Megapixel FLIM with bh TCSPC Modules

The New SPCM 64-bit Software

Abstract: Becker & Hickl have recently introduced version 9.60 of their SPCM TCSPC data acquisition software. SPCM version 9.60 not only runs on 64-bit computers, it is a real 64-bit application. It thus takes full advantage of the capabilities of 64-bit Windows. The most significant one is that a large amount of memory can be addressed. As a result, FLIM data can be recorded with unprecedented numbers of pixels and time channels. Moreover, the large memory space allows multi-dimensional FLIM procedures to be used without compromising spatial resolution. Multi-spectral FLIM can be recorded at unprecedented spatial resolution, the image area can be increased by spatial mosaic recording, Z stacks can be efficiently acquired without the need of intermediate data save actions, and fast time series of FLIM data can be accumulated. This application note gives an overview of the performance that can be achieved by running bh TCSPC FLIM modules with the new software.

Image Size in FLIM

TCSPC FLIM has been introduced in 1996 with the SPC-535 modules of Becker & Hickl. The first applications were in ophthalmology, where FLIM was shown to detect early stages of eye diseases. FLIM for laser scanning microscopy was introduced with the SPC-730 modules in 1998. The FLIM data were built up by the on-board processing logics in the memory of the TCSPC module. The size of the FLIM images was therefore limited by the size of the on-board memory. Typical images had 128 x 128 pixels, and 256 time channels per pixel. The image size increased by a factor of four with the introduction of the SPC-830 modules. The data format typically used by these modules was 256 x 256 pixels, and 256 time channels [1, 2].

A possible way to record larger images is to transfer the data into the computer photon by photon, and build up the FLIM data in the memory of the computer. A suitable operation mode, the 'FIFO Mode' [1, 2], had been implemented in bh SPC modules already in 1996. However, the bus transfer speed and the processing capabilities of the PCs of the 1990s was insufficient for this kind of operation. At count rates above 100 kHz, as they are routinely used in FLIM, the computer was not able to read the single-photon data from the SPC module and simultaneously process them to build up a photon distribution.

The situation changed with the introduction of multi-core PCs. The tasks of data transfer and data processing could now be shared between the CPU cores. Count rate in the FIFO mode was no longer a problem. bh introduced the 'FIFO Imaging' mode for FLIM [6] in 2007. Limited by the amount of memory addressable by Windows 32 bit, the typical image size was 512 x 512 pixels, 256 time channels. This is more than enough to obtain diffraction-limited microscopy images of cells and tissue samples at a time resolution (time-channel width) on the order of a few 10 ps.

However, there are FLIM applications that make it desireable to record FLIM data of larger size. Examples are imaging of a large number of cells in the same field of view, or FLIM measurements over additional parameters, such as wavelength, depth in the sample, or time after a stimulation. Such application require an amount of memory space which is only available in a 64-bit environment.

FIFO Imaging with Windows 64 bit

A typical PC running Windows 64 bit is able to use about 16 GB of memory, as compared to 4 GB for Windows 32 bit. bh therrfore released a 64-bit version of the SPCM data acquisition software. Provided the computer has enough memory SPCM 64 bit can record images as large as 2048 x 2048 pixels and 256 time channels, or 1024 x 1024 pixels and 1024 time channels. Several such data sets can be recorded simultaneously if the FLIM system has several TCSPC channels.

SPC system parameters for an image of 2048 x 2048 pixels and 256 time channels are shown in Fig. 1. Of course, the scan format of the scanner must be at least of the same size [4, 5]. Settings for the bh DCS-120 confocal scanner are shown on the right.

Fig. 1: Image format definitions for an image of 2048 x 2048 pixels and 256 time channels. Left: SPCM system parameters, Data Format and Page Control section. Right: DCS-120 scanner control panel.

An image of a convallaria sample recorded with these parameters is shown in Fig. 2. The image on the left is the entire FLIM image, the image on the right is a digital zoom into the area marked in the in the image shown left.

