The Sweet PIE (Pulsed Interleaved Excitation)

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1 Introduction to PIE

The crosstalk between two fluorescent species causes problems in fluorescence microscopy imaging, especially for quantitative measurements such as co-localization, Förster resonance energy transfer (FRET), fluorescence cross correlation spectroscopy (FCCS). Having two different colored fluorophores (e.g. F1 - blue shifted and F2 - red shifted) in the same sample, two imaging channels (Ch1 for F1: Ex1-Em1, Ch2 for F2: Ex2-Em2) are typically used: Ex1 and Ex2 are the excitation wavelengths for F1 and F2; Em1 and Em2 are the wavelength ranges of detecting F1 and F2 emission. In order to quantify the fluorescence emission from each fluorophore in the same pixel location, it requires that each channel only detect the fluorescence emission from the corresponding fluorophore. With single-photon excitation, it is often feasible to use the proper Ex2 that does not excite F1, and to set up the suitable Em1, where F2 does not emit. However, it is often difficult to completely reject the emission bleedthrough of F1 excited by Ex1 to the Em2 channel, when the two imaging channels are acquired simultaneously. An example of using the Cerulean-Venus pair is given in Figure 1.

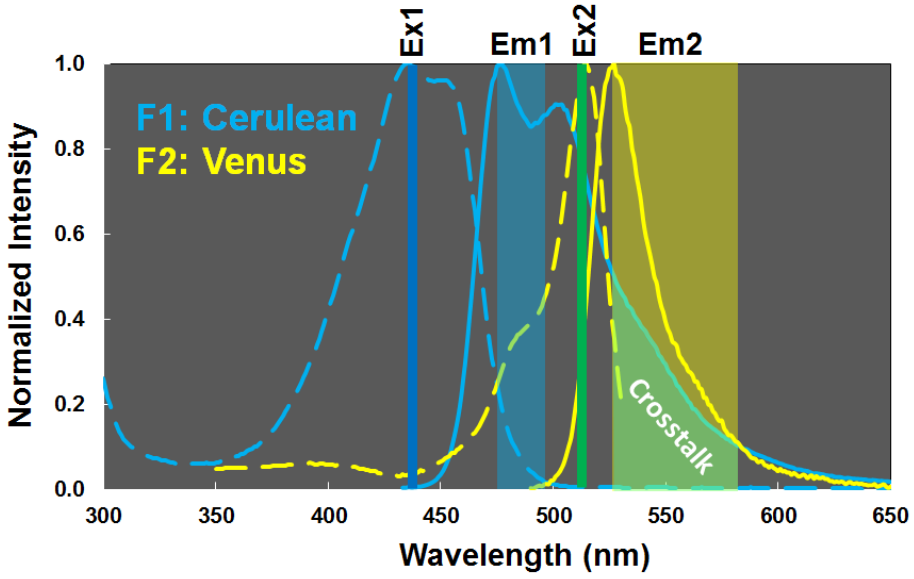

Figure 1: The bleedthrough of the Cerulean emission to the Venus channel.

The crosstalk can be taken care of by sequentially acquiring the data in the two channels. Since there is always a delay between the measurements of the two channels on the same spatial location, the length of the delay becomes

a critical factor to be considered depending upon the applications. In laser scanning confocal microscopy, the lasers can be switched on and off by the acousto-optic tunable filter (AOTF) in the microsecond scale for alternative line scanning in order to avoid the crosstalk while minimizing the time delay between two lasers on the same pixel location. This is probably sufficient for most of cellular imaging applications in the ensemble level. However, the time delay has to be shortened further to allow for the tracking of very fast dynamics at the single molecule level. Kapanidis and colleagues developed a fast alternating laser excitation (ALEX) method, where the two excitation sources were interleaved using the electro-optical modulators combined with polarizers on a timescale between 25 and 3000 μs (1). Using the ALEX technique, they were able to determine the labeling stoichiometry of individual complexes and demonstrated the improved accuracy of single molecule FRET (smFRET) measurements (2). Later, Müller and colleagues introduced the pulse interleaved excitation (PIE) method and further pushed the limit of switching two laser excitation wavelengths to the nanosecond scale (3).

