FLIM and FCS by Pulse-Interleaved Excitation with the Zeiss LSM 710/780 Intune System

Abstract: We describe FLIM and FCS by pulse-interleaved excitation (PIE) with the Intune laser and the picosecond diode laser of the Zeiss LSM 710/780 family. The diode laser is triggered by the synchronisation output of the Intune laser. The sources of the timing reference of the TCSPC FLIM modules are selected via bh USB-controlled delay switch boxes. Both TCSPC channels of the FLIM system can be synchronised from the intune laser or from the ps diode laser. Moreover, or one TCSPC channel can be synchronised from the Intune laser while the other is synchronised from the diode laser. We show the application of the system to dual-excitation FLIM and dual-excitation FCS.

Keywords: PIE, FLIM, TCSPC, FCS

Pulse-Interleaved Excitation

There are applications where it is desirable to simultaneously record time-resolved data not only in two separate detection wavelength channels but also at two excitation wavelengths. Examples are FLIM in the presence of several fluorophores with strongly overlapping emission stectra, dual-colour FCS with dyes with overlapping emission spectra, or single-molecule FRET experiments where the donor and the exeptor must be probed separately.

Most of these tasks can also be performed by laser-wavelength multiplexing: Two high-frequency pulsed lasers are multiplexed at microsecond periods, and the photons excited by each laser are routed into different photon distributions [1]. Multiplexing has a number of advantages: It is free of crosstalk, an incomplete decay model can be used for data analysis [2], there is no mutual influence by pile-up, countig loss, or detector afterpulsing, and differences in the excitation efficiencies can be compensated by different ‘on’ times of the lasers [2]. However, multiplexing requires that the lasers can be turned on and off at sub-microsecond speed. This is easy for bh ps diode lasers [5, 6] (also the versions integrated in the Zeiss LSMs) but not necessarily for other lasers, such as the Intune laser of the Zeiss LSM 710/780 microscopes.

Pulse-interleaved excitation (PIE) does not require a fast on-off switching capability of the lasers. Instead, it relies on the synchronisation of the pulse trains of two (or more) pulsed lasers. The principle is shown in Fig. 1.

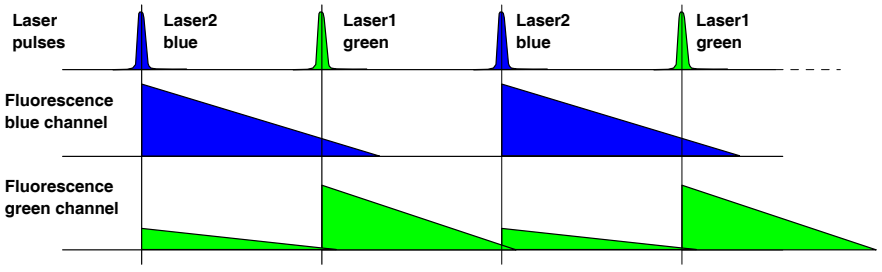

Fig. 1: Principle of PIE.

The pulses of two lasers of different wavelength, marked ‘blue’ and ‘green’ in Fig. 1, are alternating within the same signal period. The fluorescence signals, marked blue and green, are detected in separate TCSPC channels. The blue laser excites fluorescence in the blue detection channel and in the green detection channel. The green laser excites fluorescence only in the green detection channel. Unless the filters are leaky or detection wavelength intervalls are overlapping no fluorescence from the green laser is detected in the blue detection channel.

There are two ways to record data with pulse-interleaved lasers and a dual-channel TCSPC system. The synchronisation signals of both TCSPC modules can be derived from one of the lasers, or the synchronisation signals of the TCSPC modules can be derived from different lasers. The two principless are illustrated in Fig. 2.

