Fast-Acquisition TCSPC FLIM System with sub-25 ps IRF Width

Abstract: We report on a fast-acquisition FLIM system comprising a single detector, four parallel TCSPC channels, and a device that distributes the photon pulses into the four recording channels. The system features an electrical IRF width of less than 7 ps (FWHM), and a time channel width down to 820 fs. The optical time resolution with an HPM-100-06 hybrid detector is shorter than 25 ps (FWHM). The system is virtually free of pile-up effects and has drastically reduced counting loss. FLIM data can be recorded at acquisition times down to the fastest frame times of the commonly used galvanometer scanners. Fast recording does not compromise the time resolution; the data can be recorded with the TCSPC-typical number of time-channels numbers of up to 1024 or even 4096. Pixel numbers can be increased to 1024 x 1024 or 2048 x 2048 pixels. The system is therefore equally suitable for fast FLIM and precision FLIM applications.

Techniques of Fast FLIM Acquisition

There is currently a run towards faster and faster acquisition of Fluorescence Lifetime Imaging (FLIM) data. Fast-FLIM techniques normally use time-gating into only a few time windows, or a multichannel scaler process with a time-channel width in the 200-ps range or longer. Compared with TCSPC FLIM, the time resolution, both in terms of IRF width and time channel width, is much lower, and the ability to resolve multi-exponential decay profiles into their components is limited. However, in typical FLIM applications, such as FRET imaging or metabolic imaging, exactly these features are important. In FRET data, the interacting donor fraction has to be separated from the non-interacting one [1], and metabolic imaging is based on the separation of the decay components of bound and free NADH [1, 6]. Moreover, typical FLIM experiments have to be performed on samples with low fluorophore concentration and fluorophores of sub-ideal quantum efficiency. The number of photons that can be obtained from these samples is limited. Photon efficiency, i.e. the number of photons required for a given signal-to-noise ratio of the fluorescence lifetime, is therefore an important - if not the most important - parameter of a FLIM technique.

It is commonly accepted that TCSPC FLIM [1, 2] delivers the best time-resolution [1, 5] and the best photon efficiency of all FLIM techniques [1]. It is also able to record the data into a sufficient number of sufficiently small time channels so that multi-exponential decay analysis is possible [1, 2, 3]. There are also other advantages, such as the capability to record multi-wavelength data [7], simultaneous FLIM and PLIM [8], and extremely fast triggered time series [1, 3, 4]. The usual argument against TCSPC is that the ‘Pile-Up’ effect makes it impossible to achieve high count rates and short acquisition times. However, the count rate for a given pile-up error is 100 times higher than commonly believed. In contrast to the statements in most FLIM papers count rates up to 10% of the excitation rate can be used [1, 2, 16]. The often cited value of 0.1% is wrong, it goes back to a typo in the early TCSPC literature. It therefore appears unwise to discard the TCSPC technique until all options of increasing the count rate have been exploited.

In [14] we demonstrated that TCSPC FLIM of 128x128 pixel images at count rates exceeding 1 MHz can be obtained within an acquisition time of 100 to 200 ms. 256 x 256 pixel images were obtained in about 0.5 seconds. The high speed was obtained by maximising the photon efficiency of lifetime analysis via the first moment of the decay data in the pixels. The first-moment calculation not only yields the ideal signal-to-noise ratio of $\sqrt{N}$, it is also fast enough to run online FLIM up to the maximum frame rate of a galvanometer scanner [14].

TCSPC FLIM at higher count rates can be obtained by splitting the light into several parts going to different detectors, and recording the signals in several parallel TCSPC FLIM modules. We have demonstrated the technique in [15]. However, the need of an optical beamsplitter and of several detectors makes such systems uncomfortable to use. We therefore aimed at a solution which distributes the photons into several TCSPC modules without the need of using separate detectors.

The ‘Photon Spinner’

At first glance, distributing photon pulses from one detector into several TCSPC modules may appear easy. However, TCSPC is based on picosecod timing of the photon pulses, and the superior time resolution of TCSPC results from the fact that these times are obtained at extremely high precision [1]. Switching the signal path from one TCSPC module to another necessarily causes switching transients. No matter whether the rotation of the switch is performed independently of the photon detection, is synchronised with the photons, or synchronised with the laser pulses, the switching transients almost unavoidably impair the timing accuracy and the differential nonlinearity of the TCSPC process.

