bh TCSPC Systems Record FLIM with Sutter MOM Microscopes

Wolfgang Becker, Becker & Hickl GmbH

Abstract: The Sutter Instrument MOM microscope [6] is a modular platform for imaging deep within live samples. It uses multi-photon excitation by a titanium-sapphire laser in combination with non-descanned detection. Due to its pulsed excitation source and its high modularity the MOM system can easily be combined with the bh TCSPC FLIM systems. Up to four FLIM detectors can be attached to the system. The signals are processed in up to four entirely parallel TCSPC FLIM channels. Due to the parallel system architecture, high photon count rates and short acquisition times can be achieved. FLIM data can be recorded with up to 1024x1024 pixels and 1024 time channels.

System Architecture

The general system architecture of the Sutter / bh FLIM system is shown in Fig. 1. The MOM microscope is based on the usual multiphoton configuration. A femtosecond Ti:Sa laser is used as an excitation source. The laser beam is reflected down to the microscope lens by a fast galvanometer scanner. A scan lens projects the axis of the scan mirrors into the principle plane of the objective lens. The angular motion of the scan mirrors is thus converted into a lateral motion of the laser focus in the sample. The fluorescence light emitted by the sample is collected and collimated by the microscope lens. A dichroic mirror separates the fluorescence light from the laser light and projects it towards the detectors. A beam splitter assembly splits the fluorescence light into several spectral components, and projects them on the detectors. The optical setup takes advantage of the fact that multiphoton excitation is confined to a thin layer around the focal plane of the microscope lens. Therefore the fluorescence light needs not be sent back through the scanner and through a confocal pinhole. Instead, photons leaving the back aperture of the microscope lens are projected directly on the detectors. Even scattered photons from image planes deep inside the sample are thus detected and used to build up the images. Please see [2] or [3] for details.

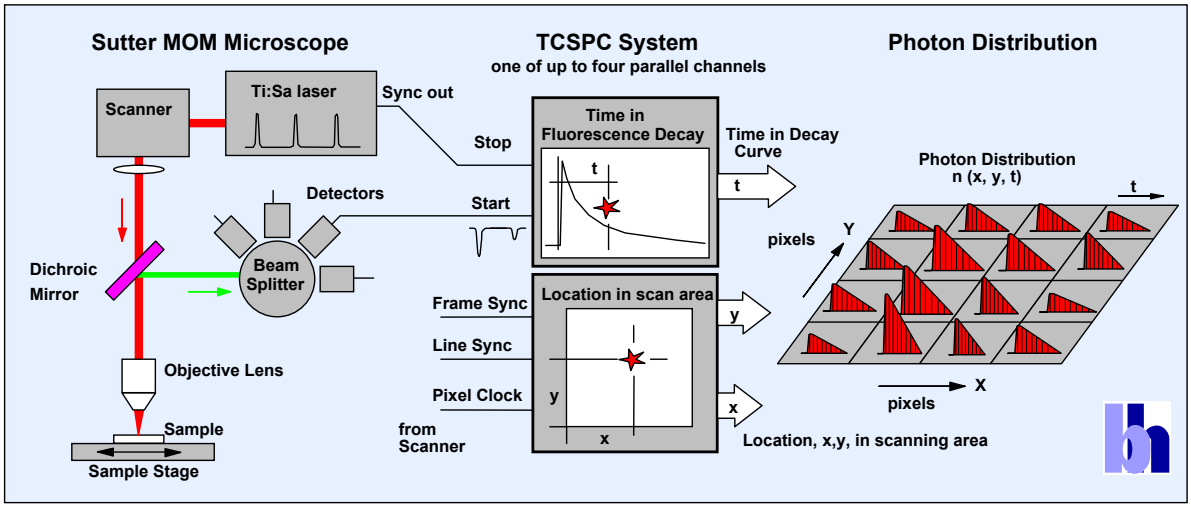

Fig. 1: General principle of the bh/Sutter FLIM systems

Up to four FLIM detectors can be placed at the beamsplitter assembly of the MOM microscope, and the signals be processed in parallel FLIM channels. A front view of the MOM system with two FLIM detectors attached is shown in Fig. 2. Due to size and weight restrictions we are using bh

PMZ-100-1 PMT modules. The active area of the detectors has 8mm diameter, thus efficiently recording the photons projected from the microscope lens to the outputs of the beam splitter assembly.