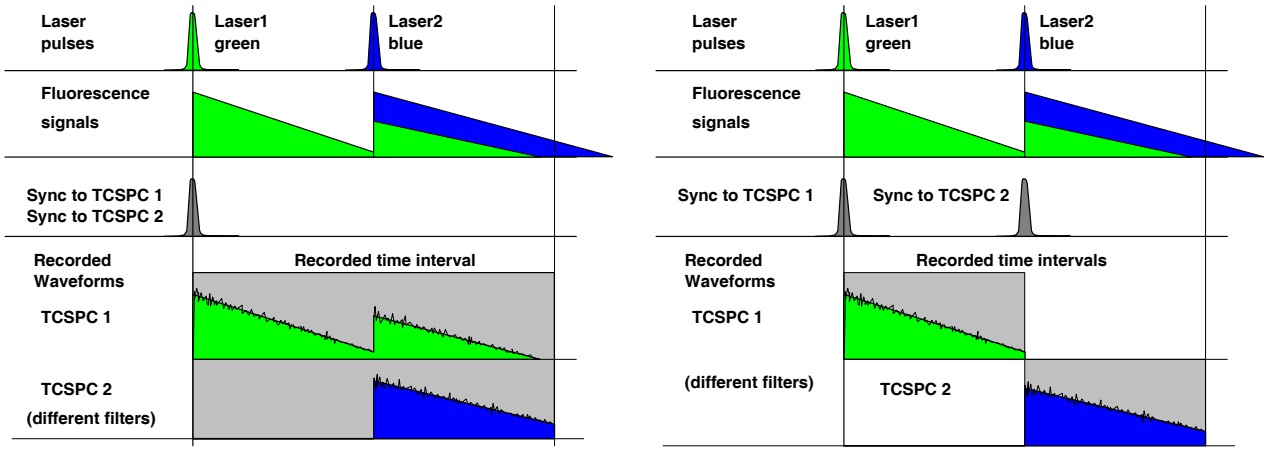

Fig. 2: Two system configurations for PIE with two TCSPC modules. Left: Full-period recording. Both TCSPC modules synchronised to Laser 1, time interval recorded is full laser period. Right: Half-period recording. TCSPC modules synchronised to different lasers, time interval recorded is half the laser period, or the time from the pulse of one laser to the pulse of the other.

In Fig. 2, left, both TCSPC modules are synchronised to one of the lasers (called laser 1 for concveniency). The recording time interval is the full laser period, e.g. the time from one pulse of laser 1 to the next. Both TCSPC modules therefore record waveforms that contain the fluorescence decay excited by laser 1 followed by that of laser 2.

In many applications only two signals are of interest: The fluorescence excited the ‘blue’ laser in the ‘blue’ spectral channel, and the fluorescence excited by the ‘green’ laser in the ‘green’ spectral channel. In that case, the TCSPC modules can be synchronised to different lasers, see Fig. 2, right. The recording time interval is half the laser period, or the time from the pulse of one laser to the pulse of the other. The advantage is that the memory space is used more efficiently; no time channels are wasted for signal components that are not needed. Moreover, data analysis is more convenient: No time gating is required to select the desired fluorescence decay in the recorded wavelforms.

PIE FLIM

Full-Period Recording

FLIM data recorded by PIE with full-period recording are shown in Fig. 3. The sample contained BPEA cells labelled with DAPI and Alexa 488. The data were recorded by a standard Simple-

Tau 152 (dual channel) FLIM system [3] attached to an LSM 710 that had both a 405 nm ps diode laser [5] and an Intune laser [7] implemented. The intune laser was tuned to 502 nm. Decay curves in selected pixels are shown at the top, lifetime images at the bottom. Both TCSPC channels received their Sync signal from the Intune laser, the 405 nm laser was synchronised to the Intune. The recording time interval of the TCSPC modules was matched to the full laser period. It thus contains the fluorescence decay excited by the Intune laser followed by the decay excited by the 405 nm laser.

In the blue channel (Fig. 3, top left) only fluorescence from the 405 nm laser is detected. The green channel (top middle and and to right) detects fluoresence excited by both lasers.

The FLIM Images are shown in Fig. 3, bottom. The images were obtained from the time intervals selected by the cursor lines in the decay waveforms shown in Fig. 3, top. Both the fluorescence lifetimes and the fluorescence intensities were calculated from the cursor interval [3]. Thus, there are three combinations of excitation and detection wavelengths: 405 nm laser and blue detection channel (left), 405 nm laser and green detection channel (middle), and 502 nm Intune excitation and green detection channel (right).