tm = 1500 ps   4000 ps
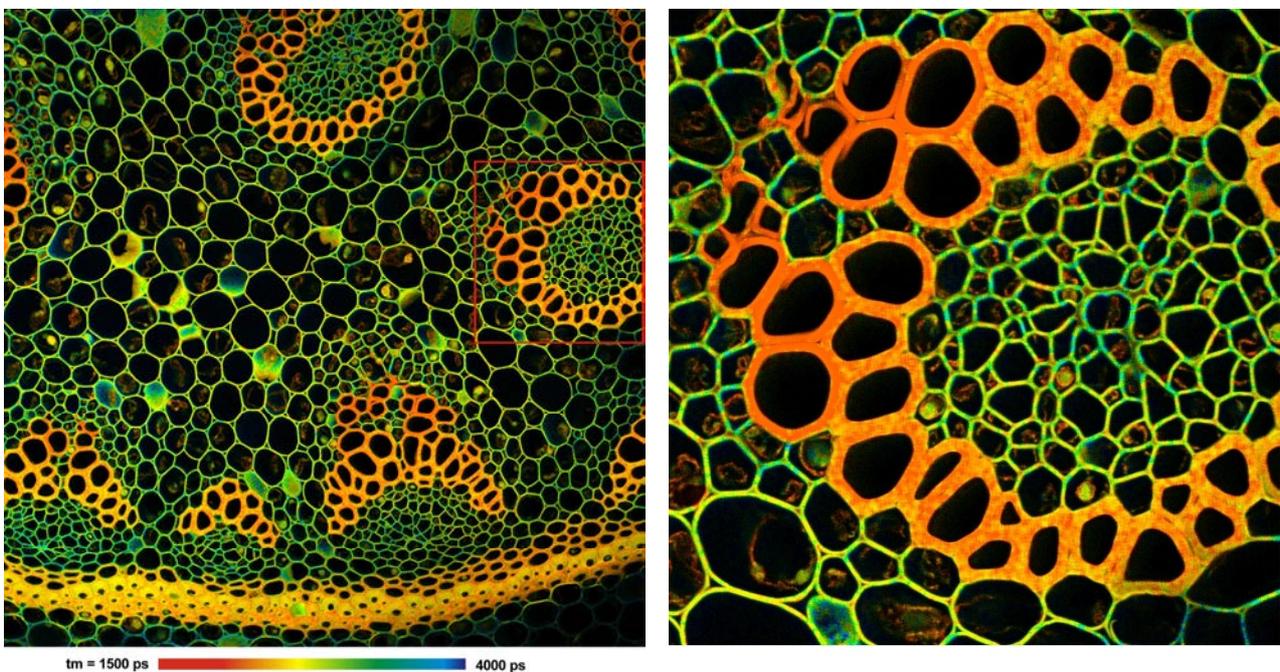
Fig. 2: FLIM recorded with 2048 x 2048 pixels and 256 time channels. Left: Entire image. Right: Digital zoom into indicated area of left image. bh DCS-120 confocal scanning FLIM system, Zeiss Axio Observer microscope, x20 NA=0.5 air lens.

FIFO imaging with the 64-bit SPCM software becomes especially powerful when several images are to be recorded simultaneously. Fig. 3 shows images recorded by a dual-channel parallel TCSPC (SPC-152) system. The sample was a BPAE cell labelled with Alexa 488 and Mito Tracker. The excitation wavelength was 473 nm. The images were recorded simultaneously in two wavelength intervals, from 484 nm to 560 nm, and 560 nm to 700 nm. The FLIM data format for both images is 1024 x 1024 pixels and 1024 time channels. The total size of the FLIM data set is 4 GB. A decay curve at a selected pixel position is shown in Fig. 4.
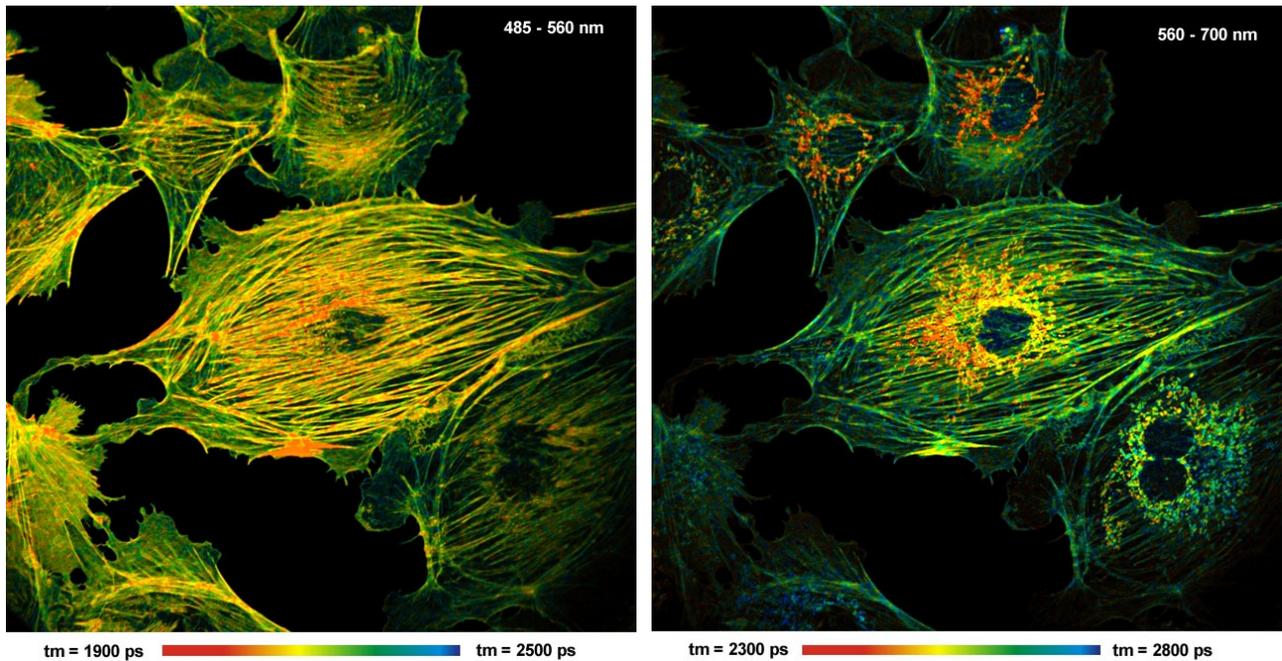
Fig. 3: Dual-channel parallel FLIM with two bh SPC 150 TCSPC modules. Both images, each with 1024 x 1024 pixels and 1024 time channels, were recorded simultaneously. bh DCS-120 confocal scanning system with Zeiss Axio Observer microscope, x63 NA=1.3 oil immersion lens.
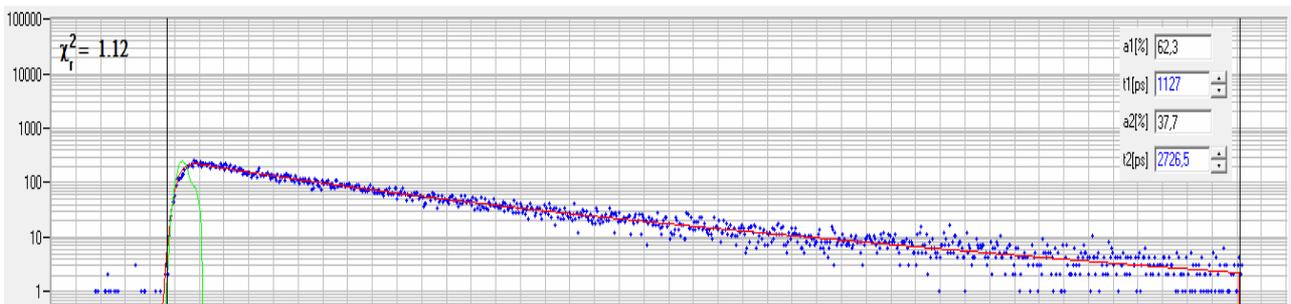
Fig. 4: Fluorescence decay function at selected position of Fig. 3, left. The decay function is recorded in 1024 time channels. Blue dots: Photon numbers in the time channels. Red curve: Fit with a double-exponential model.