Our solution to the problem is shown schematically in Fig. 1. As usual, the photon pulses from the detector are passing a constant-fraction discriminator, CFD. The output pulses of the CFD have a constant width and a time independent of the pulse amplitude of the detector pulses [1]. The pulses from the CFD control a four-way switch that distributes the pulses to four TCSPC modules. Every photon pulse from the CFD rotates the switch by one position. The trick of the solution is that every photon sets the switch not for the next photon, but for itself. To do so, the photon pulses pass a delay line. Every photon arrives at the switch a short time after the switching action has been completed. In other words, the photon sets the switch ahead of itself. Of course, a switching transient also occurs in the circuit shown in Fig. 1. But there is an important detail. Because every photon pulse arrives at the switch at a fixed time after the switching action the sum of the photon pulse and the switching transient is the same for all photons. It is also independent of the time of the photon in the laser pulse period. Therefore, the switch has no influence on the photon timing.

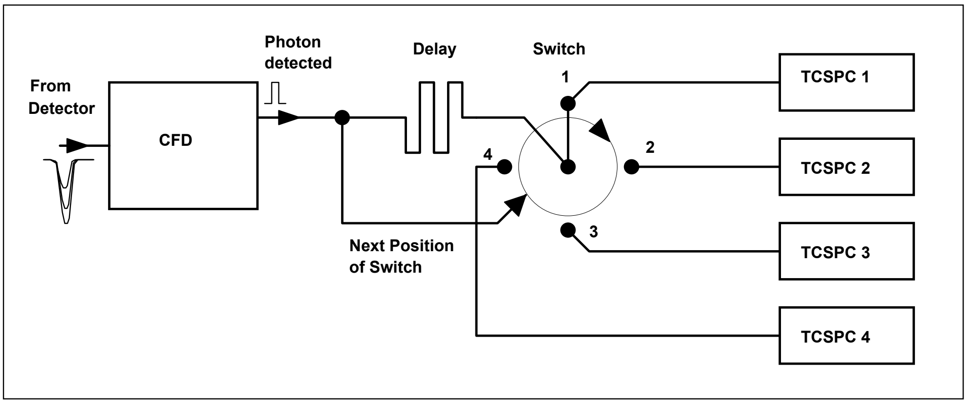

Fig. 1: The ‘Photon Spinner’. Every photon puts the position of a signal switch forward by one position. It arrives at the switch shortly after the switching action is complete, and proceeds into the next TCSPC module. Because the time between the switch set and the arrival at the switch is constant the switching transient has no effect on the photon timing.

As the switch spins around with the photons arriving, each of the TCSPC modules records 1/4 of the photons. Counting loss (by detecting a photon in the dead time of a previous one) is thus substantially reduced. The improvement is larger than for a system with four separate detectors because the distribution device (the Photon Spinner') regularises the photon arrival times. Short time intervalls between the photons therefore become less likely.

The second effect of the 'Photon Spinner' is a reduction of possible pile-up errors. The pile-up reduction larger than for a system with four detectors. If a new photon is detected in the same laser pulse period with a previous one it goes to the next TCSPC module. It thus does not cause any pile-up distortion. Only if more than *four* photons were detected within one and the same laser pulse period a pile-up error would occur. For the commonly used pulse repetition rates of 50 to 80 MHz the detection of more than four photons is extremely unlikely.

Another advantage of the Photon Spinner is that it works independently of the TCSPC modules. No feedback or 'ready' signal from the TCSPC modules is necessary. The device can therefore be built as a simple extension box to a TCSPC four-module package. Superficially, it has similarity with a 'Router'. However, unlike a router, it does not funnel the photons of several detectors in one TCSPC module but distributes the photons of one detector into four TCSPC modules.

FLIM with the Photon Spinner

To demonstrate the performance of the system we used an bh DCS-120 confocal scanning FLIM system [9] with an SPC-154N four-channel TCSPC package. A FLIM image with 128 x 128 pixels and 1024 time channels per pixel is shown in Fig. 2, left. The image was recorded within 100 ms in a single scan of the DCS system. The lifetime image was calculated by the online FLIM function of the SPCM software [14]. The average count rate over the entire image was about 12 MHz, the peak count rate certainly exceeded 20 MHz. Decay data in a 5x5 pixel area are shown in Fig. 2, middle. A decay curve over the entire scan area is shown in Fig. 2, right.

