

FLIM with Excitation-Wavelength Multiplexing

Abstract: Laser wavelength multiplexing is used for simultaneous FLIM of several fluorophores with different excitation spectra. Lasers of different wavelength are alternately switched on and off synchronously with the frames, the lines, or the pixels of the scan. The TCSPC process marks each photon with an identifier of the laser or laser wavelength that was active in the moment of the detection. It then uses this information to build up separate images for the individual lasers. Applications are simultaneous measurements of cell parameters with several spectroscopic markers and measurements of the complex behaviour of live systems over time.

Motivation

For many years, FLIM has been focusing on the determination of single cell or tissue parameters via their influence on the fluorescence-decay function of a special molecular probe. However, in the past 10 years, FLIM experiments have become considerably more complex. The task is now to record the complex behaviour of live systems as a function of time, of the environment conditions, or the phase in the cell cycle. The experiment then usually involves simultaneous recording of signals from several endogenous or exogenous fluorophores. Things remain relatively straightforward as long as the fluorophores can be excited at the same wavelength but fluoresce in different emission wavelength intervals. If this is not the case, either because there is no overlap in the excitation spectra or too much overlap in the emission spectra, different excitation wavelengths have to be used. A possible solution is to run several measurements one after another with different lasers or with the laser tuned to different wavelengths. However, laser-induced changes in the sample can then make the results difficult to compare. Moreover, it is not possible to record physiological effect on a time scale faster than a few minutes.

It is therefore desirable to multiplex the laser wavelengths at high rate, i.e. quickly switch back and forth between the laser wavelengths and record the signals into separate photon distributions [1]. Generally, there are two ways to perform this kind of measurement: The lasers can be multiplexed pulse by pulse or they can be multiplexed within a period comparable to the frame, line, or pixel times of the scan. Advantages and disadvantages of the two techniques are described in [1]. Here we refer to the second technique because it does not require a reduction of the laser pulse rate, is free of crosstalk by overlap of the tails of the decay functions, and has no intensity crosstalk by pile-up and counting-loss effects. The principle is illustrated in Fig. 1.

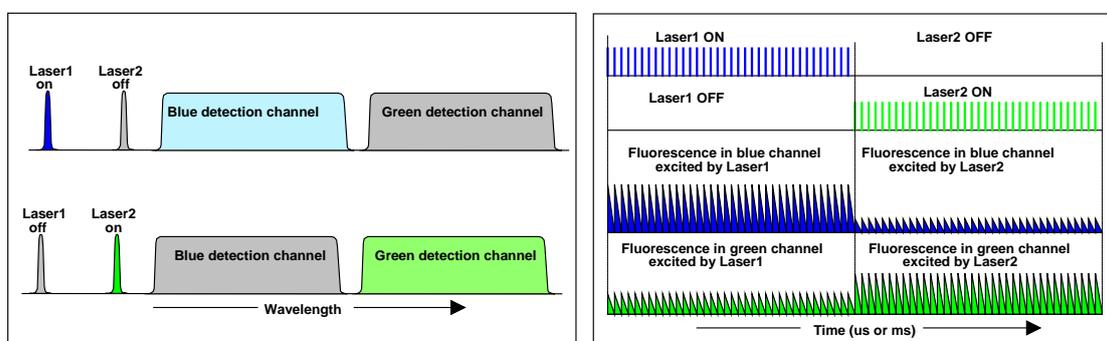


Fig. 1: Laser wavelength multiplexing by on/off modulation of lasers. Left: Laser wavelengths and detection wavelength intervals. Right: Laser ON/OFF switching and detected signals.

In Fig. 1 both laser wavelengths are shown on the short-wavelength side of the detection-wavelength intervals. However, if the right beamsplitters and filters are used one laser wavelength can also be between the two detection wavelength intervals.

