NDD FLIM Systems for Leica SP2 MP and SP5 MP Multiphoton Microscopes

bh FLIM systems for the confocal and the multiphoton versions of the Leica SP2 and SP5 microscopes are available since 2002 [5]. These systems use the X1 port of the SP2 or SP5 scan head. The multiphoton FLIM versions have now been complemented with non-descanned detection systems. Compared with detection through the confocal beam path non-descanned detection yields a considerably higher efficiency for deep sample layers. Moreover, the use of novel GaAsP hybrid detector allows you to combine non-descanned detection with SPAD-like sensitivity. The NDD FLIM detectors are attached to the reflected light (RLD) port of the microscope. The FLIM electronics is contained in an extension box of a laptop computer. The FLIM data acquisition is controlled by the scan clock signals of the microscope. Because no changes in the microscope hardware or software are required the NDD FLIM systems are easy to install on any Leica SP2 or SP5 MP microscope.

Principle of Non-Descanned Detection

The general optical principle of a laser scanning microscope is shown in Fig. 1, left. A laser beam is focused into the sample through the microscope lens. The fluorescence from the sample is collected back through the microscope lens and focused in a pinhole in the upper focal plane of the microscope. Light from outside the focal plane is not focused into the pinhole and thus substantially suppressed. Images are obtained by scanning both the laser and the detection beam via fast galvanometer mirrors [10, 14].

The principle can be used both for one-photon and for multiphoton excitation. As long as the sample is optically thin the confocal principle shown in Fig. 1, left, collects all photons emitted from a diffraction-limited volume around the laser focus into the solid angle defined by the NA of the microscope lens. The situation is different for a thick sample. The NIR laser beam of a multiphoton microscope reaches sample layers considerably deeper than a NUV or visible laser beam. The fluorescence photons from deep layers are, however, scattered on the way to the surface of the sample, see Fig. 1, middle. Scattered photons leave the back aperture of the microscope lens in a wide cone. Only a small fraction of these photons is able to pass the confocal beam path. Confocal detection, even with a wide pinhole, therefore becomes inefficient for sample layers deeper than 50 µm [11].

The solution to multiphoton imaging of deep-tissue is non-descanned detection (NDD), see Fig. 1, right. The fluorescence light is diverted from the excitation beam path by a dichroic mirror directly behind the microscope lens and collected by a transfer lens or lens system. To direct the photons to the detector the transfer lens projects an image of the back aperture of the microscope lens (not of the sample!) on the detector. Thus, scattered photons within the full acceptance cone of the transfer lens are transferred to the detector. The efficiency for scattered photons is therefore significantly higher than in a confocal detection system.
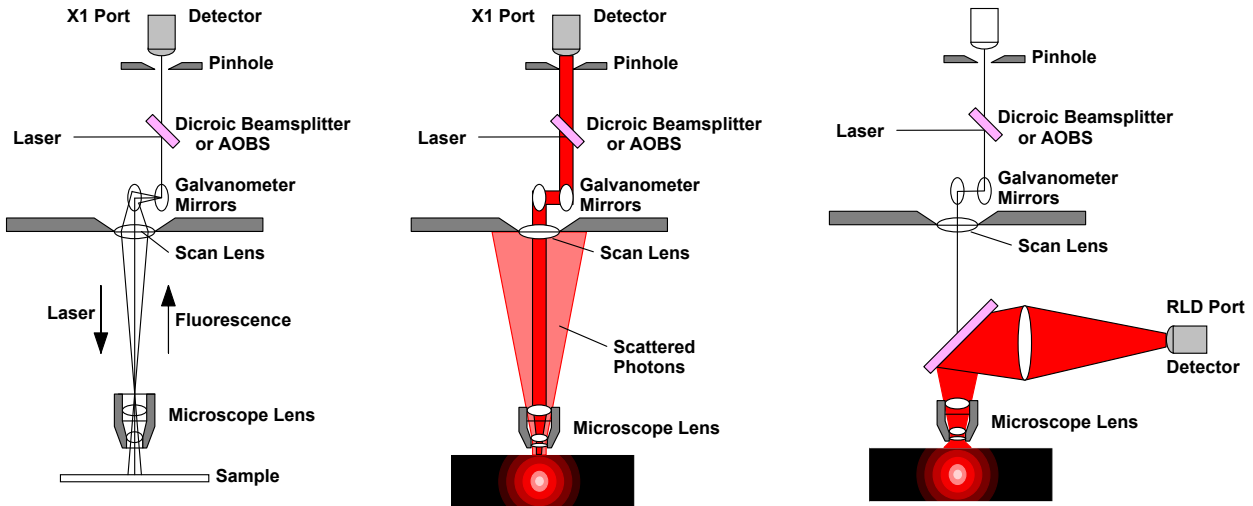
Fig. 1: Left: Principle of confocal scanning. Middle: Confocal scanning of deep layers of a thick sample by multiphoton excitation. Scattered photons are not transmitted through the confocal beam path. Right: Multiphoton excitation with non-descanned detection. The non-descanned detection collects also scattered photons.