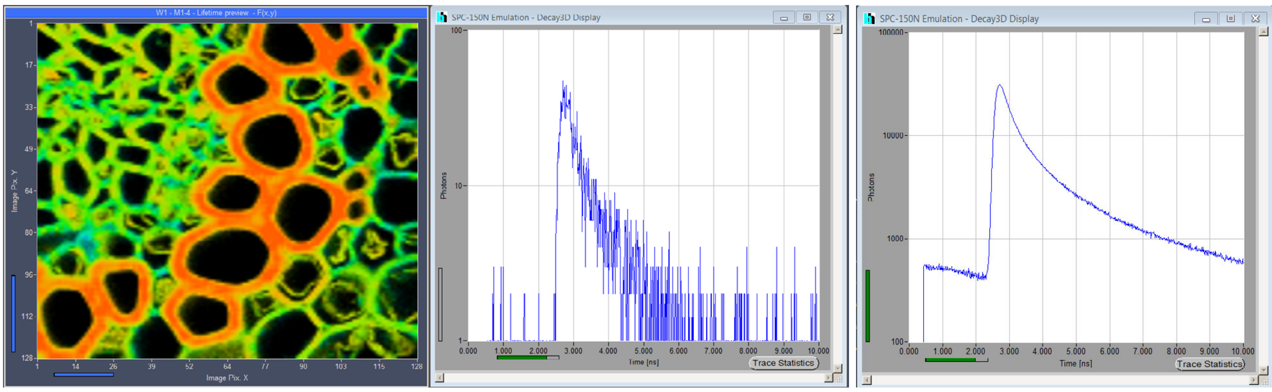

Fig. 2: FLIM of a Convallaria sample. Acquisition time 100 ms, 128x128 pixels, 1024 time channels, time channel width 12 ps. Lifetime range 1 ns (red) to 2.5 ns (blue). Middle and right: Decay curve in 5x5 pixel area and decay curve over entire image. DCS-120 system with 488 nm diode laser and HPM-100-40 detector.

An image recorded with 256 x 256 pixels and 250 ms acquisition time is shown in Fig. 3. Because the number of pixels is four times higher but the acquisition time only 2.5 time longer the pixels contain less photons than in Fig. 2. Nevertheless, a reasonable lifetime image is obtained is obtained.

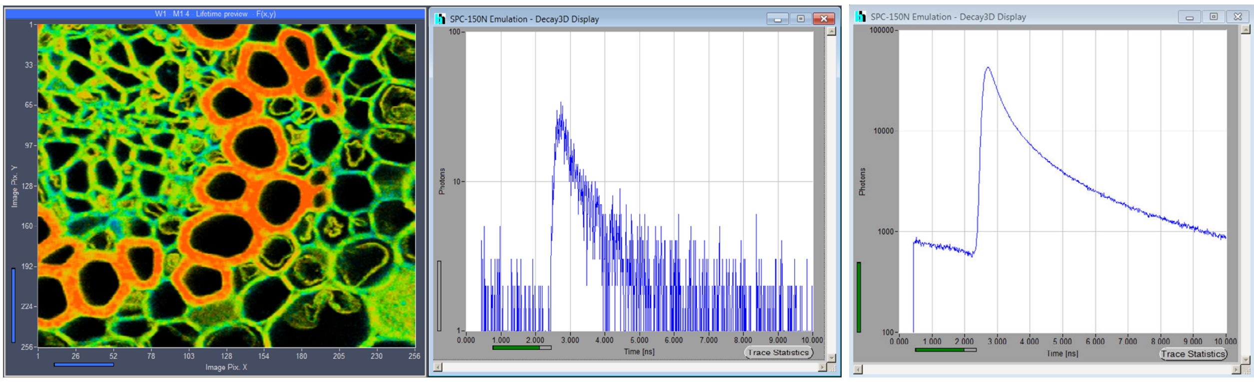

Fig. 3: FLIM of a Convallaria sample. Acquisition time 250 ms, 256x256 pixels, 1024 time channels, time channel width 12 ps. Middle and right: Decay curve in 4x4 pixel area and decay curve over entire image. DCS-120 system with 488 nm diode laser and HPM-100-40 detector.