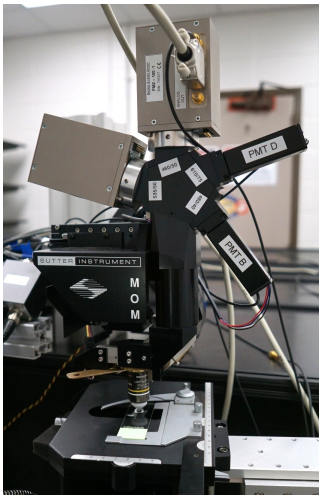

Fig. 2: Two FLIM detectors attached to outputs 1 and 2 of the MOM beamsplitter assembly

The principle of FLIM recording is illustrated in the right part of Fig. 1. The FLIM recording is synchronised with the laser via its 'Sync out' signal, and with the scanner via its pixel, line, and frame clock pulses. For every photon, the TCSPC electronics determines the time within the laser pulse period and the location of the laser focus in the sample in the moment of its detection. The instrument software uses this information to build up a photon distribution over the x and y coordinate of the scan area, and the times of the photons within the laser pulse period. The result is identical with an array of pixels over x and y, each containing a fluorescence decay function in form of photon numbers in consecutive time channels [1, 2]. TCSPC FLIM delivers a near-ideal recording efficiency and an extremely high time resolution. Normally, the recording process is run over a large number of frames of the scan. The result is then independent of the scan rate. The signal-to-noise ratio only depends on the photon rate delivered by the sample, and on the total acquisition time.

More parameters can be added to the photon distribution, such as the wavelength of the photons, the wavelength of several multiplexed lasers, the time from the start of an experiment or from a stimulation of the sample, or the time within period of a modulation of the laser. Most of these advanced FLIM modes are possible in combination with the Sutter MOM system but will not be described here. Please see [2] for details.

System Parameter Setup

The bh / Sutter system is controlled both by the software of the MOM system and the SPCM software of the TCSPC system. The MOM software controls the microscope and the scanner, the SPCM software the data acquisition. The synchronisation between the two system components is performed entirely by the scan clock signals. Start and stop of a measurement is coupled with the frame pulse. No matter whether the scanning or the acquisition is started first - the recording always starts and ends with a frame clock.

MOM Software Parameters

Fig. 3 shows the MOM main panel. Operation mode for FLIM is 'XY Movie', the laser power and the laser wavelength are selected in the middle. Image position, image rotation and focus position are selected on the right.

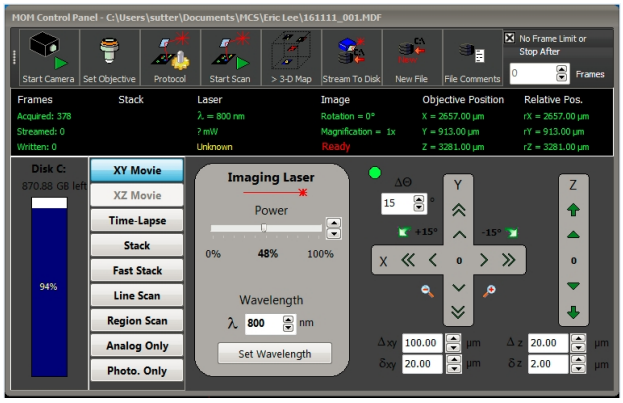

Fig. 3: Main panel of MOM software

The details of the scanning are controlled via the 'Protocol' panel. Settings for different FLIM pixel numbers are shown in Fig. 4, left, middle, and right. The number of pixels in the FLIM recording is 1/2 of the pixel number of the MOM scan. The reason is that the MOM runs a bidirectional scan along the lines. The bh FLIM systems can, in principle, record FLIM with a bidirectional scan [5]. However, in a bidirectional scan the scanner lag causes an offset between the forward and the backward scan. The resulting line shift can, in principle, be compensated in SPCM. This requires, however, that the shift and its dependence from the imaging parameters are accurately known. Since this is not (yet) the case for the MOM system the FLIM system uses only the forward scan for recording. The number of lines of the FLIM recording is therefore 1/2 of the number of lines in the scan. Fig. 4, left, middle, and right show MOM settings for FLIM recordings of 256x256, 512x512, and 1024x1024 pixels, respectively. The MOM can also run a bidirectional Y scan ('Symmetric Y Scan'). This option must be disabled for FLIM recording.

