Fast-Acquisition Multiphoton FLIM with the Zeiss LSM 880 NLO

Abstract: This application note demonstrates the performance of the bh FASTAC (fast acquisition) FLIM system in combination with the Zeiss LSM 880 NLO multiphoton laser scanning microscopes. The system is based on fast distribution of the photon pulses of a PMH-100 hybrid detector into four parallel TCSPC FLIM channels. The principle strongly reduces counting loss and pile up effects. If the sample allows, images can be recorded in excess of 10 MHz count rate, and at acquisition times down to the minimum frame times of the Zeiss LSM 880. Importantly, the system makes no compromises in terms of time resolution, time channel width, time channel number, or pixel number. The IRF width with fast detectors is less than 25 ps FWHM, and the temporal data are recorded with typically 1024 time channels per pixel. The time channel width can be made as small as 0.8 ps.

Principle

The system is based on the bh FASTAC fast acquisition FLIM system [10, 11]. The FASTAC system uses a single detector the photon pulses of which are distributed into four parallel TCSPC modules, see Fig. 1. The data of the four modules are combined into a single FLIM data set. The TCSPC modules are running the normal bh FLIM process [1, 2, 3]. Consequently, there is no need to trade time resolution or time channel width against acquisition speed. The IRF width with fast hybrid detectors is less than 25 ps (full width at half maximum) [7, 8, 10], the time channel width can be made shorter than 1 ps [1], and the number of time channels is large enough for multi-exponential decay analysis [1, 8, 13]. Moreover, the multi-dimensional features of the bh FLIM technique, such as spatial and temporal mosaic FLIM [1, 3], FLITS [4] or simultaneous FLIM / PLIM [5] remain available.

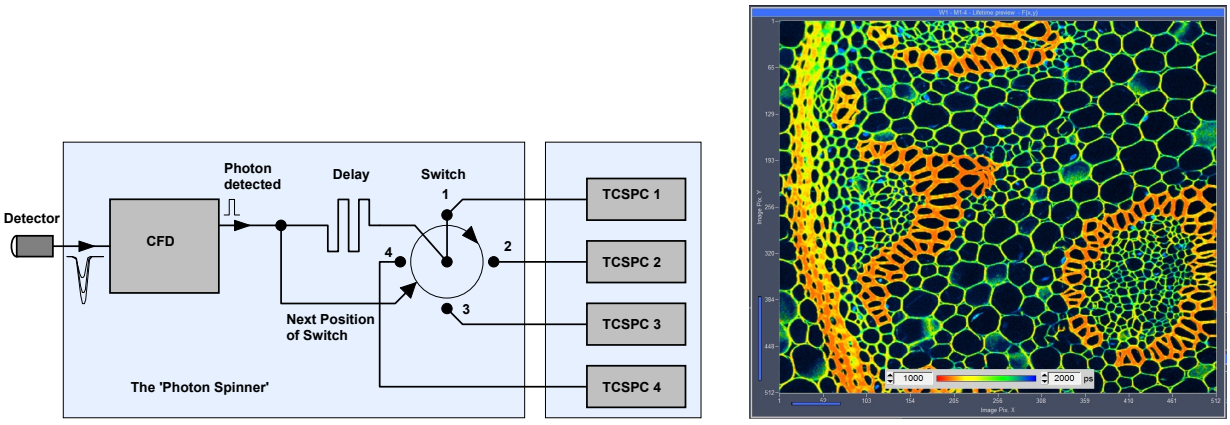

Fig. 1: Left: Principle of the bh FASTAC FLIM system. Right: Image of a *convallaria* sample, 512x512 pixels, recorded in 4 seconds.

System Architecture

For the results presented in this note we used an LSM 880 NLO multiphoton microscope with non-descanned detection. The multiphoton version was chosen because most FLIM experiments are performed on live cells and tissue [3]. The results can, however, extrapolated to one-photon systems with ps diode laser excitation [13].

