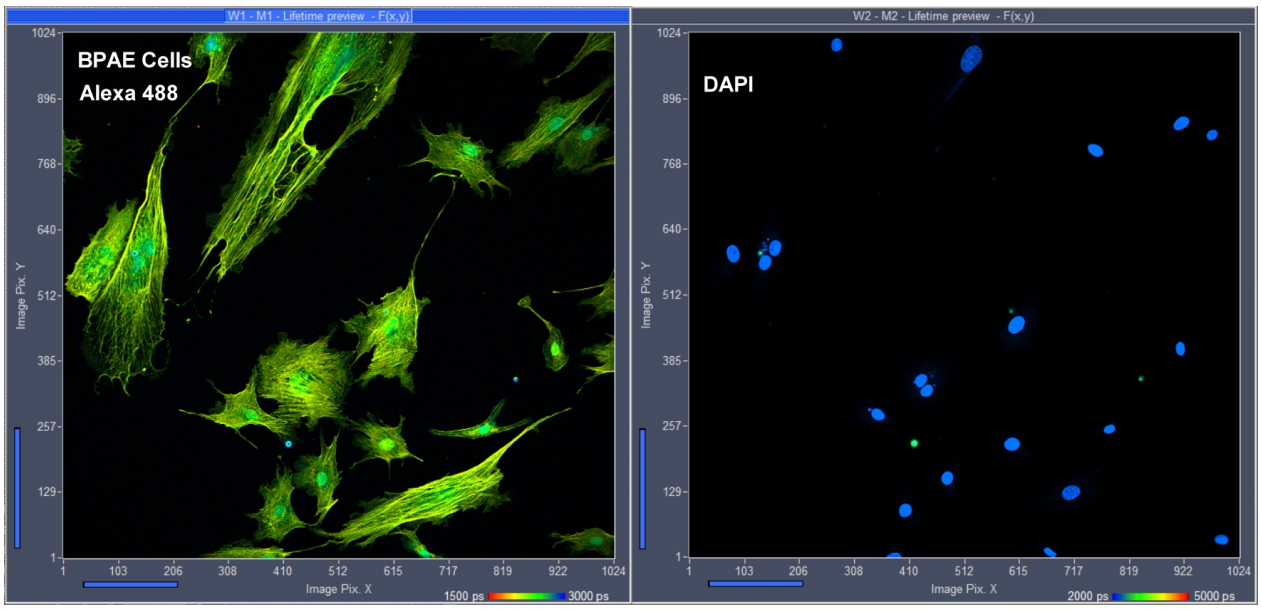

Fig. 7: Invitrogen BPAE cells, labelled with Alexa 488 and DAPI. FLIM format 1024 x 1024 pixels, 1024 time channels. Parallel detection in two channels, emission filters for Alexa 488 and DAPI. Lifetime-image display of SPCM data acquisition software.

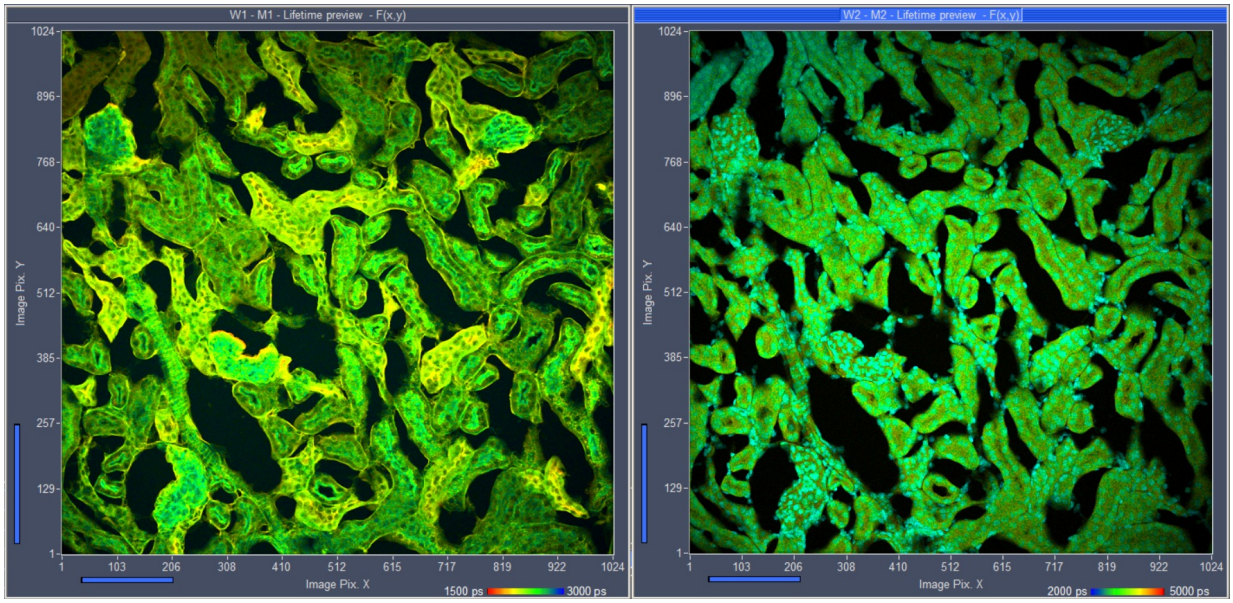

Fig. 8: Invitrogen mouse kidney sample, labelled with Alexa 488 WGA, Alexa 568-Phalloidin und DAPI . FLIM format 1024 x 1024 pixels, 1024 time channels. Parallel detection in two channels, emission filters 510 to 570 nm and 410 to 460 nm.

Summary

The 2p FLIM system presented in this application note is a simple and cost-efficient alternative to a complex Ti:Sa-based multiphoton microscope. This is especially the case if the primary application is metabolic imaging. With 785 nm excitation wavelength, the system is ideally suited for NAD(P)H FLIM. The system features extraordinarily high detection efficiency and time resolution. It provides high-quality decay data suitable for double-exponential decay analysis. It is thus capable of recording the metabolic state of cells and tissues via analysis of the amplitudes of the bound and unbound NADH components. Additionally, the system is excellently suited for recording autofluorescence FLIM from small organisms. Despite the fixed excitation wavelength the system also records FLIM from a large number of samples with exogenous fluorophores. The available excitation power is absolutely sufficient for all these applications. All FLIM images presented in this application note were recorded with no more than 6 mW in the sample plane. Higher power was not only unnecessary, but even caused destructive effects in the samples. For comparison, the laser delivers 120 mW, 40 mW of which arrive in the sample plane. Thus, there is plenty of power reserve either to destroy the sample or to record images from weakly fluorescent samples and deep sample layers.

References

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