In PIE, two pulsed lasers of different wavelengths are synchronized in the nanosecond scale, and the fluorescence emission yielded by each laser is measured in the time resolved manner. Its basic concept is illustrated in Figure 2. Both pulsed lasers of the same repetition rate excite the sample, while the two trains are delayed for ΔT within the time window T (the laser repetition time). Both detection channels are synchronized with the laser clock, such that the fluorescence decay yielded by each laser immediately follows the laser pulse. When ΔT is much longer than the lifetime of F1 to allow its complete decay, the fluorescence emission decays of F1 excited by Ex1 and F2 excited by Ex2 are then well separated in the time scale of T in Ch2. Thus, the bleedthrough of the F1 emission to Ch2 can be solidly removed by only using the time-resolved data in the "T - ΔT" time window of Ch2.

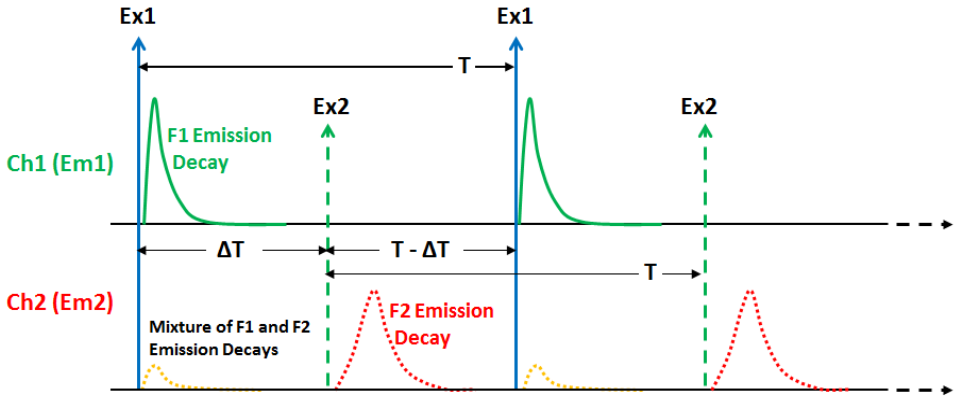

Figure 2: The basic concept of PIE.

2 Examples of PIE Configuration

Several configurations of PIE have been implemented in the Alba and Q2 laser scanning microscopy systems made by ISS using different laser sources (described below). The details of the Alba and Q2 are given on the ISS website - www.iss.com/microscopy/instruments. By default, the galvano-controlled mirrors are used for scanning the sample, although a piezo XY-stage can be another option (albeit slower). The time-resolved data acquisition is achieved by using either the time correlated single photon counting (TCSPC) or the digital frequency-domain (DFD) FastFLIM technique, or both, in the Alba or Q2, powered by ISS 64-bit VistaVision software. The typical detectors used are Hamamatsu GaAsP (H7422p) or Hybrid (R10467) photomultiplier tubes (PMTs) or Excelitas single photon counting module (SPCM) avalanche photodiodes (APDs).

2.1 TCSPC-PIE

The TCSPC electronics synchronizes the detector to the excitation pulse and records the photon arrival time relative to the excitation pulse. This time is measured by the TCSPC time-to-amplitude converter (TAC) module – the TAC timing is started by the detected photon signal trigger given to the TCSPC "constant fraction discriminator (CFD)" port, and then stopped by the corresponding laser pulse trigger given to the TCSPC "Sync" port. By accumulating the photons for a period of time, a "photon counts" histogram representing the fluorescence decay can be directly recorded; and in laser scanning microscopy the TCSPC device is also synchronized to the scanning clocks to record the decay at each spatial (XYZ) location. The high-end TCSPC device offers very high time resolution, down to a few picoseconds. However, its data collection efficiency suffers from the TAC dead time.

Figure 3 shows a TCSPC-PIE configuration using two pulsed diode lasers. Typically, both lasers are operated at 20MHz. One laser (Master) provides the reference clock signal to the TCSPC "Sync" port. The master laser also triggers the other laser (Slave) via a delay cable, which determines the delay time between the two lasers (ΔT) for about 25ns. Both lasers are combined in a laser launcher and then delivered by a single mode polarization maintained (SMPM) optical fiber to the Alba or the Q2. The photon signals of up to four detection channels are routed to the TCSPC CFD port. For each channel, the rising time of the decay is determined by the overall delay time, contributed from the laser travel time, the photon travel time and the electronic signal travel time; it can be adjusted by using the TCSPC TAC offset and changing the lengths of the cable from the laser to the TCSPC "Sync" port and the cable from the detector to the TCSPC "CFD" port.