A HPM-100-06 hybrid detector [7] was attached to the NDD port of the microscope via the usual Zeiss T adapter. The output pulses of the detector were connected to a PHDIS photon distribution

module [10]; the pulses from the four outputs of the PHDIS were connected to four SPC-150N TCSPC / FLIM modules [1]. The data acquisition in the FLIM system was synchronised with the LSM 880 via the usual pixel, line, and frame clock signals [13]. The LSM 880 was controlled by the Zeiss ZEN software, the FLIM system by bh SPCM software, version 9.78. The parameters for general FLIM acquisition, TCSPC-channel combination, and online lifetime display were chosen as described in [9], [12], and [13]. Multi-exponential data analysis was performed by bh SPCImage, version 7.1 [1, 13].

Results

Megapixel Images Obtained at Short Acquisition Time

In 2014 bh introduced 'Megapixel FLIM' with pixel formats of 1024 x 1024 and more, without compromising the temporal resolution [6]. Fig. 2 shows that Megapixel FLIM is also available for the FASTAC FLIM system. The figure shows a 1024 x 1024 pixel, 1024 time-channel image of a BPAE (bovine pulmonary artery) cell sample (stained with DAPI, Alexa 488 and Mito Tracker Red). The excitation wavelength was 800 nm. The image was recorded in 10 seconds. With 'normal' FLIM systems several minutes are required to record lifetime images of similar pixel numbers and similar signal-to-noise ratio.

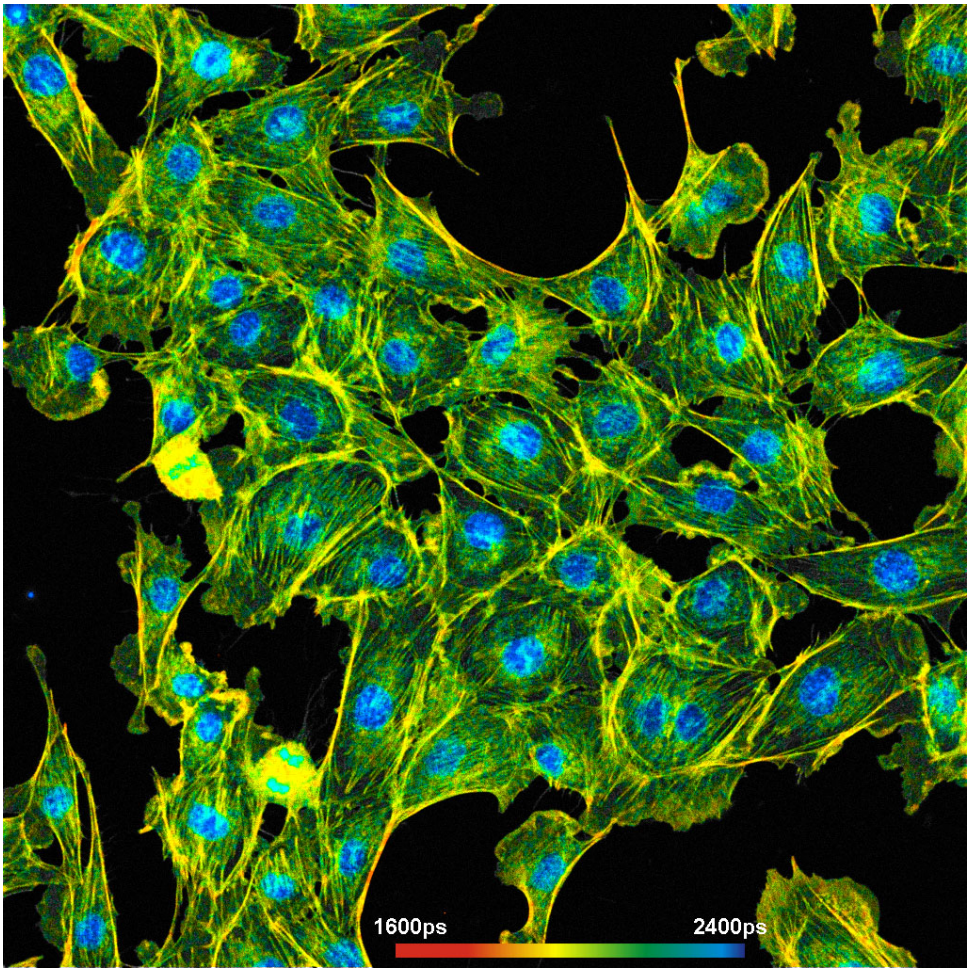

Fig. 2: FLIM of a BPAE sample. 1024x1024 pixels, 1024 time channels per pixel. Acquisition time 10 seconds

Medium-Size Images

Another lifetime image of a BPAE sample, recorded with 512 x 512 pixels and 1024 time channels, is shown in Fig. 3. The acquisition time was 10 seconds. A decay curve from an 8x8 pixel region in the centre of the image is shown on the right. Please note the fast rise of the fluorescence signal, which is a result of the short IRF width of the detector / TCSPC combination [7].