The large data formats provided by SPCM 64 bit are also desirable for multi-wavelength FLIM recordings. With Windows 32 bit, the maximum data format for the MW-FLIM 16-channel multi-wavelength detector was limited to 256 x 256 pixels, 64 time channels, or 128 x 128 pixels, 256 time channels. With Windows 64 bit and SPCM V 9.60 images with 512 x 512 pixels and 256 time channels can be obtained. This is 16 times the number of pixels obtained with Windows 32 bit.

Fig. 5 shows a multi-wavelength FLIM recording of a Convallaria sample. The data were recorded by a bh DCS-120 confocal scanning system with a bh MW-FLIM GaAsP 16-channel detector. 16 lifetime images in 16 wavelength intervals were recorded simultaneously. The data were recorded at a resolution of 512 x 512 pixels and 256 time channels per pixel. The wavelength channel width was 12.5 nm, the centre wavelengths of the channels range from 490 nm to 690 nm. The size of the entire multi-wavelength data set is 2 GB.
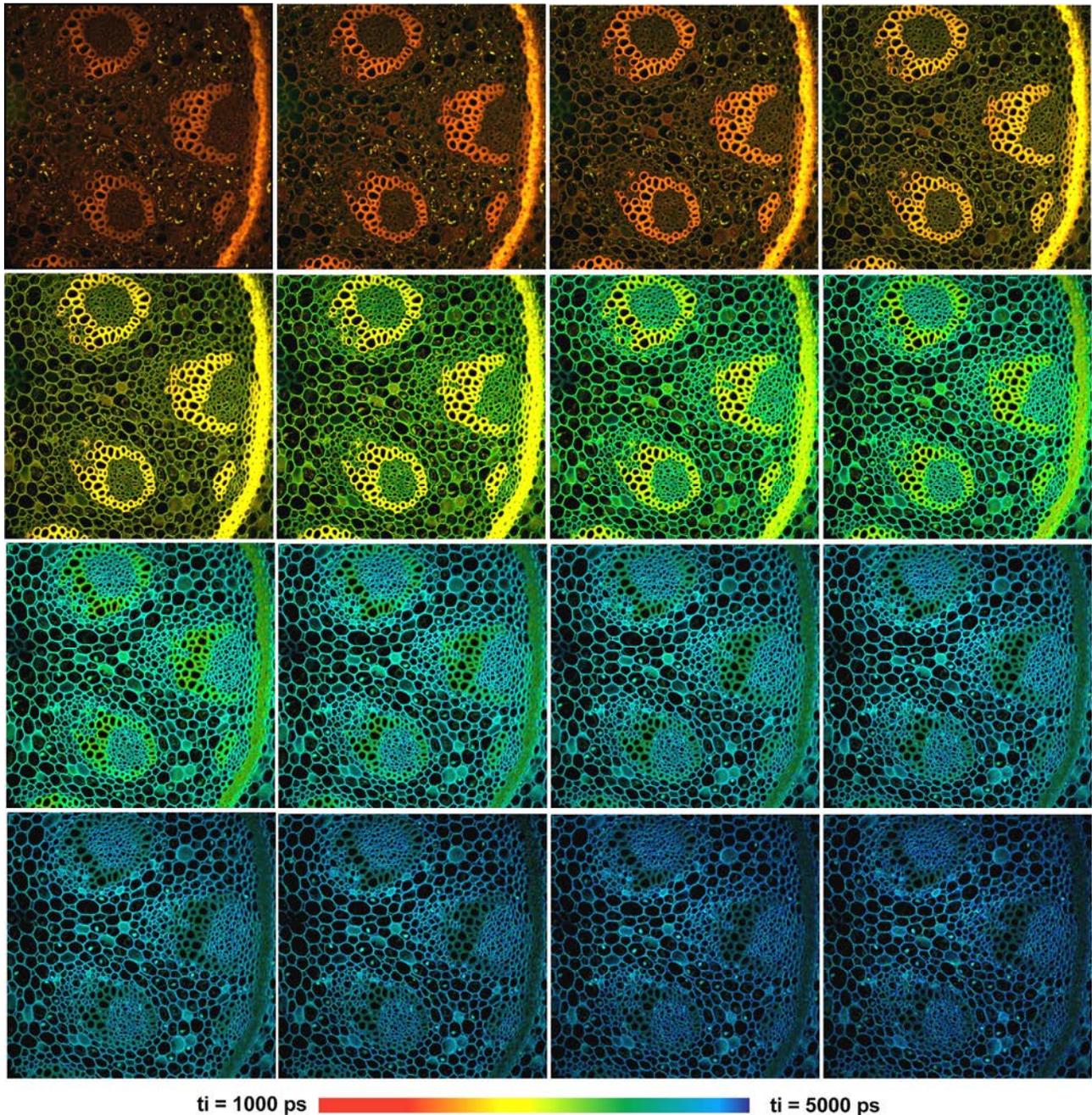
Fig. 5: Multi-wavelength FLIM with a bh MW-FLIM GaAsP 16-channel detector. 16 images with 512 x 512 pixels and 256 time channels were recorded simultaneously. Wavelength from upper left to lower right, 490 nm to 690 nm, 12.5 nm per image. DCS-120 confocal scanner, Zeiss Axio Observer microscope, x20 NA=0.5 air lens.