Implementation in the Leica SP2 and SP5 MP

The bh NDD FLIM system attached to a Leica SP5 multiphoton microscope is shown in Fig. 2. The FLIM detector is attached to the RLD port of the microscope, see right side of microscope body. The FLIM system is shown on the right. It is based on the bh 'Simple Tau' systems [2]. The complete FLIM electronics is contained in an extension box connected to a laptop computer.
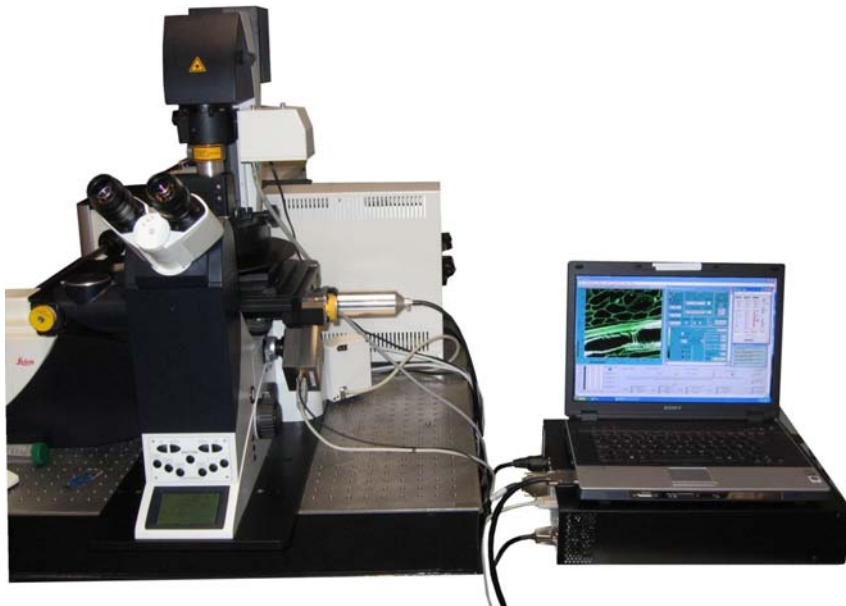

Fig. 2: Leica SP5 MP with bh NDD FLIM system. FLIM detector attached to RLD port of the microscope, data acquisition system shown on the right.

The FLIM system is based on bh's proprietary multi-dimensional TCSPC technique [1, 2]. Both hardware and software-based acquisition modes are available. Thus, high speed FLIM, high-resolution FLIM, FLIM acquisition at high pixel numbers, multi-spectral FLIM, fast FLIM time series, and precision single-point measurements are available. FLIM data can be recorded both as

photon distributions versus time, image coordinates and wavelength, or as time- and wavelength-tagged single photon data. In combination with confocal detection the bh FLIM systems also deliver FCS data. Please see [2] for details.