Recording Principle

Laser-multiplexed FLIM uses the multi-dimensional recording capability of the bh TCSPC technique [1, 2]. The principle for multiplexing two lasers is shown in Fig. 2. The lasers are on/off modulated synchronously with the pixels, lines, or frames of the scan. A routing signal is sent into the TCSPC module to indicate which laser was active at the moment when a photon was detected. The TCSPC module uses this information to direct the photons into different photon distributions. In other words, the laser wavelength is used as an additional coordinate of the photon-recording process. With two TCSPC / FLIM modules running in parallel, data for four combinations of excitation and emission wavelength are obtained, see Fig. 2. The technique is not restricted to two lasers or two TCSPC modules, in principle any number of laser wavelengths or TCSPC modules can be used [1]. To avoid interference of the laser multiplexing with the scanning the on/off modulation of the lasers is synchronised with the pixels, lines, or frames of the scan. Please see [1] for further technical details.

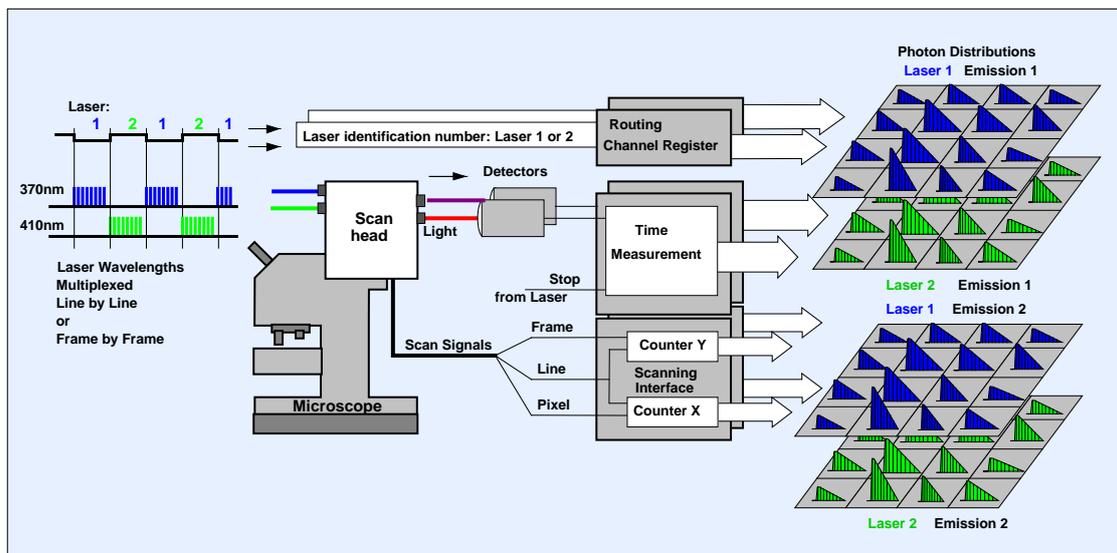


Fig. 2: FLIM with laser multiplexing. Two lasers are multiplexed in time, and the photons are recorded by two TCSPC modules. The result is four images with different combination of excitation and emission wavelength.

Laser Multiplexing with the DCS-120 System

Laser multiplexing is a standard function of the bh DCS-120 confocal FLIM systems [3]. The systems have two ps diode lasers and two TCSPC channels, i.e. the structure is exactly as shown in Fig. 2. Laser on/off control is implemented in the GVD-120 scan controller. The multiplexing function is accessible via the scan control panel, see Fig. 3, left. In the example shown, frame multiplexing was selected. To direct the photons in separate FLIM images, Routing Channels = 2 has to be selected in the SPCM System Parameters, see Fig. 3, middle. The 3D Trace Parameters were set to display images for all four combinations of laser (Routing: 1, 2) and detection wavelengths (TCSPC Module: M1, M2). The images are displayed as colour-coded lifetime images (Type: LIFET), using the online-lifetime function of SPCM.