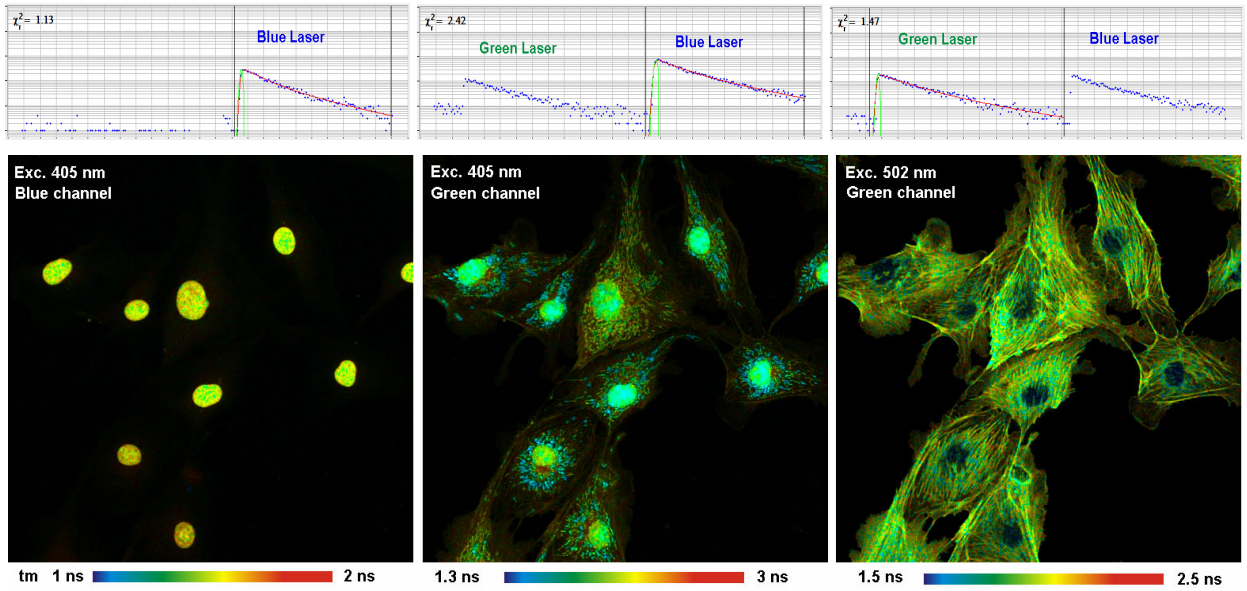

Fig. 3: FLIM by PIE with the Intune laser and a 405 nm ps diode laser, full-period recording. Left: Blue laser, blue detection channel. Middle: Blue laser, green detection channel. Right: Green laser, green detection channel. Top: Decay curves in selected pixels. Bottom: FLIM images, analysed by SPCImage, amplitude-weigthed lifetime of double-exponential decay fit, time-gated intensity

Half-Period Recording

FLIM data recorded by PIE with half-period recording are shown in Fig. 4. The sample was the same as used above for full-period recording. Decay curves in selected pixels are shown at the top, lifetime images at the bottom. One TCSPC channels received its Sync signal from the Intune laser, the the other from the 405 nm diode laser. The recording time interval of the TCSPC modules was matched to the time interval between the intune laser pulse and the diode laser pulse. The advantage of half-period recording is that the time channels within the pixels are used more efficiently: The time channels of each pixel are used for one decay curve instead for two. The disadvantage is that no image for the first laser wavelength and the second detection wavelength interval is obtained.