NDD FLIM systems are available with different detectors, see Fig. 3. The bh PMC-100-01 module is shown in Fig. 3, left. The PMC-100 is a compact PMT module containing the preamplifier, the PMT power supply, overload shutdown circuitry, and thermoelectric cooling [2]. Because the PMC-100 is easy to use, rugged and almost undestroyable its has become a standard detector for a wide range of bh TCSPC applications. For ultra-high time resolution the Hamamatsu R3809U MCP PMT is available. To provide maximum safety against overload the R3809U is operated via a shutter, see Fig. 3, middle. Since 2009 bh have added GaAsP hybrid detectors to their TCSPC systems. These detectors combine the sensitivity of a single-photon avalanche photodiode with the large active area of a PMT. They are free of afterpulsing and thus deliver an extremely high dynamic range of fluorescence decay detection [9]. Multi-spectral FLIM [3] is available by using the bh MW FLIM multi-wavelength detector assembly [2, 4], see Fig. 3, right. The multi-spectral FLIM assembly is based on bh's proprietary multi-detector TCSPC technique. It detects simultaneously in 16 wavelength channels, without any wavelength scanning or time gating.

Fig. 3: Left: bh PMC-100 detector module, with RLD adapter. Second left: HPM-100-40 hybrid detector module. Second right: R3809U MCP PMT, with shutter and RLD adapter. Right: bh MW FLIM multi-spectral detection assembly

A bh PMC-100 detector attached to a Leica SP5 is shown in Fig. 4. The detector is attached or de-attached by loosening a single clamping screw. If required, it can be swapped with the original Leica RLD detector (seen in the background) within a few seconds. The RLD adapter contains a transfer lens and a Chroma SP700 NIR blocking filter. An additional emission filter can be inserted at the output side of the adapter.

Fig. 4: PMC-100 detector attached to the RLD port of a Leica SP5 multiphoton microscope

There is only a minimum of system connections between the microscope and the FLIM system. In fact, the only electrical connection is via the scan clock signals of the SP2 or SP5 microscope. This makes the system very easy to install. The FLIM system comes with a cable that connects directly to the scan clock outputs of the SP2 or SP5 microscope, see Fig. 5.

Fig. 5: Scan control cables of the Leica NDD FLIM systems. Left: SP2. Right: SP5. The cables are the only electrical signal connection between the microscope and the FLIM system.

Updating the Setup of Existing Leica FLIM Systems

Since the introduction of the FLIM systems in 2000 the range of functions implemented in the SPCM data acquisition software has been continuously extended. However, users of bh FLIM systems embedded in Leica SP2 and SP5 microscopes often have not been provided with updated system setup files. Consequently, they do not use the full range of the hardware and software functions available to their TCSPC systems [2, 7]. For users who want to upgrade existing FLIM systems with NDD detection we therefore give a few recommendations here.

1. Configure the FLIM system for online display

Online display is obtained by defining 'Cycles' for the Scan Sync In mode, or using a 'Display Time' for the FIFO Imaging mode [2, 6]. The essential parts of the SPCM system parameter panel are shown in Fig. 6. Please see [2] for details.

Fig. 6: Parameter definitions for online display. Left: Scan Sync In mode. Right: FIFO Imaging mode.

With the settings shown, intermediate results are shown every 4 to 5 seconds during a measurement. Faster update rates are possible by may slow down the data acquisition.