Fig. 6 demonstrates the true spatial resolution of the data. Images from two wavelength channels, 502 nm and 565 nm, of the data shown Fig. 5 are displayed at larger scale and with individually adjusted lifetime ranges. The spatial resolution is comparable with what previously could be reached for FLIM at a single wavelength. Nevertheless, the decay data are recorded at a temporal resolution of 256 time channels. Decay curves for selected pixels of the images are shown in Fig. 7.
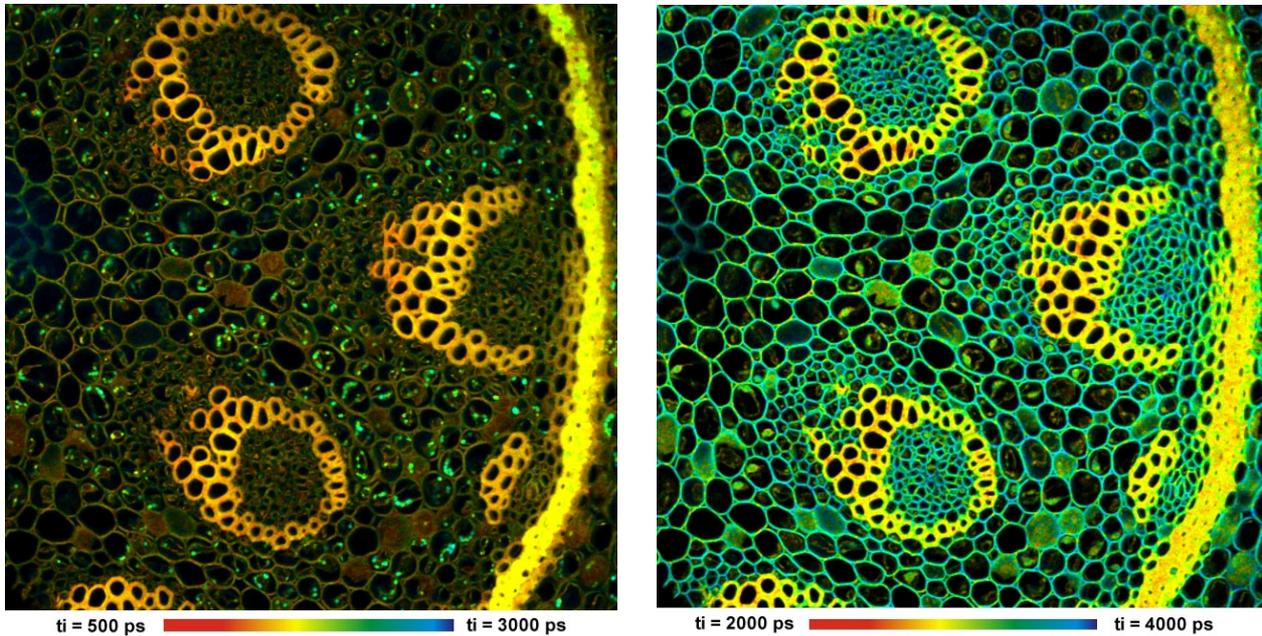
Fig. 6: Two images from the array shown in Fig. 5, displayed in larger scale and with individually adjusted lifetime range. Wavelength channels 502 nm (left) and 565 nm (right). The images have 512 x 512 pixels and 256 time channels.
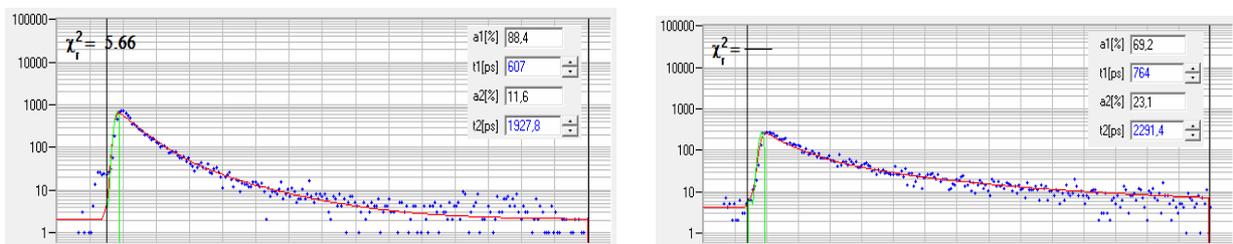
Fig. 7: Decay curves at selected pixel position in the images shown above. Blue dots: Photon numbers in the time channels. Red curve: Fit with a double-exponential model.