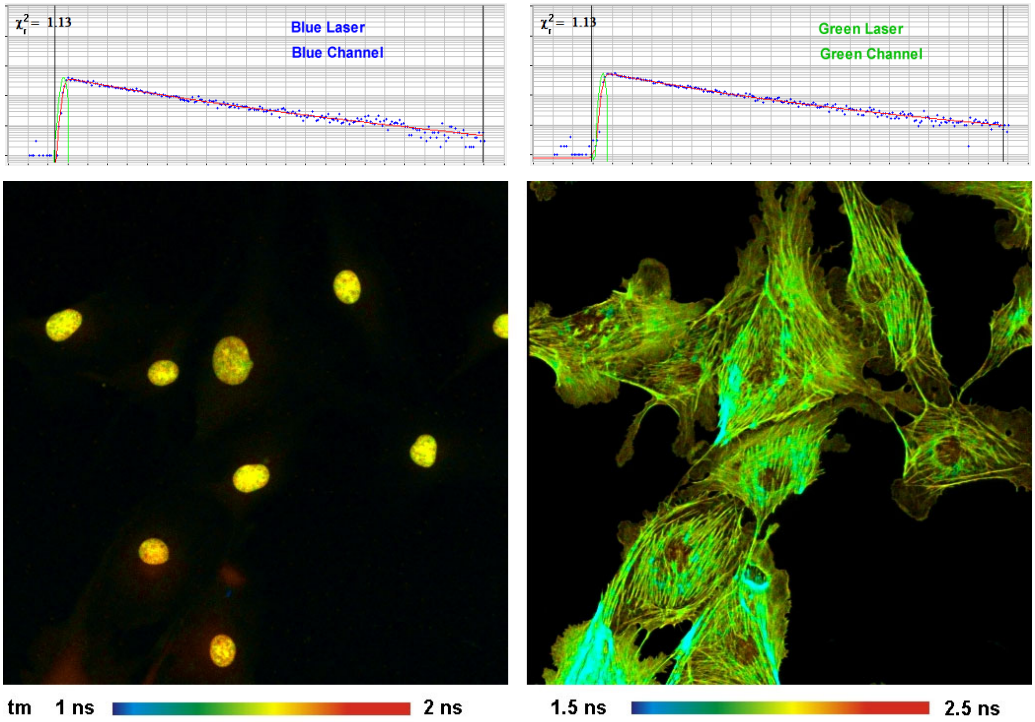

Fig. 4: FLIM by PIE with the Intune laser and a 405 nm ps diode laser, half-period recording. Left: Blue laser, blue detection channel. Right: Green laser, green detection channel. Top: Decay curves in selected pixels. Bottom: FLIM images, analysed with SPCImage, amplitude-weigthed lifetime of double-exponential decay fit.

FCS

FCS with the LSM 710/780 microscopes is performed in the 'Spot' mode of the scanner and in the FIFO / FCS mode of the FLIM system. For details of parameter setup please see [3], chapter 'FCS Measurements'. The measurements produces a stream of parameter-tagged photon data. These data can be correlated either on-line or off-line to obtain FCS curves for the signals detected in the individual TCSPC channels, or Cross-FCS curves between the channels [2]. The data can be time-gated to remove Raman emission of the solvent from the correlation data.

Full-Period Recording

Fluorescence-decay and FCS curves from full-period data are shown in Fig. 5 and Fig. 6. The sample contained Dextrane labelled with Cascade Blue and Dextrane labelled with Alexa 488. Cascade blue and Alexa 488 cannot be efficiently excitaed at the same excitation wavelength. To obtain reasonable excitation for both dyes PIE with the ps diode laser (405 nm) and the Intune laser (tuned to 490 nm) was used. The data were recorded in the Full-Period Recording configuration of the TCSPC system, and analysed with bh 'Burst Analyser' software [4].

The decay curves obtained for full-period recording are shown in Fig. 5. The decay traces shown contain both the signal from the Alexa 488 (green curve) and the signal from the Cascade Blue (blue curve). For FCS analysis the signals of the fluorophores were selected from the traces by software time-gating. Morover, Raman peaks present at the top of the decay functions were gated off.

FCS curves are shown in Fig. 6. Autocorrelation curves for the Cascade Blue and the Alexa 488 are shown left and middle. The cross correlation between both is shown right. As expected, the signals do not correlate to each other: Both dextranes were diffusing independently. Please not the different scales of the correlation coefficient.