2. Define a 'Fast Preview' mode

The fast preview is a fast repetitive measurement that delivers images at a rate on the order of one image per second. Fast imaging is obtained by defining a collection time of 0.5 to 1 seconds in the Scan Sync In mode, and activating the 'repeat' function, see Fig. 7, left and second left. In order to minimise the amount of data to be transferred and processed, 'ADC Resolution' is set to '1'. The number of pixels should be no larger than 256x256.
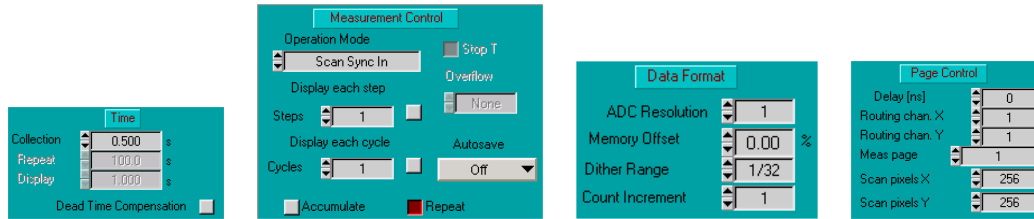
Fig. 7: Parameter definitions for fast preview

3. Use the 'Predefined Setup' panel to conveniently switch between different system setups

To facilitate the change between different instrument configurations it is recommended to put frequently used system setup files into a list of 'Predefined Setups'. The Predefined Setup panel shown in Fig. 8, left. To load a setup, click on the button left of the name. Switching between different setups is then only a matter of a single mouse click.

For editing the list of predefined setups, put the mouse cursor into the panel, and click on the right mouse key. This opens the panel shown in Fig. 8, middle. Any setup (.set) or setup/data file (.sdt) can be selected for and added to the Predefined Setup panel. Caution: To avoid overwriting the setup data unintentionally, the files should be copied into a directory 'DefaultSetups' (see Fig. 8, right) and loaded from there. The SPCM software denies to overwrite files in this directory. Please see [2] for details.
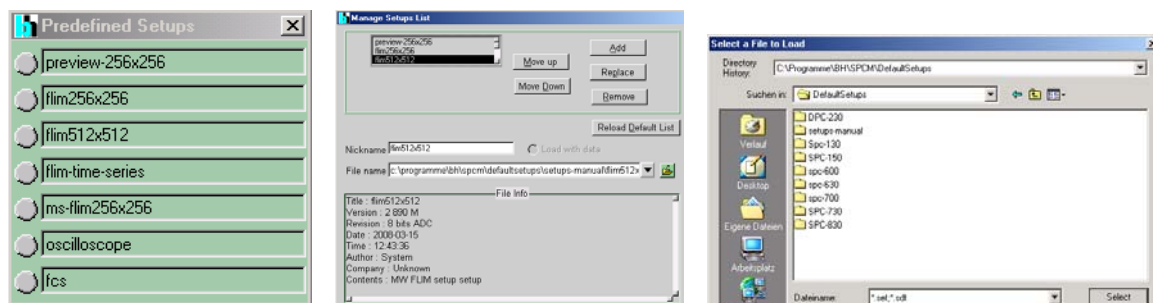

Fig. 8: Left: Predefined Setup panel. Middle and right: Editing the list of predefined setups

Running an NDD FLIM Measurement

Users of multiphoton FLIM are usually advised to acquire a confocal image via the microscope software first, adjust the focal plane and the scan area, then to switch to multiphoton imaging through the confocal beam path, adjust the focal plane again, and then switch to FLIM. A procedure cannot seriously been recommended. It is not only troublesome and circumstantial, it also causes unnecessary photobleaching even before the FLIM measurement has been started. We therefore recommend a more efficient procedure:

1. Put the sample under the microscope. Use the eyepieces to bring it into focus, and shift the sample into the desired lateral position.

2. Switch the microscope beam path to NDD detection. Load a fast preview mode from the Predefined Setup panel. Start a continuous scan in the Microscope and start the measurement in the FLIM system.

3. Look at the images continuously displayed by the FLIM system. Optimise the focal plane, and zoom into the desired scan area by the microscope software.

4. Load the setup of the desired FLIM format from the Predefined Setup panel and start the FLIM measurement. Let the FLIM acquisition run until you are satisfied by the signal-to-noise ratio.