Mosaic FLIM

To record images larger than the maximum field diameter of the microscope lens laser scanning microscopes use a 'tile imaging' function. The microscope scans an image at one position of the sample, then offsets the sample by the size of the scan area, and scans a new image. The process is repeated, and the images of the individual frames are stitched together. This way, a sample area larger than the field of view of the microscope lens can be imaged.

X-Y Mosaic

The large memory size available in the 64 bit environment allows tile imaging to be combined with FLIM. In the memory of FLIM system, a two-dimensional mosaic of FLIM data blocks is defined.

The number and arrangement of the data blocks is the same as for the tiles in the imaging procedure of the microscope. The FLIM data structure can thus be considered one large FLIM image that contains all tiles in the correct arrangement. Moreover, for each element of the FLIM mosaic a number of frames is defined which is identical with the number of frames per tile defined in the microscope. When the microscope starts the scan the FLIM system starts to record an image in the first mosaic element. It continues to do so until the defined number of frames has been completed, and then switches to the next mosaic element. This way, the FLIM system switches through all mosaic elements, filling all of them with data of the corresponding tiles. The procedure ends when the microscope has completed the last frame of the last tile.

An example of a Mosaic FLIM is shown in Fig. 8. The image was recorded by a Zeiss LSM 710 Intune (tuneable excitation) system in combination with a bh Simple-Tau 150 FLIM system. The mosaic has 4x4 elements, each element has 512 x 512 pixels with 256 time channels. The complete mosaic has thus 2048 x 2048 pixels, each pixel holding 256 time channels. The sample area covered by the mosaic is 2.5 mm x 2.5 mm.
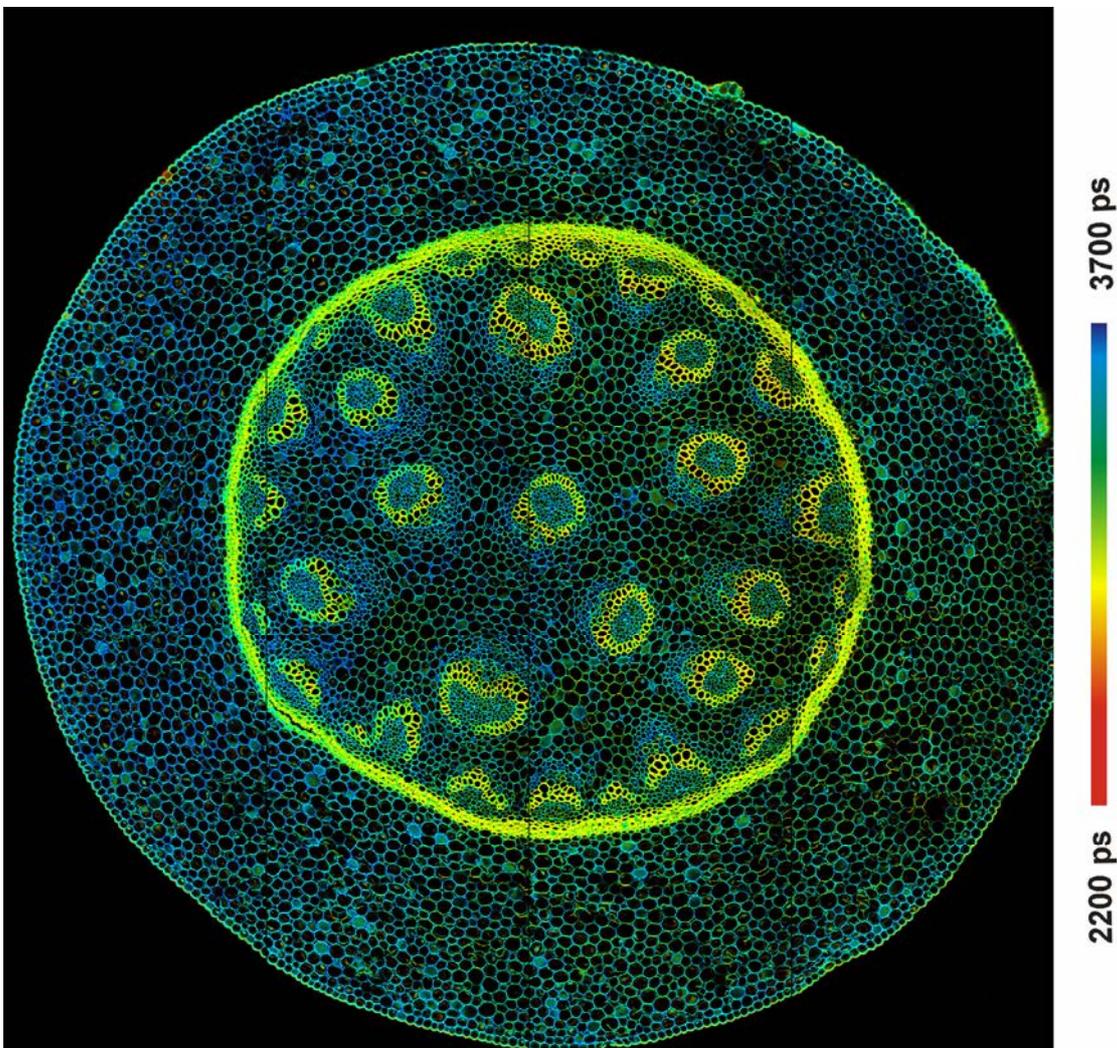
Fig. 8: Mosaic FLIM of a Convallaria sample. The mosaic has 4x4 elements, each element has 512x512 pixels, each pixel has 256 time channels. Zeiss LSM 710 Intune system with bh Simple-Tau 150 FLIM system.