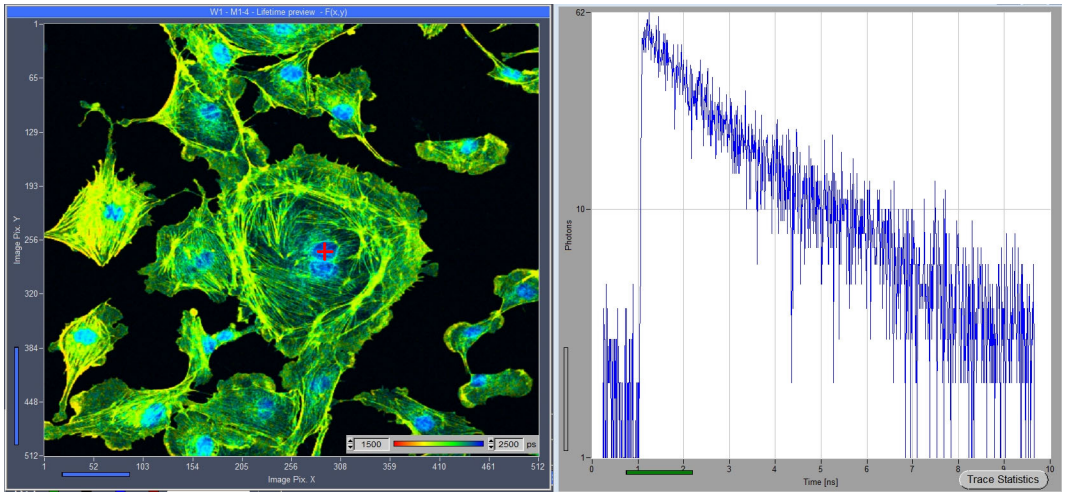

Fig. 3: FLIM image of a BPAE sample, 512 x512 pixels, 1024 time channels, excitation wavelength 800 nm. Acquisition time 10 seconds. Left: Lifetime image, created by online lifetime function of SPCM. Right: Decay curve from 8x8 pixel area around the spot marked in the image. Note the fast rise of the fluorescence, which is a result of the extremely fast IRF of the system.

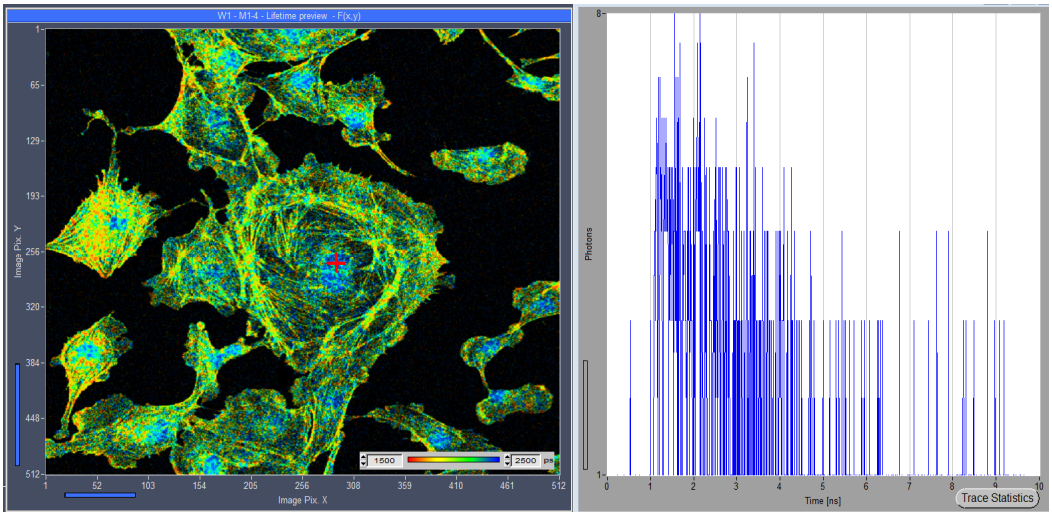

Fig. 4: Same sample and sample area as in Fig. 3, but image recorded within 0.6 seconds