5. Stop the measurement. Stop the scan. Save the data. Send the data to the SPCImage data analysis software for closer inspection.

Examples of FLIM Measurements

An example of a multiphoton NDD FLIM measurement is shown in Fig. 9. A plant tissue sample was scanned with 512x512 pixels, and 256 time channels per pixel. The average count rate over the whole image was about 10^6 photons/s, corresponding to about $5 \cdot 10^6$ photons/s in the brightest pixels. The total acquisition time was 60 seconds. Fig. 9 shows the main panel of the SPCM data acquisition and control software. All panels of the SPCM software are configurable for different applications and instrument configurations [2]. The configuration used in Fig. 9 shows a gated intensity image, the display parameter panel, the detector control panel, and the 'predefined setup' panel for convenient changing between different instrument configurations. The lower part of the panel shows essential system setup and status parameters.
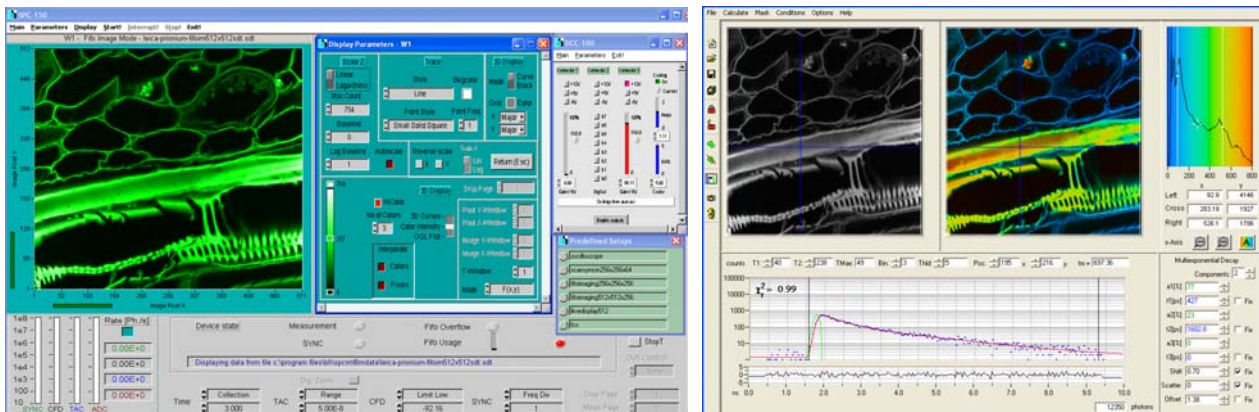

Fig. 9: Left: Data acquisition panel of the FLIM software. Intensity image in selectable time window, display parameter panel, detector control panel, and predefined setup panel for quick changing between instrument configurations. Right: Data analysis panel of the FLIM software. Intensity image, colour-coded lifetime image, lifetime histogram, decay curve in selected spot, and decay parameters. Lifetime range shown is 0 to 800 ps. Plant tissue sample, Leica SP5 MP, NA=1.3 water immersion lens, Laser Power 3%, PMC-100 detector, image size 512x512 pixels, 256 time channels, average count rate 10^6 photons/s.