Mosaic FLIM for Z-Stack recording

The same FLIM procedure can be used to record z-stacks of FLIM images. The number of mosaic elements is made identical with the number of z planes the microscope scans. The number of frames per mosaic element is selected according to the number of frames per z plane. Compared with a z-stack FLIM procedure by a record-and-save sequence [2, 7] mosaic FLIM has the advantage that the synchronisation between the microscope and the FLIM system is easier, and no time has to be reserved for the saving actions. Moreover, the data of all z planes are recorded in one and the same photon distribution. The data analysis can therefore be run for the data of all z planes together. This makes it easier to maintain exactly the same fit conditions and model parameters for all z planes, and to extract global parameters form the entire array. An example is shown in Fig. 9.
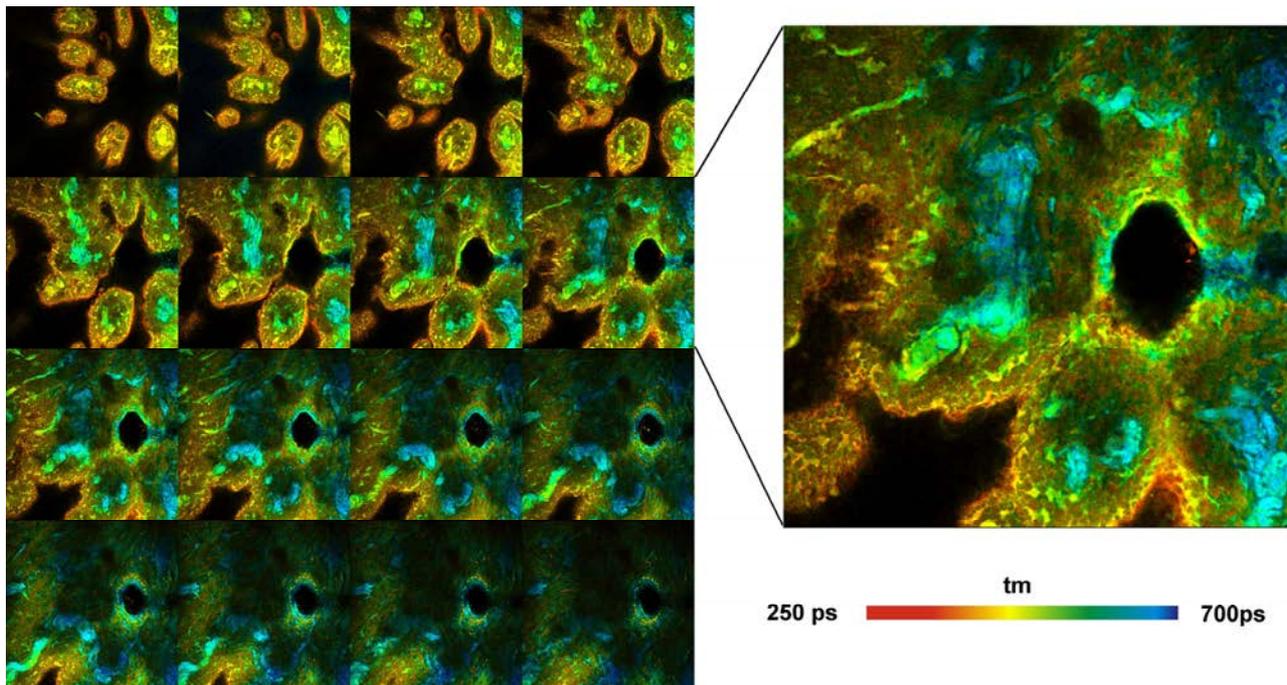
Fig. 9: Z-stack recorded by Mosaic FLIM. Pig skin stained with Indocyanin Green. Zeiss LSM 7 MP OPO system, 16 planes from 0 to 60 µm from top of sample, each plane 512x512 pixels, 256 time channels. Amplitude-weighted lifetime of double-exponential decay.

Temporal Mosaic FLIM

The usual way to record time series by FLIM is to record a sequence of images and save the subsequent images in consecutive files [1, 2, 4, 5]. Mosaic FLIM provides a second way to record time series. In that case, every element of the mosaic represents one step of the series. Each element can be a single frame or a defined number of frames of the scan.