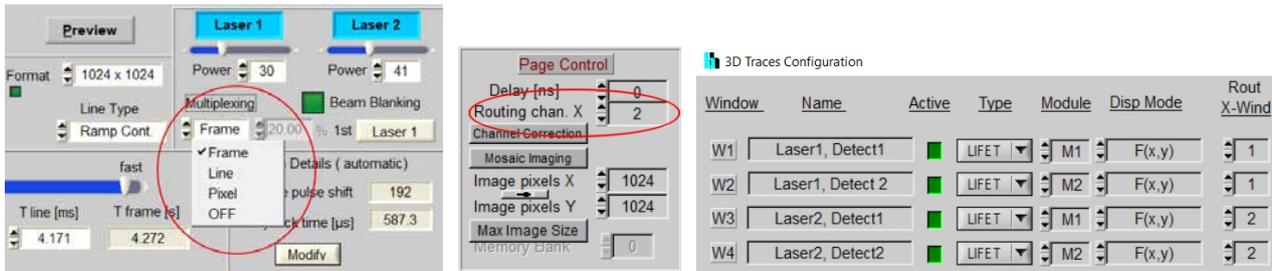


Fig. 3: Parameters for Laser Multiplexing. Left: Scan parameters, Frame Multiplexing selected. Middle: Page Control in SPCM System Parameters: Two routing channels defined. Right: 3D Trace parameters: 4 images defined, for four combinations of lasers (Routing Window) and TCSPC modules (M1, M2). Images are displayed as colour-coded lifetime images (Type = LIFET).

Fig. 4. demonstrates laser multiplexing on a BPAE sample (available from Invitrogen). The sample contains DAPI (excitable at 405 nm) and Alexa 488 (excitable at 488 nm). For the result shown in Fig. 4 the sample was excited by lasers of 405 nm and 480 nm in the frame-multiplexing mode. The image on the left is the Alexa image, excited by 480 nm and detected from 510 nm to 580 nm. The middle image shows the DAPI, excited by 405 nm, and detected from 430 nm to 470 nm. The image on the right shows data excited at 405 nm but detected in the ALEXA emission range. It shows emission both from DAPI and from Alexa 488. The fourth image (not shown), excited at 488 nm and detected from 430 nm to 470 nm, does not contain data because the detection wavelength is shorter than the excitation wavelength.

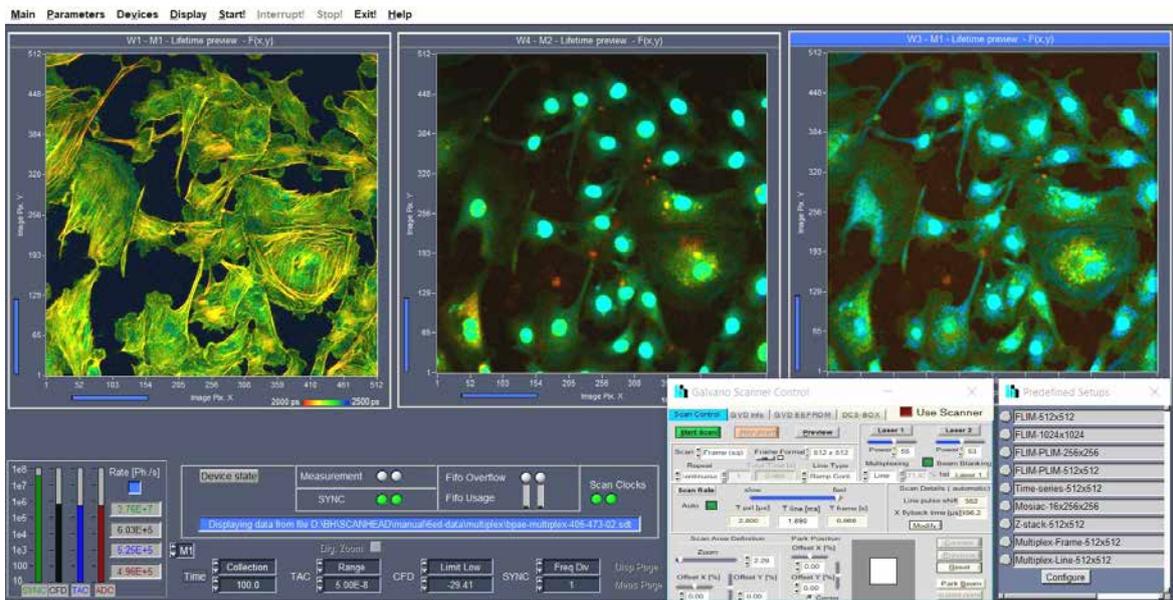


Fig. 4: Excitation wavelength multiplexing, SPCM main panel showing images of three combinations of excitation and detection wavelength. Online-lifetime display of SPCM software.