High-resolution images with 1024 x 1024 pixels are shown in Fig. 4 and Fig. 5. The sample was a BPAE cell slide from Invitrogen. The average count rate over the entire scan are was 6 MHz, the peak count rate in the brightest pixels about 15 MHz. Fig. 4 was recorded in a single frame of the scanner, with 2 seconds acquisition time. Even within this short time, a reasonable FLIM image was recorded. (Please note that the lifetime range is only 400 ps wide).

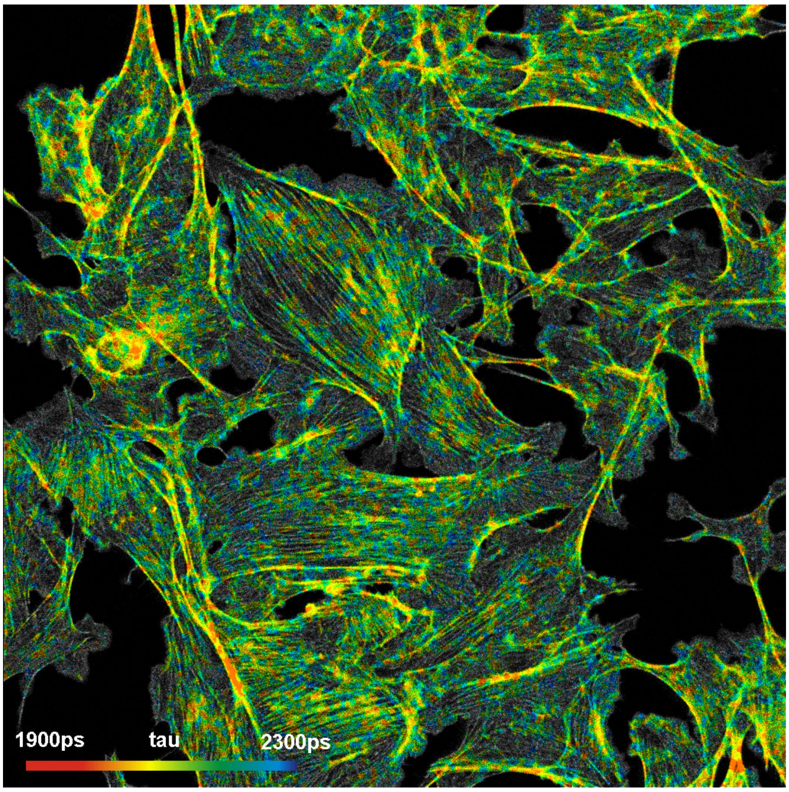

Fig. 4: FLIM of a BPEA sample, 1024x1024 pixels, 1024 time channels, acquisition time 2 seconds. DCS-120 system with 488 nm diode laser and HPM-100-06 ultra-fast hybrid detector.

An image of the same sample recorded with an acquisition time of 10 seconds (5 frames of the scanner) is shown in Fig. 5. The decay data in a 10x10 pixel spot and a decay curve integrated over the entire image are shown in Fig. 6. The image and the decay curves show that the system is able to record FLIM data of extraordinary quality within relatively short acquisition times.

