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Picosecond Laser Scanning Microscopy

Upgrade Your Leica TCS SP-2 for Lifetime Imaging

Fluorescence Lifetime Imaging (FLIM) has become a new powerful method to investigate molecular interactions, metabolic reactions and energy transfer in cells and subcellular structures. These effects cause changes in the fluorescence quantum efficiency and thus in the fluorescence lifetime. Since the fluorescence lifetime does not depend on the unknown dye concentration it is a direct measure for the quantum efficiency. It therefore gives a more direct access to the investigated effects than the fluorescence intensity. Furthermore, the fluorescence lifetime can be used to separate the fluorescence of different luminophores in the cells if the components cannot be distinguished by their fluorescence spectra..

Recording time-resolved fluorescence images can be achieved by combining the Leica TCS SP2 Laser Scanning Microscope with Ti:Sa laser excitation and a new Time-Correlated Single Photon Counting (TCSPC) Imaging technique introduced by Becker & Hickl.

Fig. 1: The Leica SP-2 Laser Scanning Microscope and the Becker & Hickl SPC-730 TCSPC Imaging Module

TCSPC Imaging

The principle the SPC-730 TCSPC Imaging module is shown in fig. 2.

The module employs an advanced TCSPC technique featuring both high count rate and low differential nonlinearity. It contains the usual building blocks (CFDs, TAC, ADC) in the ‘reversed start-stop’ configuration together with a scanning interface and a large histogram memory integrated on one board.

The TCSPC module receives the single photon pulses from the photomultiplier (PMT) of the microscope, the reference

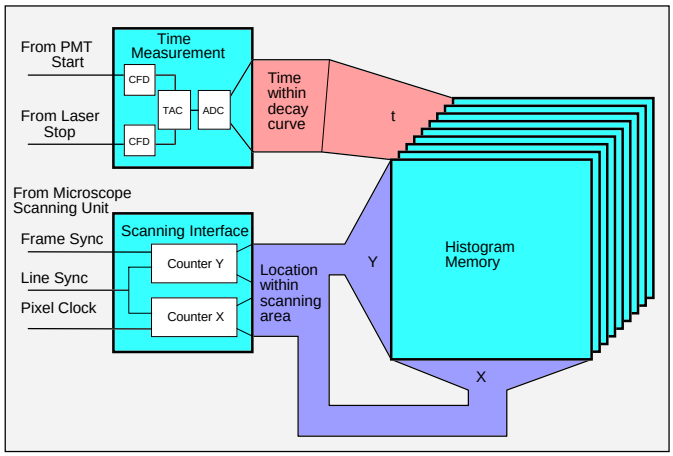

Fig. 2: Basic principle of the SPC-730 TCSPC Imaging module

pulses from the laser and the Frame Sync, Line Sync and Pixel Clock signals from the scanning unit of the microscope. For each PMT pulse, i.e. for each photon, the TCSPC module determines the time of the photon within the laser pulse sequence and the location within the scanning area. These values are used to address the histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over x, y, and the time within the fluorescence decay function builds up. The result can be interpreted as a two-dimensional (x, y) array of fluorescence decay curves or as a sequence of fluorescence images for different times (t) after the excitation pulse.

Interestingly, there is practically no loss of photons in the TCSPC imaging process. As long as the photon detection rate is not too high all detected photons are processed and accumulated in the histogram, thus providing maximum sensitivity. This is a key advantage of the TCSPC Scanning Microscope over gated photon counting or gated image intensifiers which gate away the majority of the fluorescence photons.

The TCSPC Laser Scanning Microscope

The general setup is shown in figure 3. A Spectra Physics MAI TAI Ti:Sa laser delivers femtosecond pulses over a wavelength range from 780 nm to 920 nm. The repetition rate is 80 MHz, the typical pulse width is below 160 fs FWHM. The short excitation pulses in conjunction with the high power density in the focus of the microscope lens enable effective two-photon excitation of typical fluorescence marker dyes and of the autofluorescence of biological samples. The near-infrared excitation light easily penetrates deeply into the sample. Fluorescence images can be recorded from sample layers as deep as 100 um. Furthermore, living cells are surprisingly stable under the NIR radiation of the Ti:Sa laser.