The system parameter settings for line and pixel multiplexing are, by and large, similar to the settings shown above. However, the lasers are now running alternately in every second line or every second pixel. Laser one is running in the uneven lines (pixels), laser 2 in the even lines (pixels). 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 combination is defined in the More Parameters panel of the SPCM system parameters, see Fig. 5, right. With the setup parameters shown the recording process splits the data of a 512 x 512 pixel scan with line multiplexing into two 256 x 256 pixel images for

the individual lasers. For details, please refer to [3], chapter ‘Advanced Techniques and Procedures’.

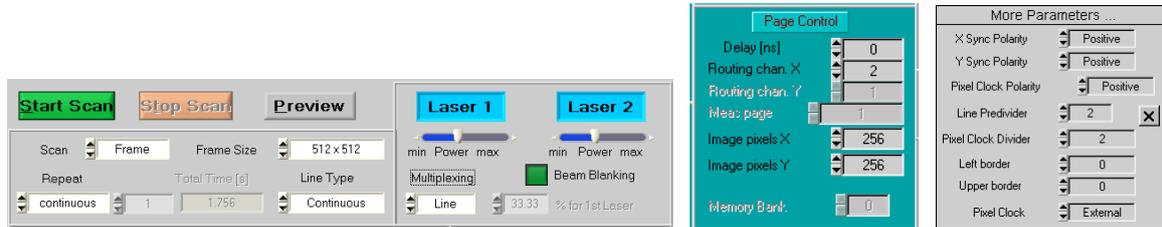


Fig. 5: Parameter setup for laser multiplexing, line by line. The data of a 512 x 512 pixel scan are split into two 256 x 256 pixel images for the individual lasers.

Simultaneous FLIM of NADH and FAD

An important (if not the most important) application of excitation-wavelength multiplexing is simultaneous recording of NADH and FAD FLIM. The decay functions of NADH and FAD change with the metabolic state of the cells or tissues. More reductive metabolism, as it exists in tumors, increases the amplitude of the fast decay component of NADH and decreases the amplitude of the fast decay component of FAD. More oxidative metabolism, as it is typical of normal cells, has the reverse effect [1]. Because the effect for NADH and FAD goes in different directions FLIM data are meaningless unless the signals are clearly separated. However, exactly this is the problem. Both the excitation spectra and the emission spectra are strongly overlapping. A separation of the signals can be achieved, however, if NADH is excited at 370 nm and detected from 420 to 475 nm, and FAD is excited between 410 to 450 nm and detected at 490 nm and higher [1]. For reasons of data compatibility it is desirable to record the NADH and FAD images simultaneously, hence the solution is FLIM with excitation-wavelength multiplexing. An example is shown in Fig. 6.