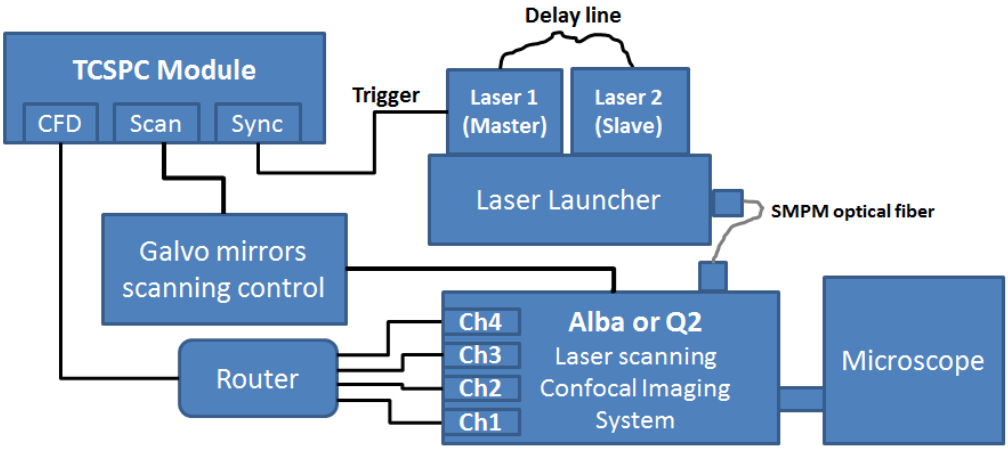

Figure 3: A TCSPC-PIE configuration of using two pulsed diode lasers.

As shown in Figure 4A, the rising time of the decay in each channel is set to follow the corresponding laser pulse immediately. However, this setup gives trouble in selecting a time window (TAC range) to remove the crosstalk due to Ex1 in Ch2, because the TAC settings affect both channels equally. By adjusting the cable length from the Ch2 detector to the router, the rising time of the F2 decay due to Ex2 in Ch2 can be aligned with that of the F1 decay due to Ex1 in Ch1, so that both decays are now in the time window, while the crosstalk in Ch2 is well separated from this time window (Figure 4B). Thus, the crosstalk in Ch2 can be removed completely by gating off the crosstalk time window using the proper TAC settings (Figure 4C). Figure 4D shows an example of using the TCSPC-PIE setup to measure the mixture of Fluorescein and Alexa568 in HPLC water at pH 7.6: Ch1: Ex1, 488-nm, Em1, 525/30-nm; Ch2: Ex2, 561-nm, Em2, 624/40-nm.