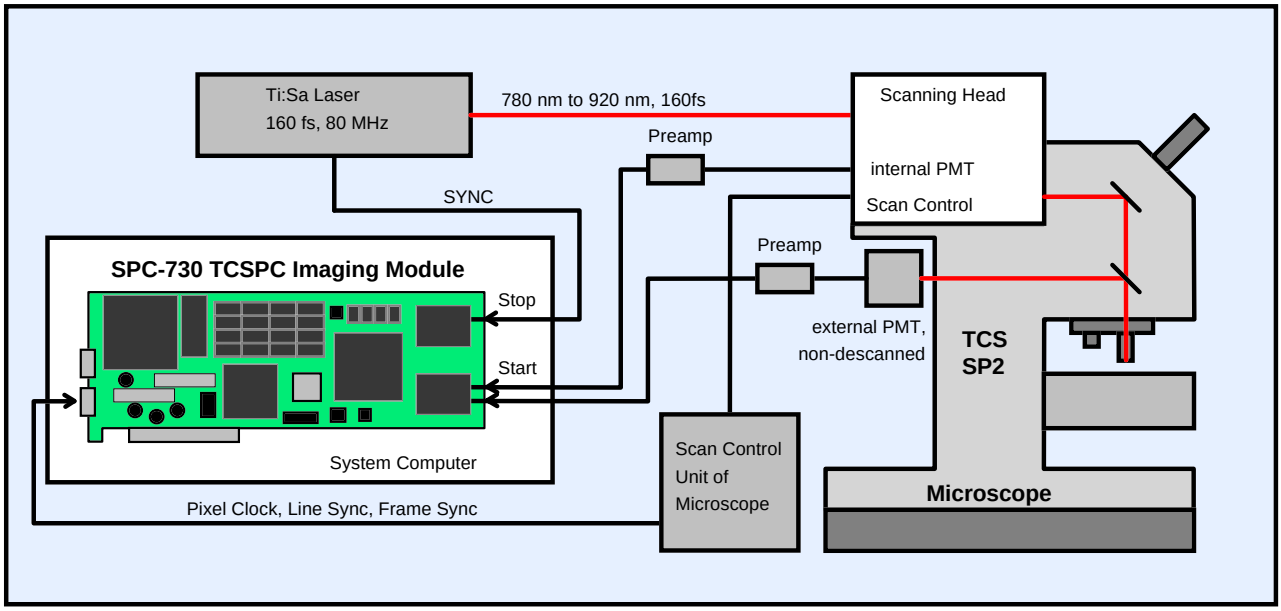

Fig. 2: Basic setup of the TCSPC Laser Scanning Microscope

The SP-2 microscope scans the sample in the x-y plane giving an image of the sample in the focal plane of the objective lens. By changing the depth of the focus in the sample, 3 D imaging of the sample is achieved. Furthermore, the full flexibility of the SP-2 scanning modes can be used.

The scanning head of the SP-2 microscope has several detection channels equipped with separate confocal pinholes, monochromators, and photomultipliers (PMTs). The PMTs are small side-window tubes which give good sensitivity but are not optimised for time resolution. With a Becker & Hickl HFAC-26-10 preamplifier the detectors can be used in the TCSPC mode, but the time resolution is only 300 to 500 ps FWHM.

For two-photon excitation, which does not require a pinhole, attaching a fast detector to the non-descanned port of the microscope is therefore a better solution. We recommend the PMH-100-1 detector head of Becker & Hickl (resolution 150 ps FWHM) and the Hamamatsu R3809U-50 (resolution < 30 ps FWHM). To get best performance from the R3809U-50 a Becker & Hickl HFAC-26-01 preamplifier is used. The TCSPC instrument response functions for these detectors are shown in fig. 4 and fig. 5.