An example is shown in Fig. 10. The sample was a fresh moss leaf, the microscope a bh DCS-120 confocal FLIM system [4]. The time runs from lower left to upper right. The excitation light causes a non-photochemical transient in the chlorophyll fluorescence [8], resulting in a decrease in the fluorescence lifetime over the time of exposure.
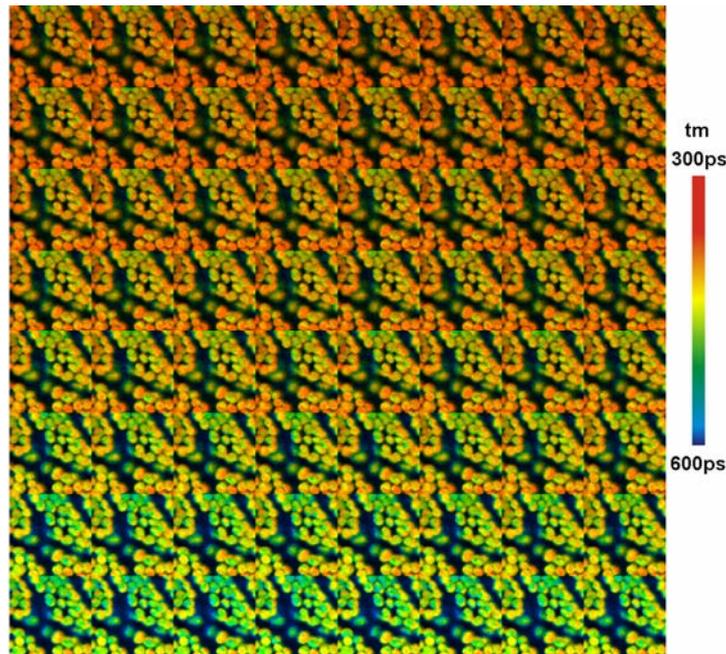
Fig. 10: Time series recorded by mosaic imaging. Non-photochemical transients in chloroplasts of a moss leaf. 64 mosaic elements for consecutive times after turn-on of excitation light. Acquisition time per element 1 second, total time of sequence 64 seconds, image size of each element 128 x 128 pixels, 256 time channels. Time runs from lower left to upper right. bh DCS-120 confocal FLIM system with SPC-150 TCSPC modules, SPCImage data analysis software.

Mosaic time series recording has two advantages over the conventional record-and-save procedure. First, the transition from one mosaic element to the next occurs instantaneously. There are no time gaps between the steps of the sequence. Therefore, very fast time series can be recorded.

The biggest advantage is, however, that mosaic time series data can be accumulated: A sample would be repeatedly stimulated by an external event, and the start of the mosaic recording be triggered by the stimulation. With every new stimulation the recording procedure runs through all elements of the mosaic, and accumulates the photons. Accumulation allows data to be recorded without the need of trading photon number and lifetime accuracy against the speed of the time series. Consequently, the time per step (or mosaic element) is only limited by the minimum frame time of the scanner. For pixel numbers on the order of 128x128 pixels and small scan areas galvanometer scanners reach frame times on the order of 50 milliseconds. Resonance scanner are even faster. This brings the time-series resolution into the range where even lifetime changes induced by Ca^{2+} transients in neurons can be recorded. Previously, this was only possible by FLITS, i.e. recording the decay functions within the pixels of a line scan [3].

Fig. 11 shows a mosaic FLIM recording of the Ca^{2+} transient in cultured neurons after stimulation with an electrical signal. Oregon Green was used as a Calcium sensor. A Zeiss LSM 7 MP multiphoton microscope was used to scan the sample. The scan format was 64 x 64 pixels. With 64 x 64 pixels and a zoom factor of 5, the LSM 7 MP reaches a frame time of 38 ms. The FLIM data were recorded by a bh SPC-150 module. A FLIM mosaic size of 8 x 8 elements was defined. The stimulation period was 3 seconds. 150 milliseconds before every stimulation a recording through the entire 64-element mosaic was started. With the frame time of 38 ms, the acquisition thus runs through the entire mosaic in 2.43 seconds. 100 such acquisition runs were accumulated. The result shows clearly the increase of the fluorescence lifetime of the Ca^{2+} sensor in the mosaic