Fig. 4 shows an image of the same area of the same sample, but recorded within a single frame of 0.6 seconds. Necessarily, the image gets more noisy than the 10-second image. Nevertheless, the online-lifetime calculation algorithm of SPCM [9] clearly reproduces the lifetime variation between different regions of the cells.

The images shown in Fig. 2, Fig. 3 and Fig. 4 were recorded at an excitation power of 4% of the available laser power. At this power, the BPAE sample delivered a count rate of about 4 MHz averaged over the entire sample. The peak count rate probably reached 10 MHz. This is significantly less than the FASTAC system can process. In principle, the count rate and thus the

signal-to-noise ratio could be increased by increasing the laser power. However, laser power levels of 5% and more caused rapid photobleaching and even photodamage in the sample.

Higher count rates can be obtained from samples which contain higher concentrations of fluorophores. A typical representative is the *convallaria* sample (stained with acridine orange) often used for microscope demonstrations. Fig. 5 shows two images of a convallaria sample from Lieder. Both images have 512 x 512 pixels and 1024 time channels per pixel. The left image was recorded in 0.6 seconds, the right image in 4 seconds. Despite the short acquisition time, the lifetime noise in the 0.6-second image is barely visible. In the four-second image it is not visible at all.

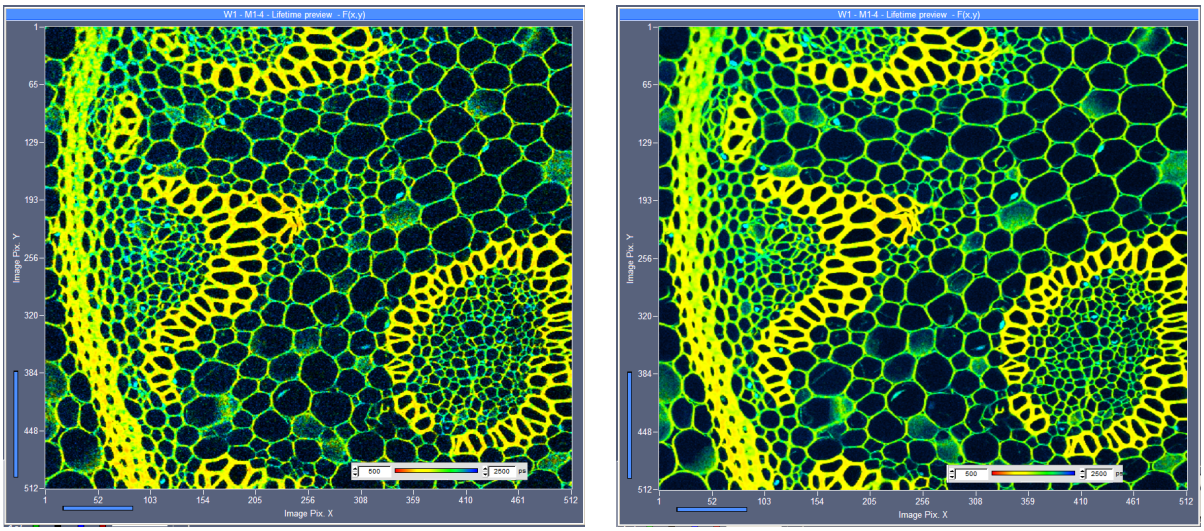

Fig. 5: Convallaria sample, 512 x 512 pixels, 1024 time channels. Left: Acquisition time 0.6 seconds. Right: Acquisition time 4 seconds. Images calculated by online-FLIM algorithm of SPCM.

The data quality of the 4-second image is high enough for double-exponential decay analysis. Fig. 6 shows images of the amplitude-weighted lifetime, tm, and the amplitude-ratio, a1/a2. Lifetime images of the fast and the slow decay component of the double-exponential decay are shown in Fig. 7.