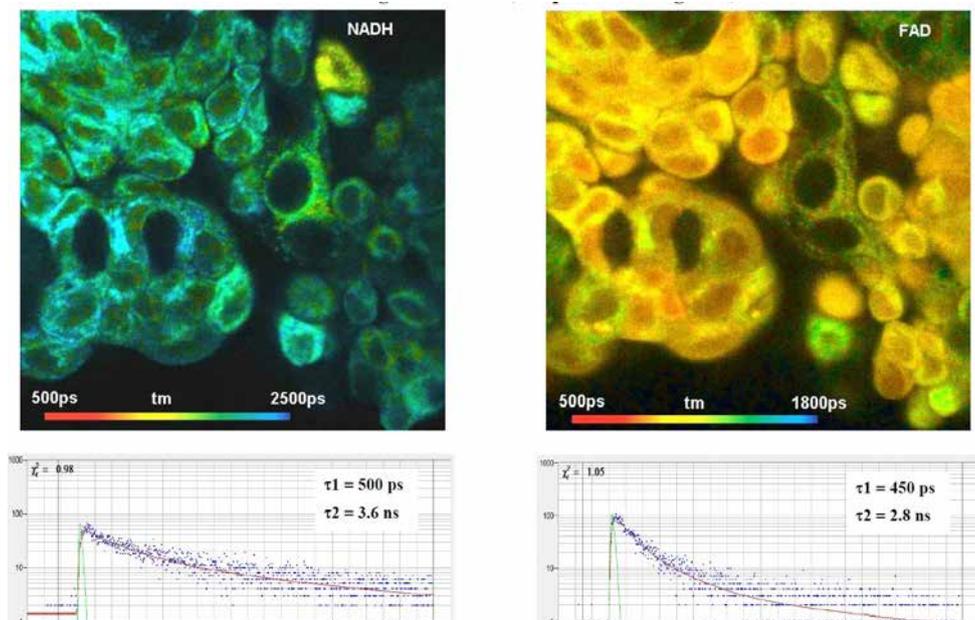


Fig. 6: Simultaneous FLIM of NADH (left) and FAD (right) by laser-wavelength multiplexing and dual-channel detection. Fluorescence decay curves in selected spots shown at the bottom.

The data were recorded with a DCS-120 confocal scanning FLIM system in the configuration shown in Fig. 2. The laser wavelengths were 370 nm for NADH excitation and 410 nm for FAD excitation.

Please see [1, 4] for details. For application of the system and comparison of FLIM results with histology please see [1], chapter ‘autofluorescence FLIM of cells and tissues’, and [7, 8].

Laser Multiplexing with the LHB-104 ‘Laser Hub’

The LHB-104 ‘Laser Hub’ contains four bh BDS-SM series picosecond diode lasers [5]. The optical outputs of the lasers are combined into one single-mode fibre. Part of the LHB-104 electronics is a multiplexing module (MPM). The MPM can be used to multiplex each two of the four lasers synchronously with the pixel, line, or frame clock pulses of a laser scanning microscope. Therefore, multiplexing is not restricted to the DCS-120 or other systems that contain a bh GVD-120 scan controller. Multiplexing of lasers by the MPM can be used in combination with any laser scanning microscope, in particular with the Zeiss LSM 980 [6]. The principle of the MPM is shown in Fig. 7. A toggle flip-flop is triggered by one of the scan clocks, Frame, Line, or Pixel. Every time when a clock pulse arrives, the flip-flop changes its state. The outputs of the flip-flop switch two lasers, Laser 1 and Laser 2, on and off alternately. Simultaneously, the MPM sends a routing bit to the TCSPC system to route photons excited by different lasers into separate memory blocks. The multiplexing mode, i.e. frame, line, or pixel multiplexing, is selected by the Mode switch. For details please see [5] and [6].

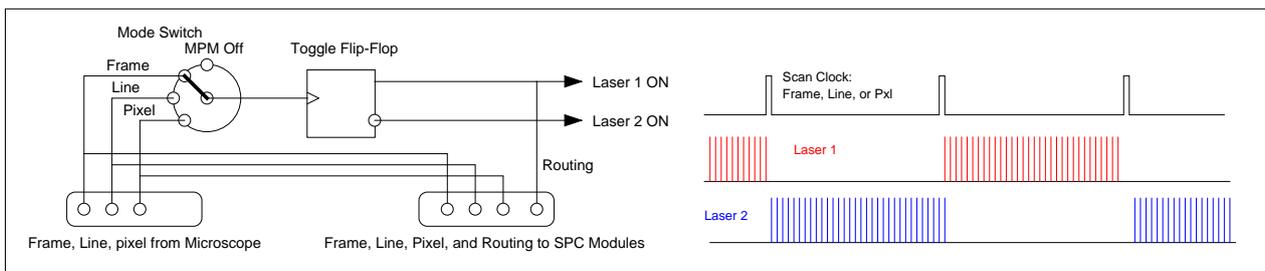


Fig. 7: Principle of the Laser Multiplexing Module in the LHB-104

An example is shown in Fig. 8. A convallaria sample was excited at 480 nm (left) and 405 nm (right). The lasers were multiplexed frame by frame. Both images were detected in one spectral channel, from 510 to 590 nm.