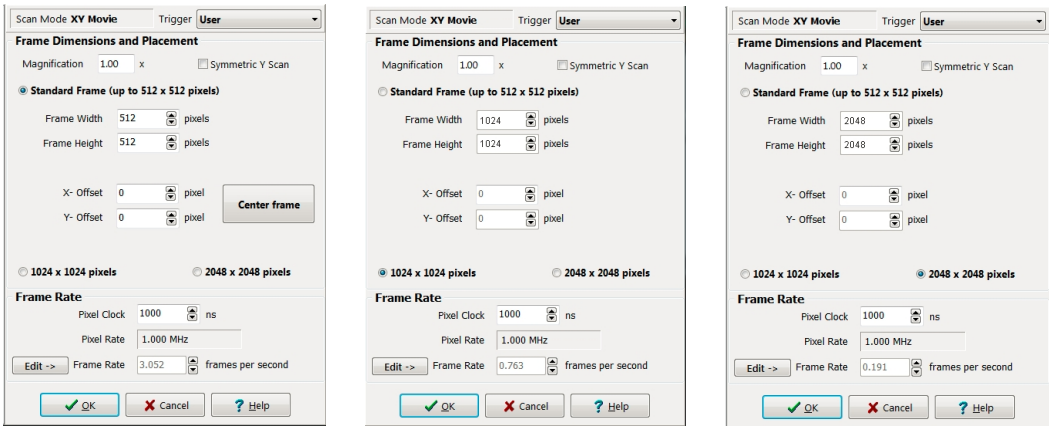

Fig. 4: 'Protocol' panel of MOM software. Left to right: For FLIM images of 256x256, 512x512, and 1024x1024 pixels.

'Magnification' is identical with the 'Zoom' factor in other scanning microscopes. It is reversely proportional to the amplitude of the scan. A smaller amplitude results in a smaller scan area, and thus of a higher magnification. The FLIM system automatically records an image of the correct

size, because the recording is synchronised via the pixel and line clocks. An image with higher 'Magnification' simply has smaller pixels.

SPCM system parameters

The general function of the SPCM parameters is described in [2]. A suitable setup for a FLIM pixel number of 512 x 512 is shown in Fig. 5, left. Note that the setup requires a pixel number of 1024 x 1024 in the MOM software, see above. FLIM pixel formats of 256 x 256 and 1024 x 1024 can be used as well. They require MOM pixel numbers of 512 x 512 and 2048 x 2048, respectively. The number of time channels ('ADC Resolution') for FLIM recordings of 1024 x 1024 pixels can be up to 1024, for the smaller image formats even up to 4096.

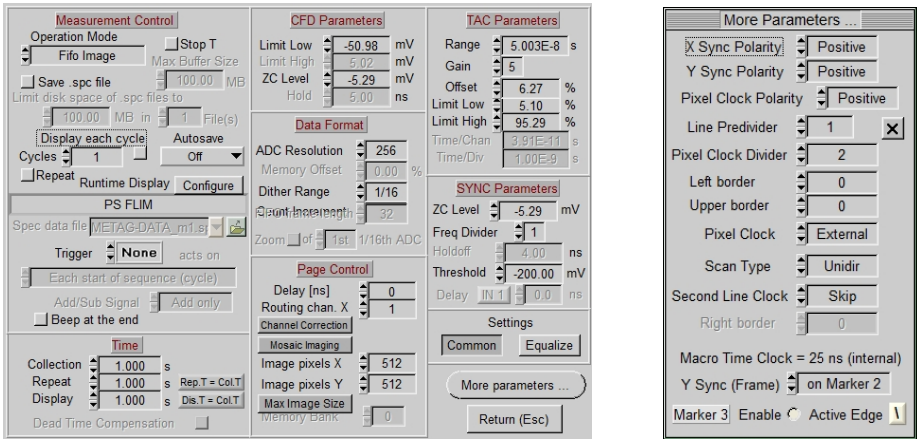

Fig. 5: SPCM Main panel (left) and More Parameters panel with scan interface parameters (right)

For the coupling with the MOM the scan interface parameters are important. They are accessible via 'More parameters', see Fig. 5, right. Line predivider is 1, pixel clock predivider is 2. 'Scan Type' must be 'unidirectional', 'Second Line Clock' is set to 'skip'.