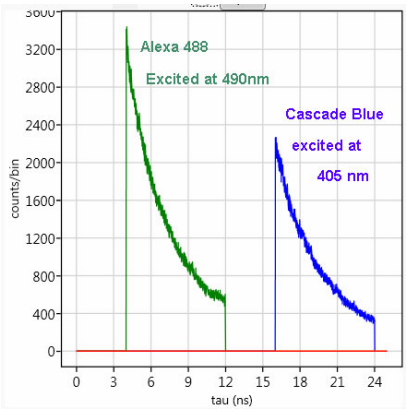

Fig. 5: Decay curves from Alexa 488 (green, excited at 490 nm) and Cascade Blue (blue, excited at 405 nm). Full-period recording. Raman peaks sitting on top of the curves were cat off by software time gating.

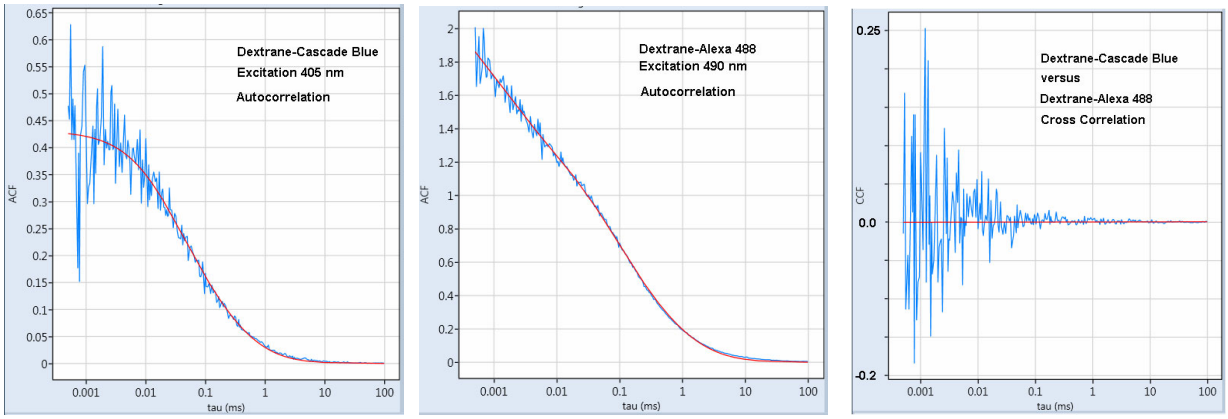

Fig. 6: FCS cuves from Dextrane-Cascade Blue (left), Dextrane-Alexa 488 (middle) and cross-correlation of both (right). Full-period recording, time-gated intensities. Please note different scales of correlation coefficients.

Half-Period Recording

With half-period recording each TCSPC channel records the signal of one fluorophore excited by one of the lasers only. This makes FCS and Cross FCS very simple: No selection of signals by time gating is required. The data can be correlated online by the SPCM data acquisition software.

FCS obtained by half-period recording is shown in Fig. 7. The decay curves are shown on the left. The decay data contain Raman light, forming the sharp peaks on top of the decay curves. Raman peaks can be gated off by the ‘Limit High’ parameter of the TCSPC modules, see [2] or [3], chapter ‘FCS Masurement’.

The FCS curves are shown on the right. The green curve is from the Dextrane-Alexa 488, the blue curves form the Dextrane-Cascade blue. Please see [2] or [3] for system parameter setup.