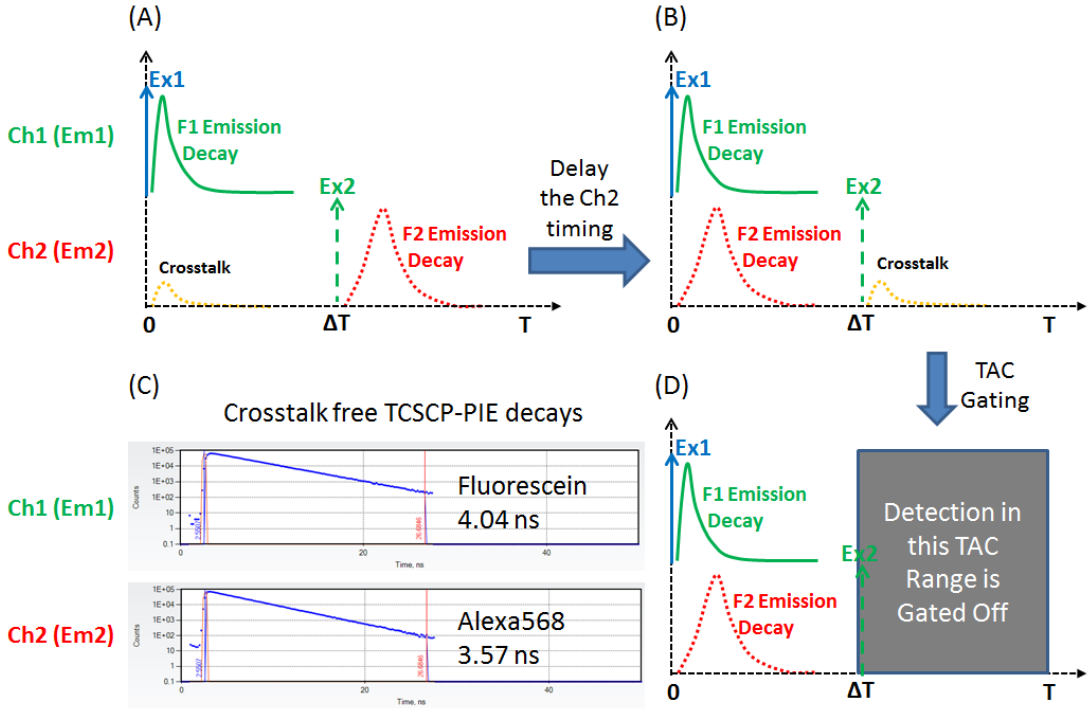

Figure 4: Crosstalk-free TCSPC-PIE decays by TAC gating.

2.2 FastFLIM-PIE

FastFLIM (www.iss.com/microscopy/components/FastFLIM.html) records the photon counts in a number of cross-correlation phase bins called the phase histogram; the digital Fourier transform of the phase histogram is then applied to calculate the phase delay and the demodulation ratio for the frequency-domain FLIM data analysis (4). FastFLIM provides four independent data input channels, allowing data acquisition from four detectors simultaneously. Compared to the TCSPC technique, FastFLIM has nearly no dead time for data acquisition, giving the higher photon counting efficiency and in turns the shorter data acquisition time. Another distinct feature of FastFLIM is that the device is easily programmable to fit into different applications, such as measuring sub-nanosecond fluorescence lifetimes to microsecond phosphorescence lifetimes. This feature also makes FastFLIM very useful for PIE.

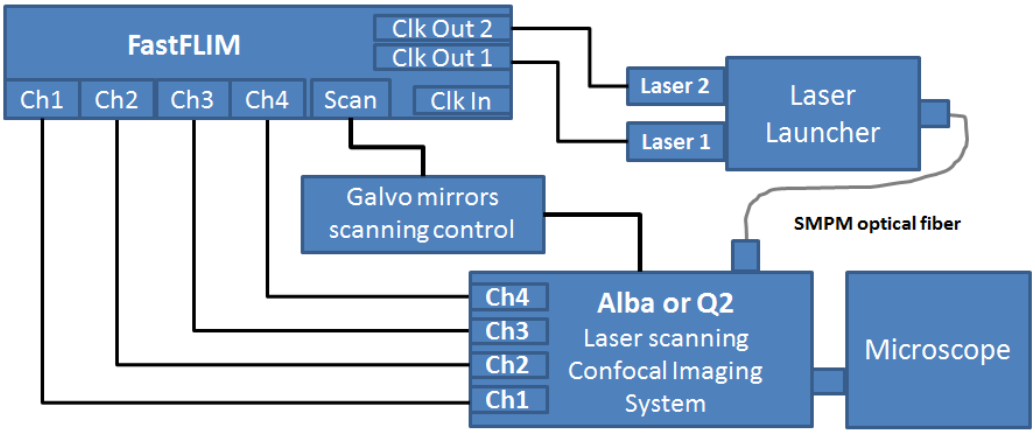

Figure 5: A FastFLIM-PIE configuration of using two pulsed diode lasers.