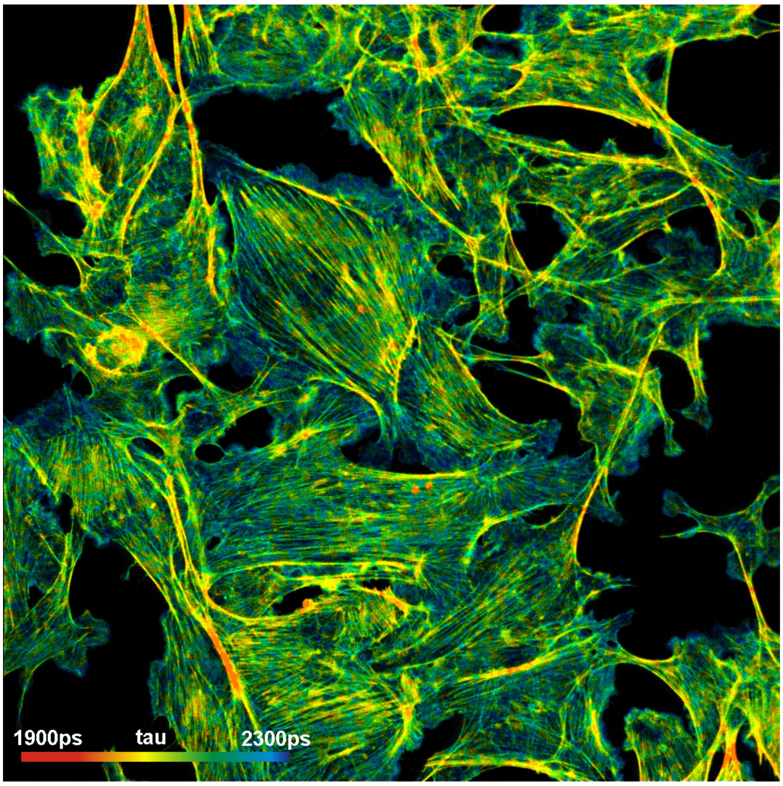

Fig. 5: FLIM of a BPEA sample, 1024x1024 pixels, 1024 time channels, acquisition time 10 seconds. DCS-120 system with BDL-SMN 488 nm ps diode laser and HPM-100-06 ultra-fast hybrid detector.

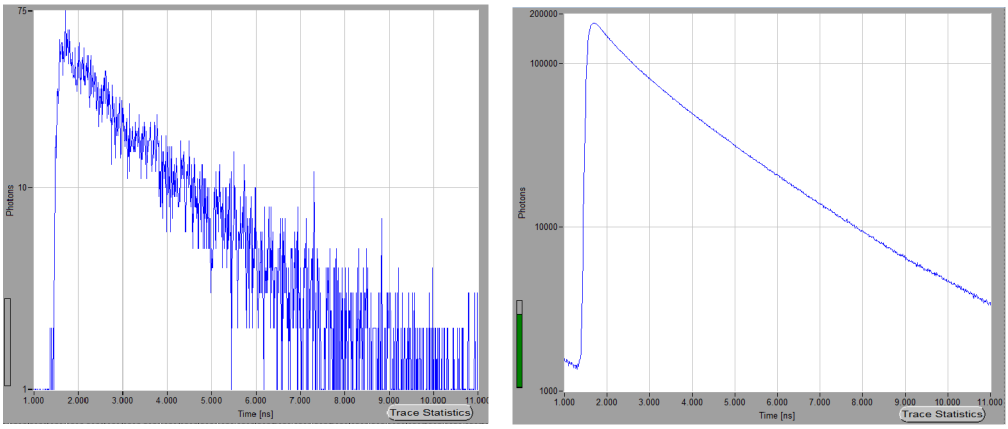

Fig. 6: Decay curves from the data shown in Fig. 5. Left: Decay data in a 10x10 pixel spot. Right: Decay curve from the entire image.

Time Resolution

The data shown in Fig. 4, Fig. 5, and Fig. 6 were recorded with an HPM-100-06 ultra-fast hybrid detector. With the bh SPC-150N modules, this detector delivers an IRF of about 20 ps FWHM (full width at half maximum) [1, 7]. The IRF width in Fig. 6 is about 60 ps, due to the pulse width of the BDL-SMN 488 nm picosecond diode laser. The question is how fast an IRF can be obtained with the Photon Spinner when a fast laser is used.

Fig. 7, left, shows the electrical instrument response functions of the four SPC 150N modules with the PHDIS-04 Photon Spinner. Surprisingly, the IRF width of the individual modules is 6.7 to 6.9 ps, i.e. only insignificantly longer than the IRF of the modules themselves (typically 6.6 ps).