Please note that these settings need not be defined every time you change the image sizes. The parameter sets are just defined once, and then put into a panel of predefined setups. From there, they can be loaded into the system by a single mouse click [2].

For setup of the SPCM display please see [2], 'Display Parameters' and '3D Trace Parameters'. Since September 2016 SPCM has a fast online FLIM display function, please see [4].

Typical Results

Fig. 6 shows a FLIM image of a Convallaria sample. The FLIM data were recorded with 1024 x 1024 pixels.

The spatial resolution of the data is excellent. No out-of-focus blur or ghost images are seen. Decay curves in two selected pixels are shown in Fig. 6, right. The decay data are clean, without any traces of optical reflections, leakage of excitation light, or other imperfections.

The convallaria image demonstrates the image quality of the bh / MOM system. Due to its high fluorophore concentration and low scattering it is, however, not representative of a typical FLIM sample. A more typical sample is shown in Fig. 7. It shows a salmon louse (*Lepeophtheirus Salmonis*), a parasite that lives and feeds on salmons. It causes substantial economic damage in salmon farms, and studying its life cycle and metabolism is of practical interest. The sample was excited at 750 nm, and the fluorescence was measured in the interval from 440 to 480 nm. The

fluorescence in this interval mainly comes from NADH and Keratin. As can be seen from Fig. 7, the FLIM data are extremely rich in detail. The biological meaning of the data and possible changes with the metabolic state remain subject of further investigation.

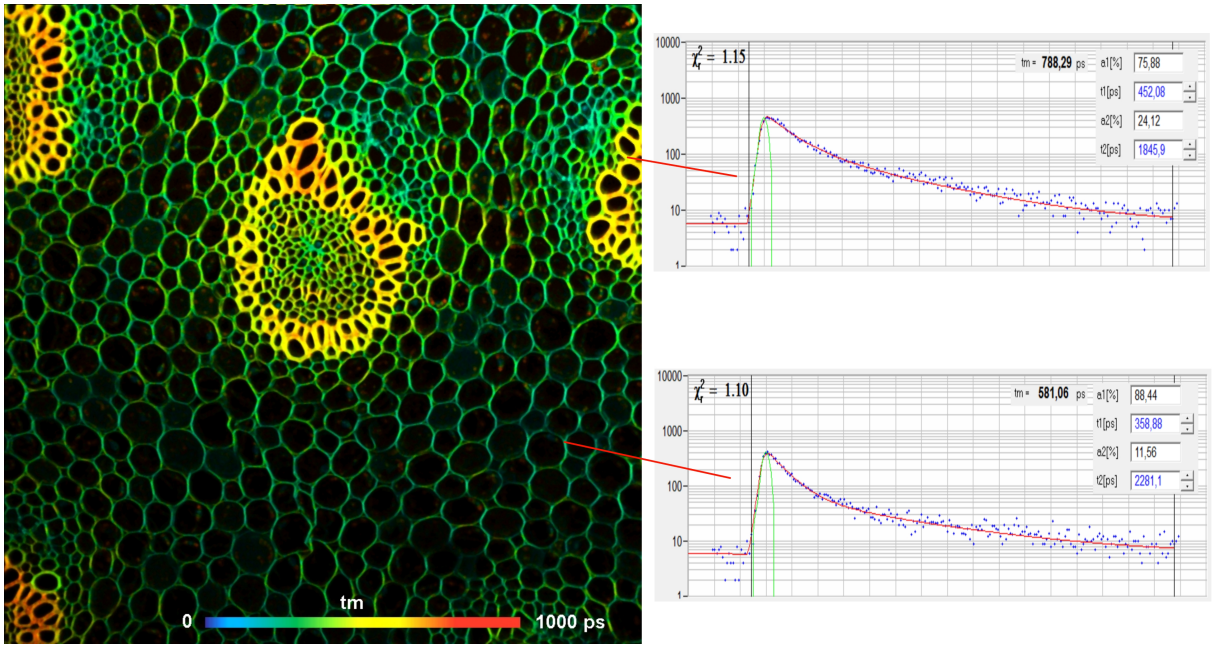

Fig. 6: Left: FLIM image of a convallaria sample, 1024x1024 pixels. Excitation wavelength 800 nm, detection wavelength 510 to 550 nm. Double-exponential decay model, amplitude weighted lifetime of decay components. Right: Decay curves in selected pixels.