Figure 5 shows a FastFLIM-PIE configuration of using two pulsed diode lasers, although a CW laser can also be used together with an electro-optic modulator. Both lasers are driven by FastFLIM using two different clock outputs, at the same repetition rate but with a delay. Both lasers are combined in a laser launcher and then delivered by a SMPM optical fiber to the Alba or Q2 system. Up to four detectors can be directly fed to the four input channels of FastFLIM for simultaneous data acquisition. To remove crosstalk, each channel can be gated independently by FastFLIM. More importantly, the laser repetition rate, the delay between the two lasers and the gating window of each channel are tunable by FastFLIM using different firmware, to optimize the photon collection efficiency upon the application. An example of FastFLIM-PIE with gating enabled is shown in Figure 6.

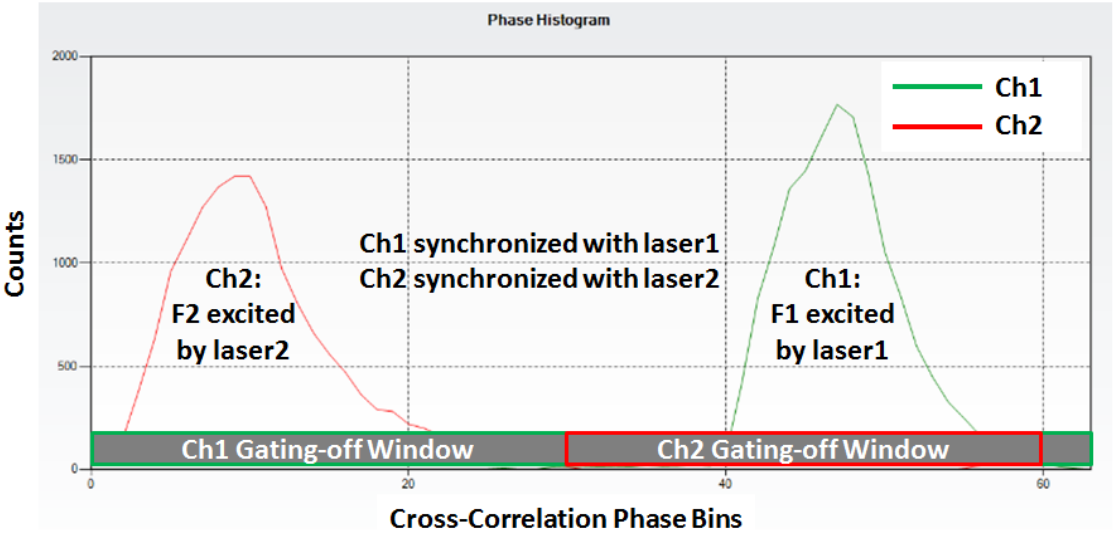

Figure 6: The gated phase histograms of the two channels in FastFLIM-PIE

2.3 PIE using the supercontinuum laser source

The supercontinuum laser source offers unlimited choices for the single-photon excitation wavelengths, and has great characteristics for time-resolved imaging, such as the very narrow pulse width (only a few picoseconds). It is easy to select multiple laser wavelengths from the supercontinuum laser source using AOTF or optical band-pass filters; however, to have two different wavelengths in the PIE mode, an optical delay between them is required. Figure 7 shows a suitable implementation of PIE using the supercontinuum laser. The hybrid laser launcher takes the input from the supercontinuum laser source, selects the excitation wavelengths, and delivers two different excitation wavelengths to the Alba or Q2 system via two SMPM optical fibers – the difference of the lengths of the two fibers determines the delay time between the laser wavelengths for PIE. The two laser wavelengths are finally combined in Alba or Q2. In this case, the laser wavelengths are selected using the optical band-pass filters installed on the motorized filter wheels (FW1 and FW2) and the intensity of each laser wavelength is independently controlled by a motorized variable neutral density filter (VND). They (FW1, FW2 and VND) are not needed, when AOTF is used together with the supercontinuum source.