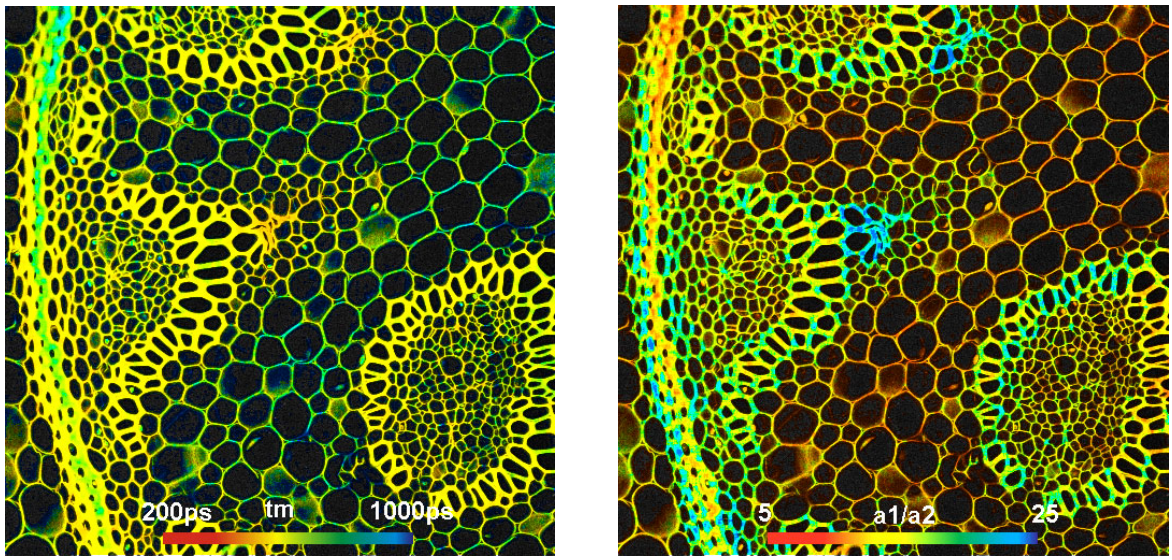

Fig. 6: Same data as in Fig. 5, right. Double-exponential FLIM analysis by bh SPCImage. Amplitude-weighted lifetime, tm, (left) and amplitude ratio of decay components, a1/a2 (right)

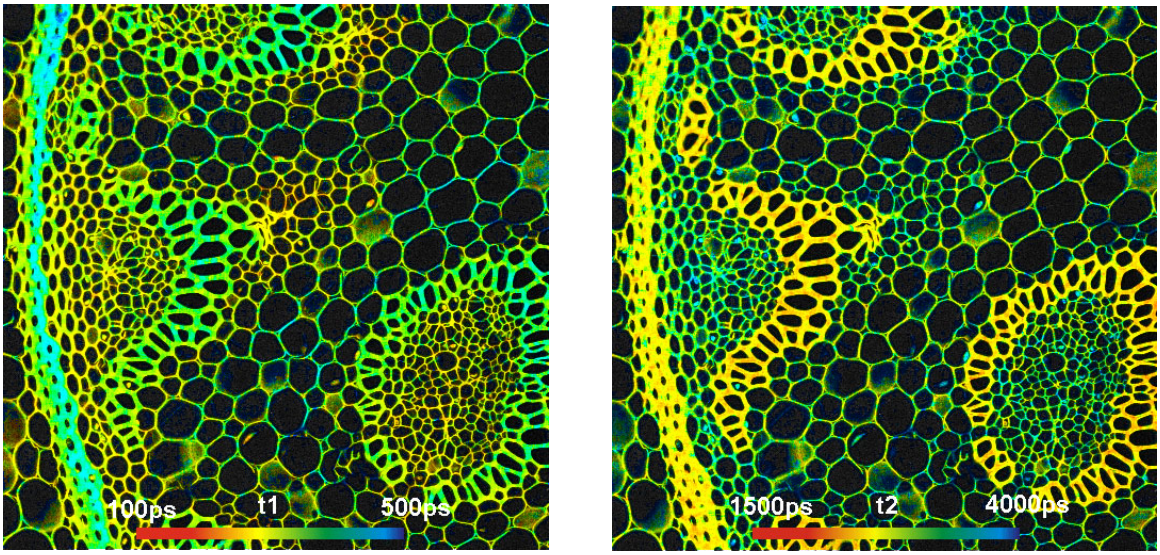

Fig. 7: Lifetime image of fast decay component, t1, (left) and lifetime image of slow decay component, t2, (right)