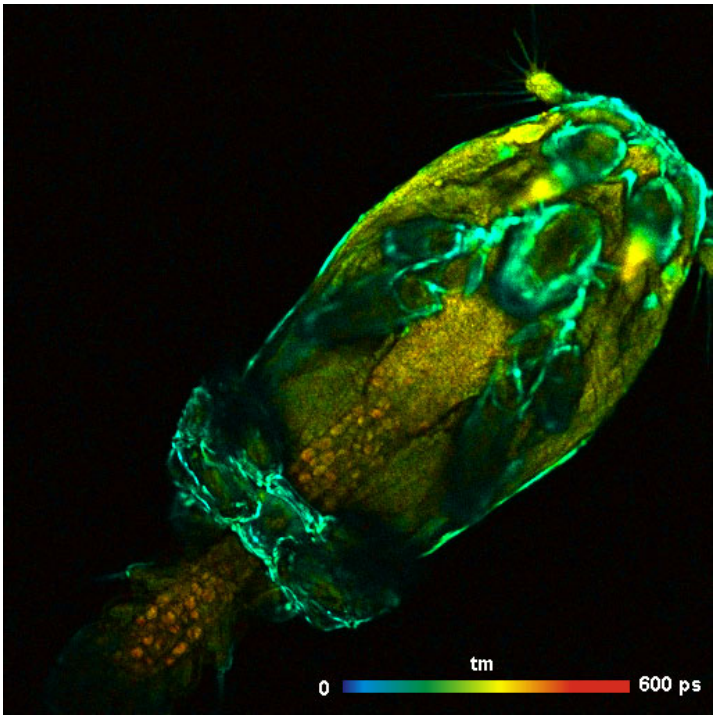

Fig. 7: Salmon louse (*Lepeophtheirus Salmonis*), 512 x 512 pixels. Autofluorescence, excitation wavelength 750 nm, detection wavelength 440 to 480 nm. Amplitude-weighted lifetime of double-exponential decay.

Remarks

As an argument against the use of FLIM at the MOM microscopes it is sometimes stated that the movable-objective function causes an unpredictable shift of the decay data and thus errors in the recorded fluorescence decay times. This is not correct. bh SPCImage data analysis uses a synthetic IRF that is calculated from the decay data themselves [2]. The results are thus independent of a possible change in the optical path length. If a measured IRF has to be used for whatever reason it should be recorded with the same objective position as the FLIM data. Of course, the FLIM setup parameters must guarantee that the decay curves stay within the time interval recorded by the FLIM system. The transit time changes caused by an objective move are in the range of a few 100 ps, so this is not a problem.

As all non-descanned detection systems, the MOM is prone to pick up environment light. This is caused by the fact that non-descanned detection collects photons from a large area of the sample. Daylight background substantially reduces the accuracy of fluorescence lifetimes derived from FLIM data, and has therefore to be avoided. The microscope must be completely covered, and operated in darkness or under dimmed light. A large amount of light can also enter the microscope lens through the sample, from the bottom of the microscope. We therefore recommend to cover the back of the sample when FLIM is recorded.

Conclusions

The combination of the bh TCSPC-FLIM system with the Sutter MOM microscope is an efficient and flexible solution to fluorescence lifetime imaging of live cells and live tissues. The instrument can be operated with up to four parallel FLIM channels, each recording FLIM images with up to 1024x1024 pixels and 1024 time channels per pixel. Multiphoton excitation and non-descanned detection make the system especially useful for FLIM of live cells and tissues. Typical applications are metabolic imaging by recording the fluorescence of NADH and FAD, protein interaction experiments by FLIM-FRET techniques, and ion concentration measurements with environment-sensitive fluorescent dyes.

References

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Contact:
Wolfgang Becker
Becker & Hickl GmbH
Berlin, Germany
becker@becker-hickl.com
www.becker-hickl.com