Due to transit time differences in the Photon Spinner and in the connecting cables the IRFs of the four TCSPC channels appear slightly shifted, see Fig. 7, left. The shift can be corrected by using separate TAC offsets for the individual TCSPC modules [1]. Fine alignment is achieved by tweaking the 'Zero Cross' levels of the CFDs of the modules. Different Zero Cross shifts the trigger point up and down the leading edge of the spinner output pulses, and thus acts as an extremely fine delay adjustment. The variation in the Zero Cross has no influence on the IRF width because the output pulses of the Photon Spinner have no amplitude jitter. The IRFs of the SPC-150N modules after delay alignment are shown in Fig. 7, middle, the combined IRF of the four modules is shown in Fig. 7, right. The combined IRF still has 6.8 ps FWHM, much faster than the transit time spread of any commonly available photon detector.

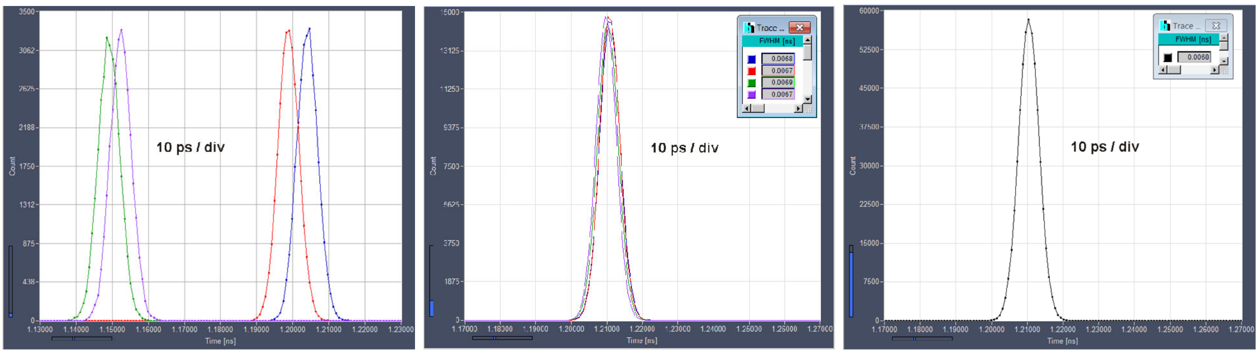

Fig. 7: Electrical IRFs of the four SPC-150N modules. Before (left) and after delay alignment (middle). The combined IRF of the four SPC-150N is shown on the right. The FWHM of the combined IRF is 6.8 ps.

The optical IRF of the entire system with an HPM-100-06 detector is shown in Fig. 8. It was recorded with a Toptica Femto Erb femtosecond fibre laser in the test setup described in [5].

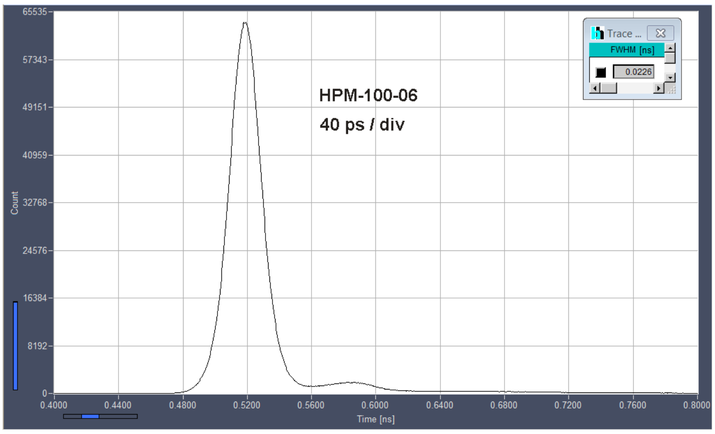

Fig. 8: Instrument response function of an SPC-154N four-module package with the Photon Spinner and an HPM-100-06 hybrid detector. FWHM is 22.6 ps.

Summary

The system described here is able to record FLIM at high count rates and short acquisition times. Importantly, the system reaches high count rate and short acquition time without any reduction in time resolution. FLIM data can still be recorded at sub-ps time channel with, and sub-25 ps IRF width, thus fully exploiting the time resolution of the bh TCSPC FLIM modules and ultra-fast hybrid detectors. It is therefore equally suitable for fast FLIM and precision FLIM applications. The system can be used in combination with the bh DCS-120 confocal and multiphoton scanning systems [9], but also with laser scanning microscopes of other manufacturers [10, 11, 13, 12].

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