The data analysis part of the FLIM software is shown in Fig. 9, right. It shows an intensity image, a colour-coded lifetime image, a histogram of the lifetime over the pixels, and the decay curve at the selected cursor position with the corresponding decay parameters. As it is typical for tissue fluorescence, the decay profiles are not single-exponential. The data were therefore analysed by a double-exponential model. The lifetime shown in the colour image is an amplitude-weighted average of the two decay components. Images of the individual lifetime components and of the lifetime and amplitude rations are shown in Fig. 10.
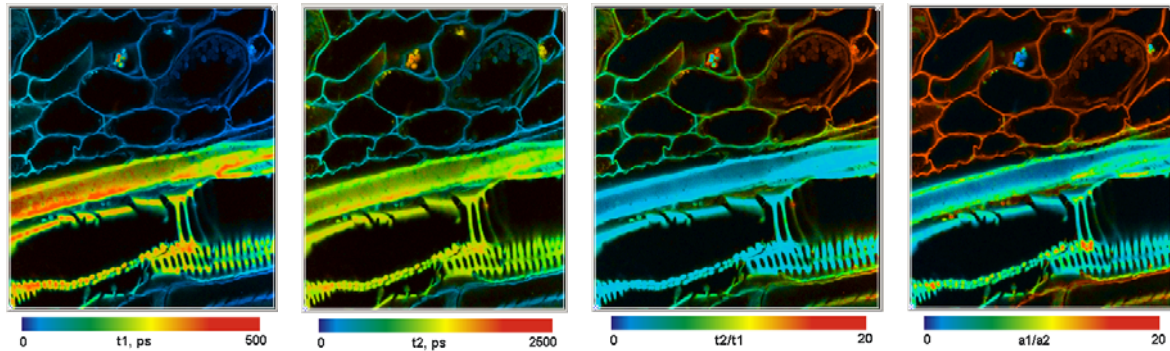
Fig. 10: Decay components of the sample shown in Fig. 9. Left to right: Lifetime of the fast component, t1, lifetime of the slow component, t2, ratio of the fast and the slow lifetime component, and ratio of amplitudes of both lifetimes.

The results of a FRET measurement is shown in Fig. 11. The figure shows a HELA cell expressing two interacting proteins. One protein was labelled with CFP (donor), the other with YFP (acceptor). The image was recorded through a filter of 480±15 nm, i.e. only the donor fluorescence was recorded. The data were analysed by a double-exponential model. A lifetime image of the amplitude-weighted lifetime is shown left. The average lifetime decreases in the areas where the proteins interact. The decay profile in the spot selected by the cursors is shown in the middle. As it is typical for FRET measurements, the decay is double-exponential. There is a slow decay component from the non-interacting donor molecules, and a fast decay from the interacting donor. The double-exponential decay analysis delivers the amplitudes of the two decay components [1]. The amplitude ratio directly represents the ratio of interacting and non-interacting proteins, see Fig. 11, right.
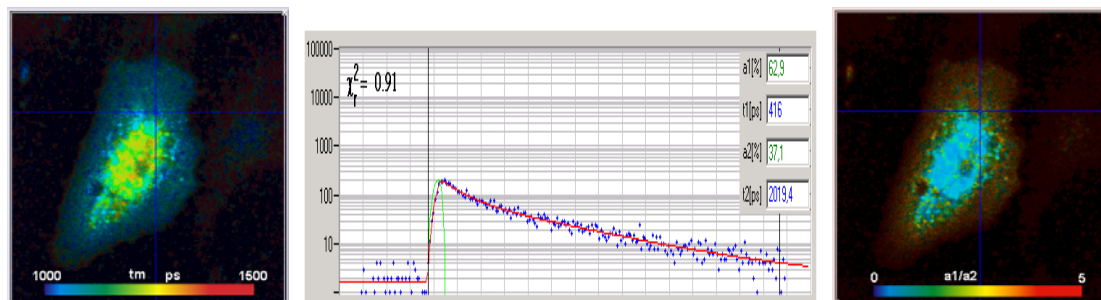

Fig. 11: FRET Measurement. HELA cell expressing two interacting proteins labelled with CFP and YFP. Image taken at donor emission wavelength. Left: Amplitude-weighted average lifetime of double-exponential decay. Middle: Decay curve at cursor position. Right: Ratio of amplitudes of decay components. Image 512x512 pixels, 256 time channels.

Other FRET applications are described in [8, 13, 16, 17]. bh NDD FLIM systems for the Leica SP2 and SP5 have also been used to measure the refractive index and the viscosity inside cells [12, 18]. Fluorophores attached to silver nanoparticles have been studied in [15]. For details and more references to bh FLIM applications please see [2].

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