elements 4 to 6, and a return to the resting state over the next 10 to 15 mosaic elements (380 to 570 ms).
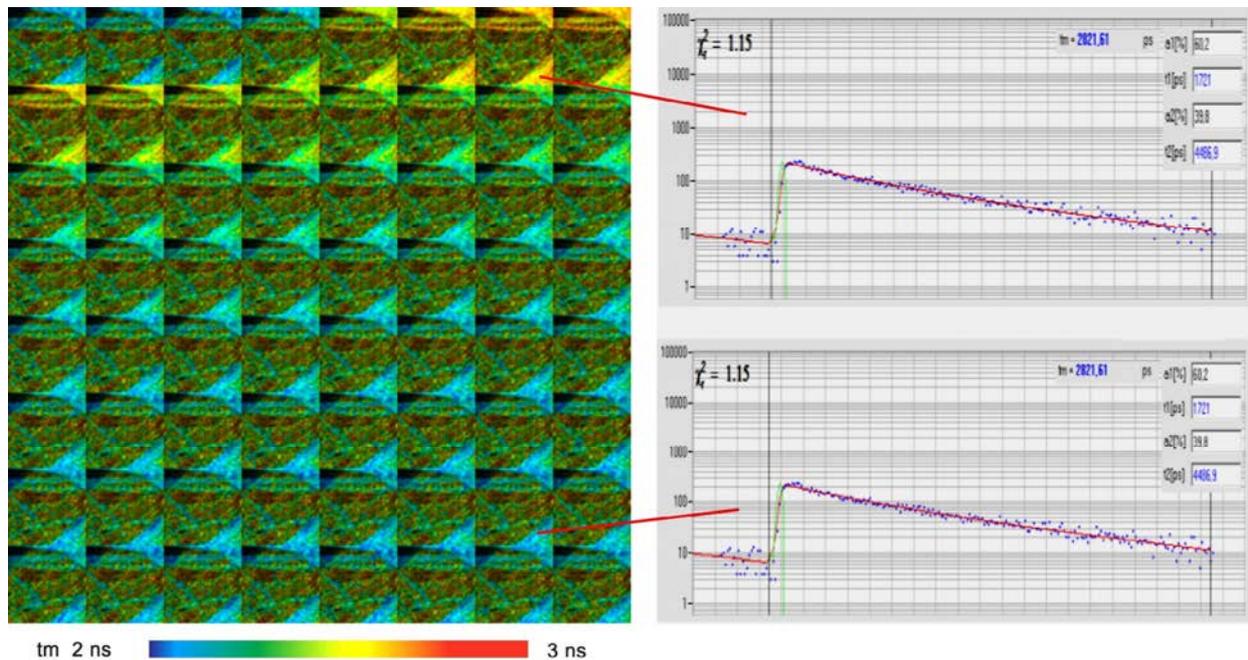
Fig. 11: Temporal mosaic FLIM of the Ca^{2+} transient in cultured neurons after stimulation with an electrical signal. The time per mosaic element is 38 milliseconds, the entire mosaic covers 2.43 seconds. Experiment time runs from upper left to lower right. Photons were accumulated over 100 stimulation periods. Recorded by Zeiss LSM 7 MP and bh SPC-150 TCSPC module. Data courtesy of Inna Slutsky and Samuel Frere, Tel Aviv University, Sackler Faculty of Medicine.

Data Acquisition and Data Analysis Times

Acquisition times for megapixel FLIM data are naturally long. To obtain a given signal-to-noise ratio, a 1024 x 1024 pixel image needs 16 times the photons of a 256 x 256 pixel image. Assuming that the 256 x 256 image is recorded in 30 seconds the 1024 x 1024 image would need 8 minutes of acquisition time to obtain the same number of photons per pixel and the same lifetime accuracy. Fortunately, in practice the acquisition time often does not scale linearly with the pixel number. The reason is that images with large pixel numbers are usually over-sampled. Under these conditions, the lifetime data in adjacent pixels (i.e. the pixels within the Airy-disc diameter) are highly correlated. It is then possible to use pixel binning for the lifetime data in the SPCImage data analysis. The photon number per pixel required for a given lifetime accuracy is than much smaller. Please see chapters on data analysis in [2], [4], or [5].

Data analysis times for large FLIM data sets can be long as well. Note that a 1024 x 1024 image with 1024 time channels is actually a stack of 1024 one-megapixel images! Because the analysis procedure has to run through all pixels and time channels the processing time scales linearly with the numbers of pixels and time-channels. To speed up the data analysis we recommend to select 'Multi-Thread Processing' in the 'Options' section. Please also make sure that you do not analyse the data with conflicting parameters: For instance, fitting a data set with a multi-exponential model and a free 'Shift' parameter causes a conflict between a possible change in a fast decay component and a change in the shift. The data analysis time then increases dramatically.

Before starting the final analysis of a large image, we recommend to analyse a small region of interest, and set appropriate model parameters. The final analysis will then run smoothly, and need not be done twice. For a fast preview of the lifetimes, we recommend to use the 'Moments' analysis of SPCImage, see 'Options', 'Model'.

Please note that recent SPCImage versions have a 'Batch Processing' function [2]. It is used to analyse several FLIM data sets with the same model and fit parameters. Once the process has been started, batch processing does not require user interaction. Several megapixel FLIM data sets can then be analysed 'over night'.

Conclusion

The bh FLIM systems with version 9.6 SPCM 64 bit software or later are able to record images of unprecedented numbers of pixels and time channels. One may argue that these pixel numbers are far beyond the number required to resolve a single cell at reasonable spatial oversampling. This is certainly correct. There are, however, FLIM applications that make it desirable to scan much larger fields of view than the one filled by a single cell. A typical case is when the fluorescence-decay signature of a large number of cells is to be compared. Being able to record many cells in the same field of view is then a clear advantage. A large field of view is also an advantage for tissue imaging. The pixel numbers obtained by the new software are on the same order as the maximum useful pixel numbers for the best microscope lenses when these are used at their maximum field of view. Another advantage of the large memory space available by SPCM 64 bit is that FLIM data with additional dimensions can be recorded without compromising spatial resolution. This allows the user to record multi-spectral FLIM at unprecedented spatial resolution, to increase the image area by spatial mosaic recording, and to efficiently acquire Z stacks and fast time series of FLIM data.

Compatibility

The FIFO imaging mode in the 64 bit version of the SPCM software can be used for all FLIM systems based on the bh SPC-150, SPC-152, and SPC-154 TCSPC modules, the Simple-Tau 150, 152, and 154 systems, similar SPC-160-based systems, and for SPC-830-based systems later than May 2007, or serial number 3D0178. The computer used must have 64 bit architecture, and run Windows 7, 64 bit. To take full advantage of the 64 bit capabilities the computer should be equipped with 16 GB of main memory. Free upgrades of the SPCM software are available from www.becker-hickl.com.

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