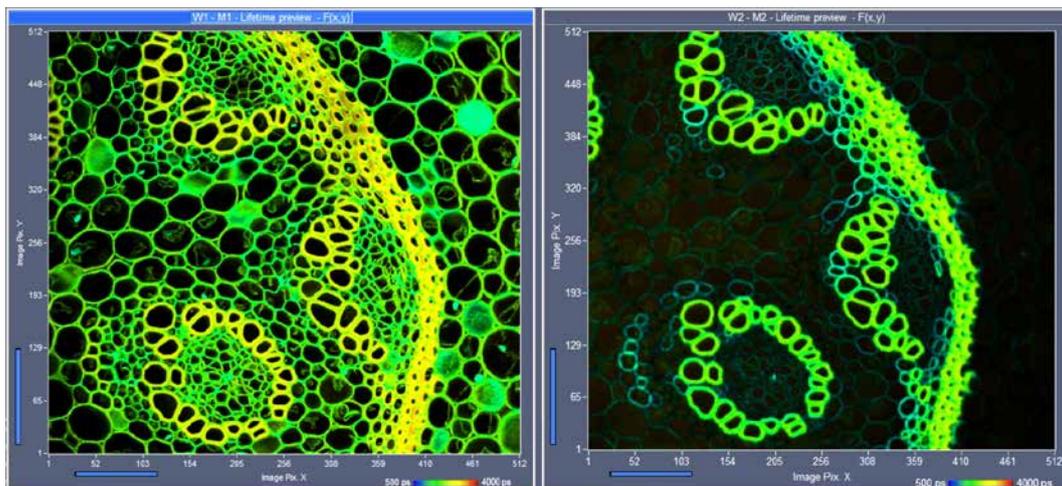


Fig. 8: FLIM of a convallaria sample, multiplexed excitation at 488 nm and 405 nm

Excitation Multiplexing with Femtosecond Fibre Laser

Multiplexed excitation can be performed also with a number of other lasers. One of them is the Toptica Femto Fibre Dichro. This laser generates two wavelengths, 785 nm and 880 nm. The pulse width is less than 200 fs. Each of the two beams passes an AOM to regulate the intensity or to switch on and off the beams. At the output of the laser the two beams are combined by a dichroic beam combiner. With its two wavelengths, its short pulse width, and its ability to on/off modulate the outputs the Femto Fibre Dichro is ideally suited for 2-photon NADH / FAD FLIM, please see [1]. Two-photon images of pig skin recorded with the Femto Fibre Dichro and the DCS-120 MP system [3] are shown in Fig. 9. The data quality is so high that the parameters of the decay components can be extracted by double-exponential decay analysis. The NADH data are shown in the upper row, the FAD data in the lower row. From left to right, the images show the amplitude of the fast decay component, a_1 , the lifetime of the fast decay component, t_1 , and the lifetime of the slow decay component, t_2 .