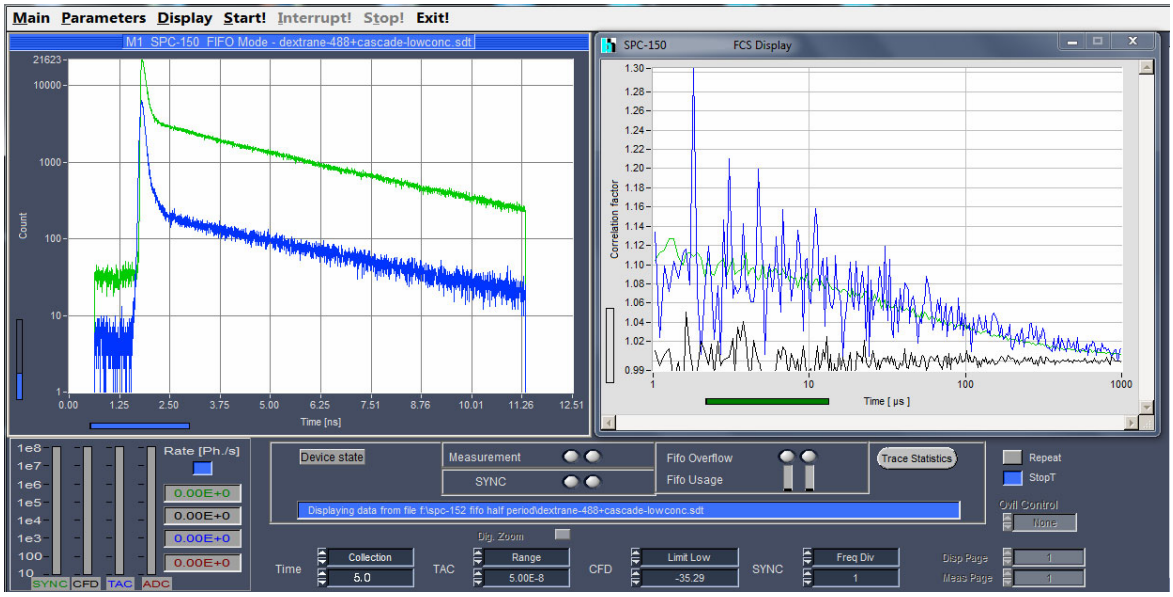

Fig. 7: Half-period recording, online FCS by SPCM data acquisition software. Left: Decay curves, with Raman peaks. Right: FCS curves. Dextrane-Cascade Blue (blue), Dextrane-Alexa 488 (green), Cross-Correlation (black).

Hardware Architecture

TheTCSPC system described in this application note uses a standard bh Simple-Tau 152 dual-channel FLIM system for the Zeiss LSM 710/780 family [3]. However, efficient working with PIE requires that the source of the Sync signals of the TCSPC modules and that the Sync delay can be selected according to the wanted configuration. Sync switching and Sync delay selection is therefore performed by two bh DEL-32 USB-controlled delay switch boxes [2]. The system parameters setup for the individual configurations is selected from the 'Prefefined Setup' panel of the SPCM software. If a new configuration is loaded the entire TCSPC system, including the delay switch boxes, reconfigures automatically. The Sync distribution for the different PIE modes and the normal FLIM modes is shown in Fig. 8 through Fig. 11.

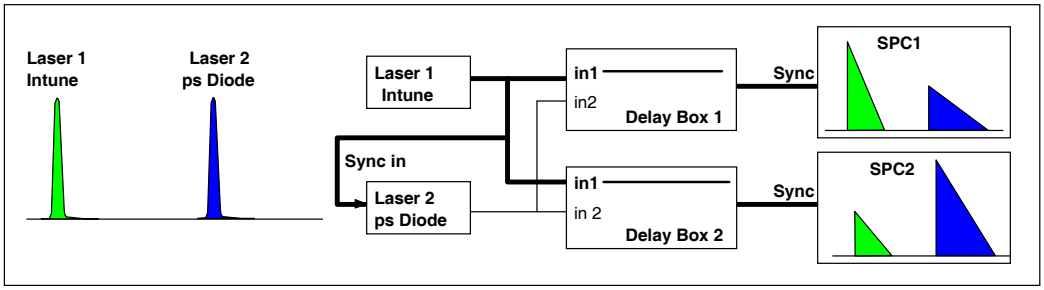

Fig. 8: PIE, full-period recording. Sync source of both SPC modules is Laser 1, Intune.

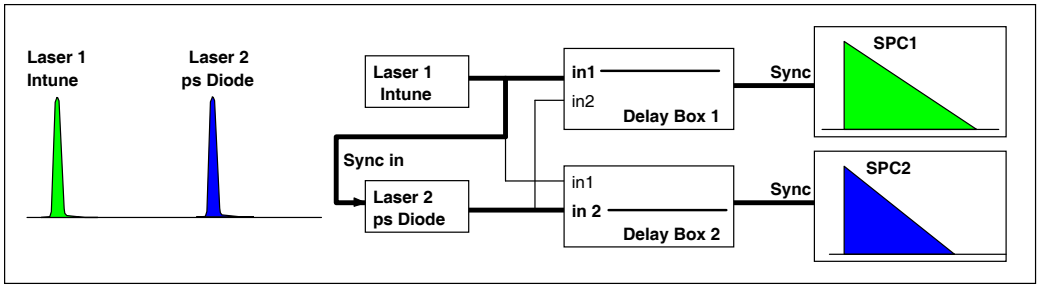

Fig. 9: PIE, half-period recording. Sync source of SPC module 1 is Laser 1, Intune, Sync source of SPC Module 2 is the ps Diode laser.