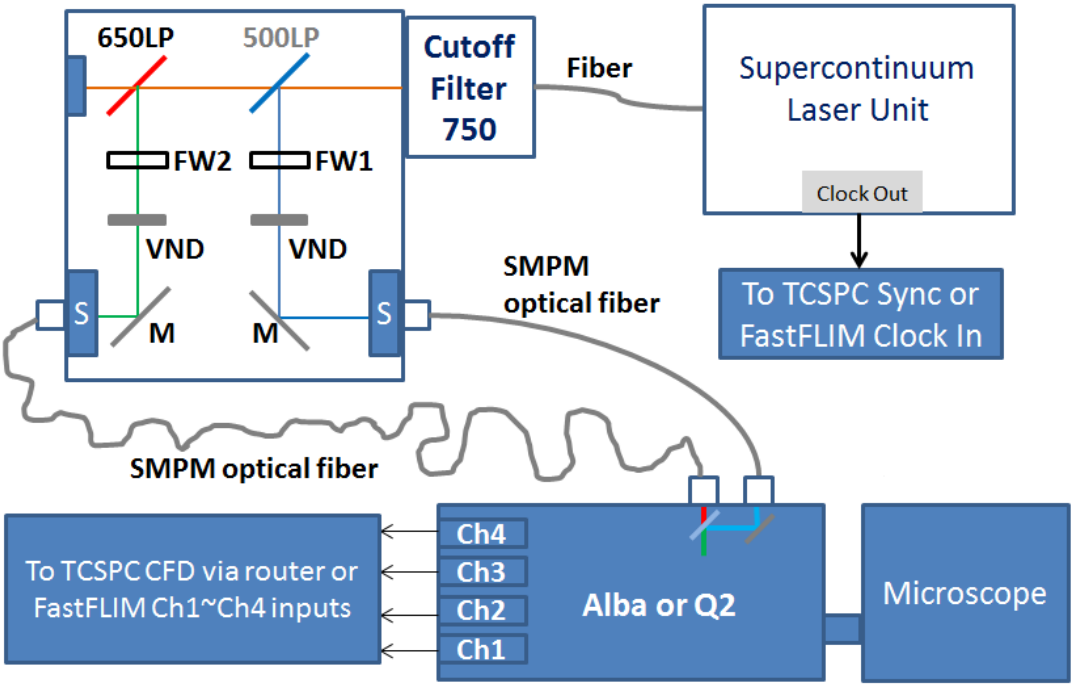

Figure 7: A hybrid laser launcher for implementing PIE with the supercontinuum laser source.

3 The sweetness of PIE, tasted by a few examples

Recent advances in fluorescence microscopy imaging have greatly extended the limit, enabling measurements of the dynamic and mechanistic properties of single molecules. These single molecule experiments have opened new opportunities for investigating molecular motors, enzyme reactions, protein dynamics, protein-protein interactions, DNA transcription, etc., especially by using the advanced FCCS and FRET techniques.

3.1 PIE eliminates the false positive cross correlation in the FCCS experiments

Fluorescence correlation spectroscopy (FCS) is a powerful biophysical technique that allows for measuring molecular motilities and concentrations from pM to μM concentrations. In FCS, the fluorescence fluctuations due to fluorescent molecules moving through a small diffraction-limited confocal volume (femtoliter) are recorded as a function of time and then statistically analyzed by correlation analysis, to obtain accurate information about concentrations, mobility, interactions and internal dynamics of molecules. FCCS (two-color FCS) simultaneously measures the fluctuations of two fluorescent species, and in addition their cross-correlation function, which can be evaluated to study molecular interactions. One major issue in FCCS experiments is that the F1 bleedthrough to the F2 emission channel causes a false positive cross correlation - this can be effectively removed by using PIE. Figure 8 shows the comparison of the FCCS measurements of the mixture of Atto425 and RhodamineB dyes in water without and with PIE at 20MHz – Ch1 (Atto425): Ex1, 440nm, Em1, 482/35-nm; Ch2 (RhodamineB): Ex2, 514nm, Em2, 550/49-nm; Nikon CFI 60X / 1.2NA water objective. In this FCCS experiment, no cross correlation between Atto425 and RhodamineB is expected, because they freely diffuse in water. However, a strong correlation was observed from the FCCS data acquired without PIE (Figure 8B-left); it was caused by the bleedthrough of Atto425 to the RhodamineB emission channel (Figure 8A). Using PIE, the false cross correlation is completely removed (Figure 8B-right), even though the bleedthrough was significant.