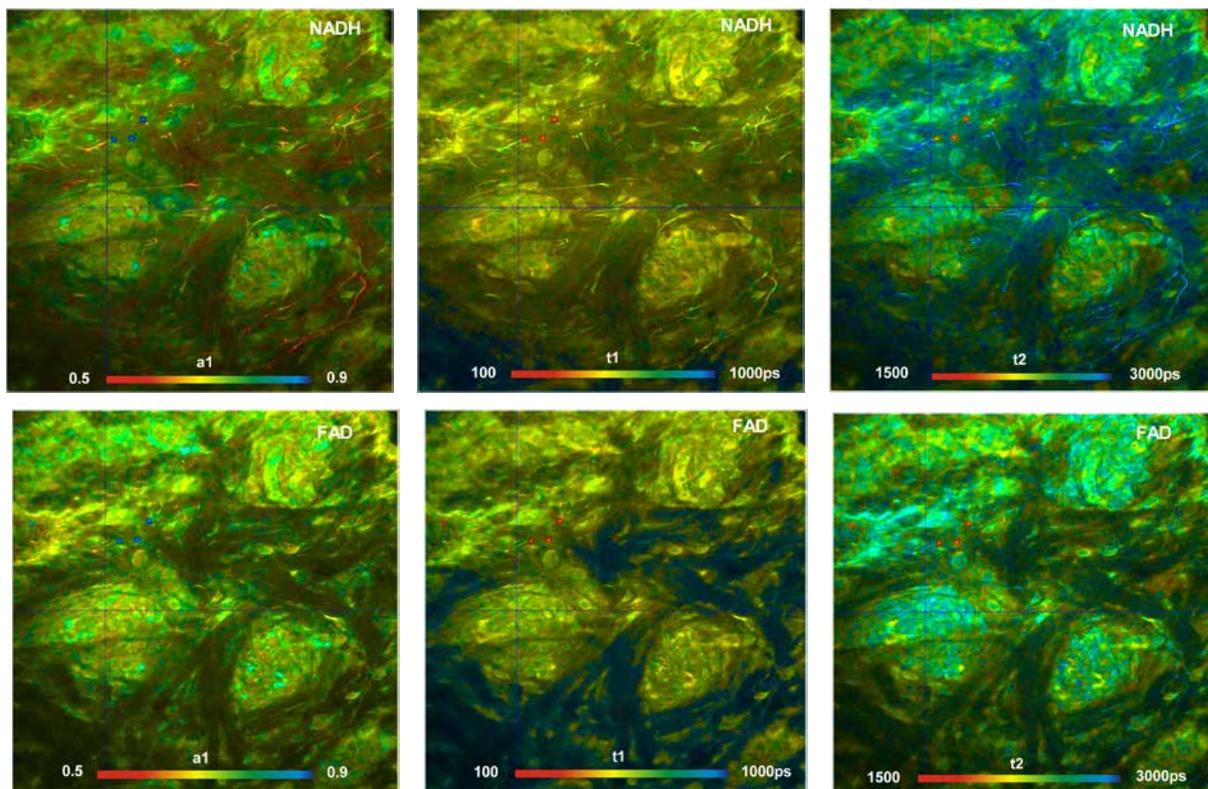


Fig. 9: FLIM images of pig skin recorded with a Femto Fibre Dichro connected to a DSC-120 MP system. Upper row: NADH images, lower row FAD images. Left to right: amplitude of the fast decay component, a_1 , lifetime of the fast decay component, t_1 , and lifetime of the slow decay component, t_2 . Data analysis by SPCImage NG.

Supercontinuum Lasers with AOTF

Wavelength multiplexing can also be performed with a supercontinuum laser and an acousto-optical filter (AOTF). Please see [1] for a demonstration of the method. A problem in fluorescence applications can be side-band leakage of the AOTF. The leakage usually has to be removed by cleaning filters in the excitation beam path, with the effect that the flexibility of wavelength selection by the AOTF is lost. AOTF leakage is less a problem on diffuse-optical imaging

applications. A system that multiplexes 8 wavelengths via an AOTF and records the diffusely reflected light has been described in [1], chapter ‘Diffuse Optical Tomography’.

Summary

Excitation-wavelength multiplexing is an efficient way to record FLIM data of several fluorophores with different excitation spectra simultaneously. Compared with sequential recording at different excitation wavelength the advantage is that possible laser-induced changes, focus drift, or motion in the sample have comparable effects on the FLIM data of all laser wavelengths. So far, excitation-multiplexed FLIM has been shown to be a promising technique for metabolic imaging [1, 7, 8]. However, the technique has an even larger potential. The multiplexing rate can be the order of 1 Hz, 1 kHz, or several 100 kHz for frame, line, and pixel multiplexing, respectively. Multiplexing therefore does not conflict with time-series recording. Even temporal mosaic FLIM with a resolution in the range of 50 ms per image appears feasible. FLIM with excitation-wavelength multiplexing is therefore a solution for the investigation of physiological effects, effects of environment conditions or drugs on the cell metabolism, or photodynamic-therapy effects.

References

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