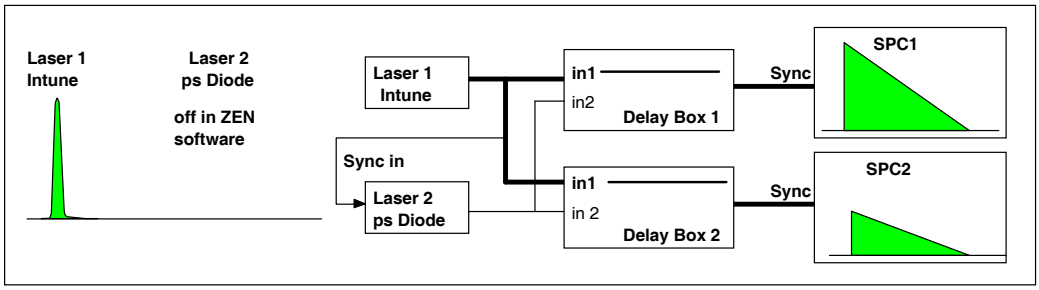

Fig. 10: Standard mode, Intune excitation: Sync Souce of both SPC modules is Intune

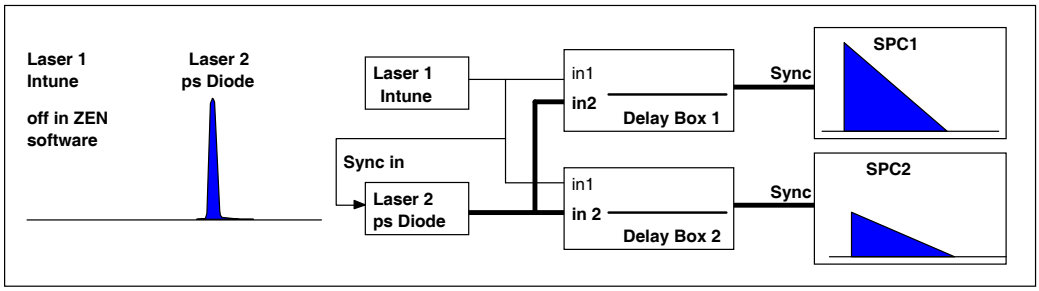

Fig. 11: Standard mode, ps Diode Laser excitation: Sync Souce of both SPC modules is ps diode laser

References

1. Becker, W., Advanced time-correlated single-photon counting techniques. Springer, Berlin, Heidelberg, New York, (2005)
2. Becker, W., The bh TCSPC handbook, 5th edition. Becker & Hickl GmbH (2012), available on www.becker-hickl.com
3. Becker & Hickl GmbH, Modular FLIM systems for Zeiss LSM 510 and LSM 710 family laser scanning microscopes, user handbook, 5th edition. Becker & Hickl GmbH (2012), available on www.becker-hickl.com
4. Becker & Hickl GmbH, Burst Analyser Software for Single-Molecule Burst Analysis, Single-Molecule FRET, and FCS, available on www.becker-hickl.com
5. Becker & Hickl GmbH, BDL-375-SMC, BDL-405-SPC, BDL-440-SMC, BDL-473-SMC Near-UV and blue picosecond diode lasers, user handbook, www.becker-hickl.com
6. BDL-SMN Picosecond Diode Lasers, user handbook, www.becker-hickl.com (2013)
7. Tuneable Excitation FLIM with the LSM 710 Intune System, Application note, available on www.becker-hickl.com
8. Becker & Hickl GmbH, Megapixel FLIM with bh TCSPC Modules - The New SPCM 64-bit Software. Application note, available on www.becker-hickl.com