In many instances, images of the decay times and the amplitudes of the components give a far better insight into molecular parameters of a sample than simple lifetime images. It is therefore important that a fast FLIM system delivers such data, and that it does so at high precision. High time resolution (< 25ps FWHM IRF width with fast detectors) substantially improves the accuracy of multi-exponential decay analysis, as has been shown recently for metabolic FLIM [8].

Small Images

Small images (with small number of pixels) can be recorded with even shorter acquisition time. One reason is that, at a given count rate, the number of photons per pixel increases with decreasing pixel number. Correspondingly, the signal-to-noise ratio of the lifetime increases [3]. Another reason is that an optical scanner scans a smaller image in a shorter period of time. The minimum frame time of the scanner depends on the number of lines, not on the number of pixels along the line. Therefore, if a scanner is operated at maximum speed and the frame format (Pixels X · Pixels Y) is reduced the pixel dwell time *increases*. In the FLIM recording, the number of photons per pixel increases and so does the signal-to-noise ratio of the lifetime. A few examples are shown in Fig. 8.

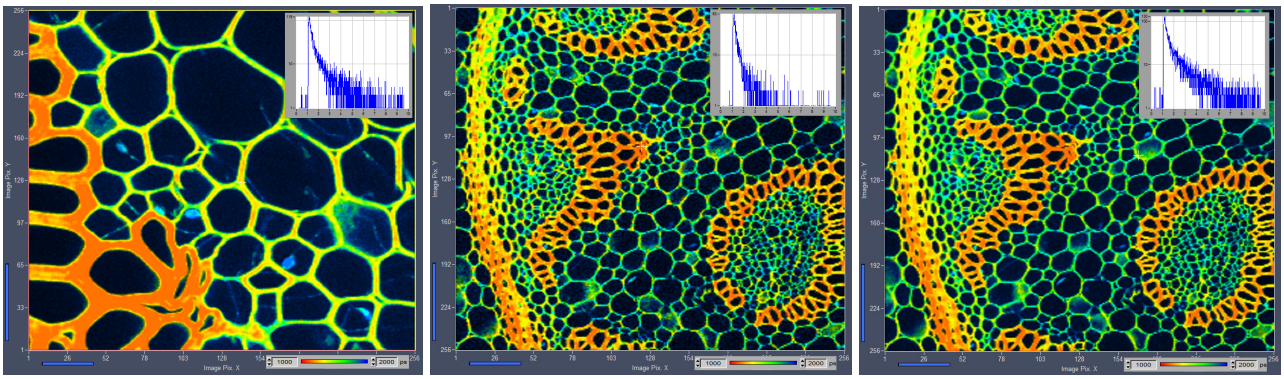

Fig. 8: FLIM data format 256 x 256 pixels, 1024 time channels. Left to right: Acquisition time 0.16 s (zoom to obtain short frame time), 0.5 s, 2.5 s. Insert: Decay data in 5x5 pixel area around the cursor position. Online lifetime display of SPCM.

Conclusions

The combination of the Zeiss LSM 880 NLO with the bh FASTAC FLIM system records FLIM images at acquisition times down to the fastest frame rates of the LSM 880 scanner. There is no compromise in terms of time-channel width, time-channel number, or pixel numbers. The system can therefore be used both for fast-acquisition FLIM applications and for precision FLIM. It should be noted, however, that fast acquisition is only possible if the sample is able to feed the system with a sufficiently high photon rate [11]. This is certainly the case for samples that contain high amounts of bright fluorophores. It may not always be the case in molecular imaging experiments, metabolic FLIM, or other applications where the fluorophores are linked to highly specific targets within the cells. However, even under sample-limited conditions the results are at least as good as with a standard bh FLIM system.

References

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