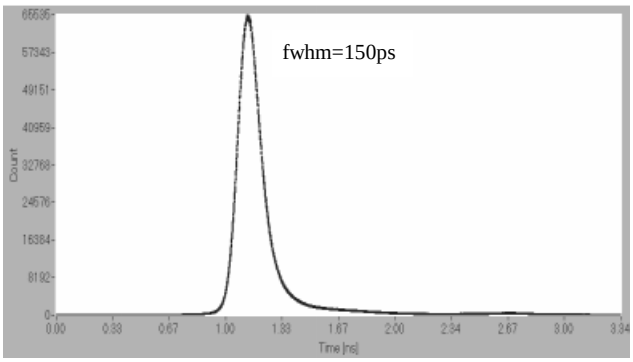

Fig 4: System response for the PMH-100 detector

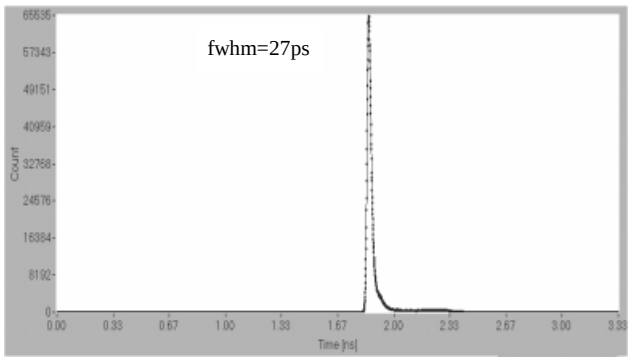

Fig 5: System response for the R3809U-50 MCP

Liftime data acquisition is accomplished by the Becker & Hickl SPC-730 TCSPC Imaging module. The CFD input receives the single photon pulses from the PMT. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from the reference photodiode of the Ti:Sa laser.

The SPC-730 module gets the scan control signals, Pixel Clock, Line Sync, and Frame Sync, from the scan controller of the microscope. Connecting the TCSPC module does not require any modifications in the microscope hardware and software. The scan parameters are controlled in the usual way via the microscope software. The normal scan speed, the zoom function, region of interest setting, etc. can be used also when recording lifetime images. Furthermore, the SPC-730 can be run in parallel with the standard image recording electronics of the microscope. Basically, steady state images and lifetime images in different spectral ranges can be obtained at the same time. Due to the simple interfacing the SPC-730 TCSPC Imaging module can be adapted also to existing microscopes including the SP-1, provided that pulsed excitation is available.

Results

Fig. 6 (next page) shows the fluorescence images of a nematode containing GFP. The sequence shows eight subsequent images within time windows of 0.5 ns starting from the excitation pulse. The decay curves over a horizontal stripe of the image are shown in fig. 7 (next page).

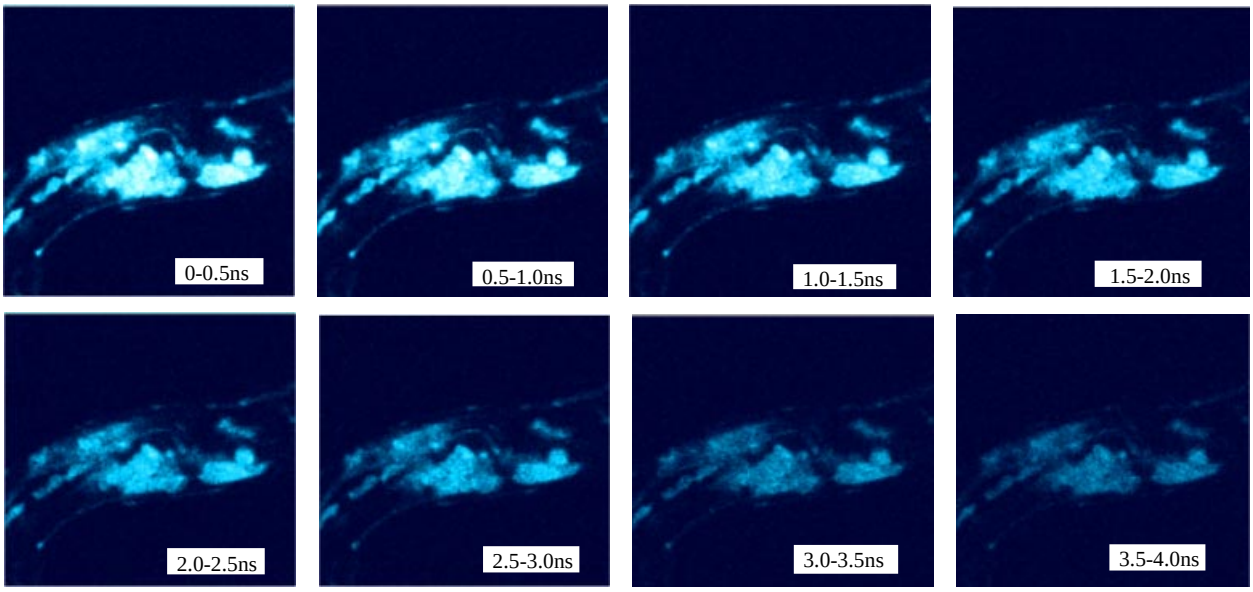

Fig. 6: Sequence of fluorescence images in intervals of 0.5ns starting from the excitation

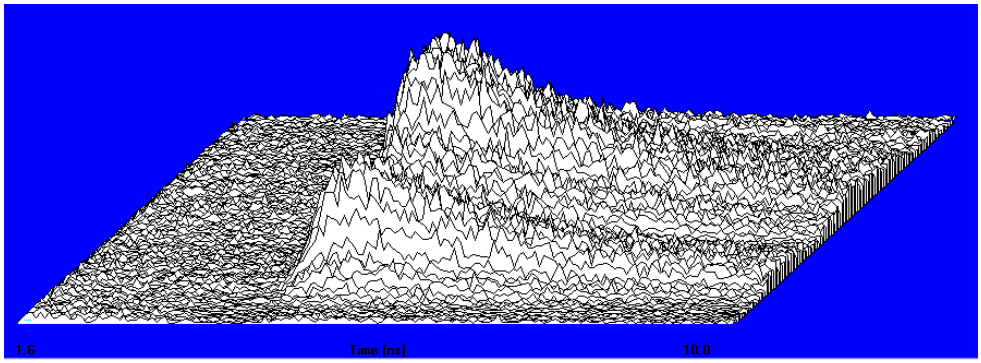

Fig. 7: Fluorescence decay curves over a 20 pixel wide horizontal stripe of the image

Fig. 8 shows a lifetime image of cortex neurons after Calcium Green injection. The image data were processed by the Becker & Hickl SPCImage lifetime imaging software. The fluorescence intensity is displayed as brightness, the fluorescence lifetime as colour. The lifetime differences are clearly visible. The lifetime of Calcium Green is a direct measure of the calcium ion concentration. Therefore, lifetime images can avoid the intensity calibration normally used for Ca imaging.

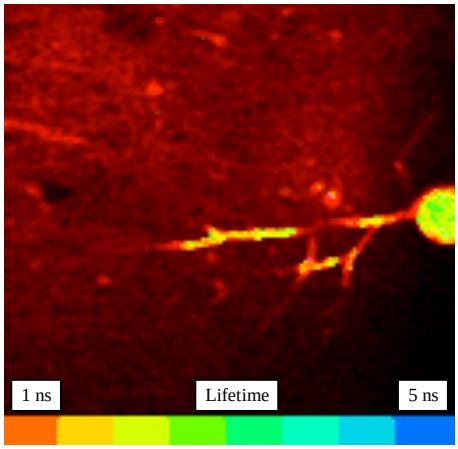

Fig. 8: Ca lifetime image of rat cortex neurons. Leica SP-1, 2-photon excitation

Conclusions

The results show the potential of TCSPC Laser Scanning Microscopy as a new method of fluorescence lifetime imaging. The field of application covers FRET (resonance energy transfer) measurements, Ca imaging, separation of multiple fluorescent labels and autofluorescence components, and other fluorescence imaging applications of microscopic samples.

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