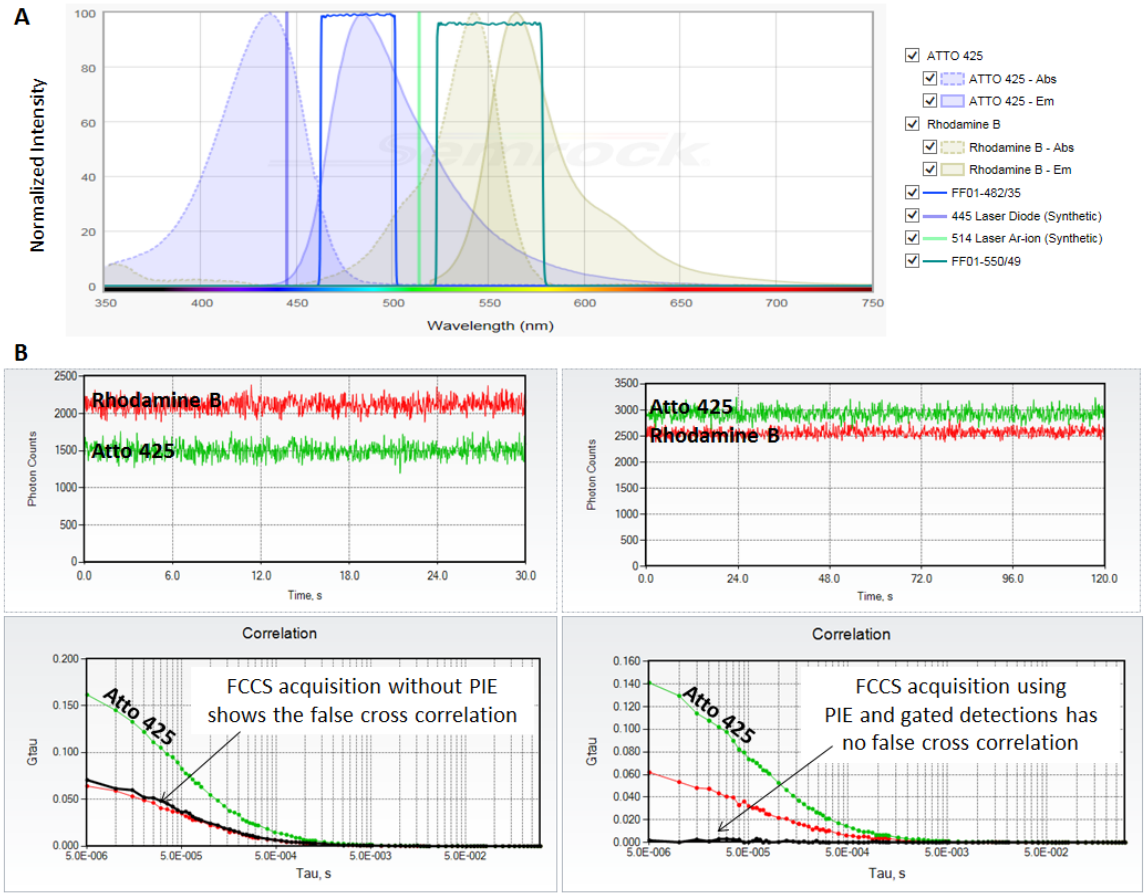

Figure 8: Comparing FCCS measurements of the mixture of Atto425 and RhodamineB dyes in water without vs. with PIE.

3.2 PIE allows simultaneous measurements of the lifetimes in two channels

As shown in Figure 8A, there is a significant overlap between the emission spectra of Atto425 and RhodamineB. The bleedthrough of Atto425 to the RhodamineB channel would complicate the fluorescence decay kinetics measured in the RhodamineB channel. This problem is solved by using PIE. As shown in Figure 9, both lifetimes of Atto425 and RhodamineB were accurately resolved by FastFLIM-PIE from the data acquired in their imaging channels (see Section 3.1). The FLIM results are presented using the phasor plots, which is a powerful approach for the FLIM data analysis. The details of the phasor plot method are described in our application and technical notes available at the following links.

http://www.iss.com/resources/pdf/appnotes/Phasor_Plot_And_Beyond.pdf

http://www.iss.com/resources/pdf/technotes/FLIM_Using_Phasor_Plots.pdf

Another example is given by Figure 10 to simultaneously measure the lifetimes of the Convallaria, Lily of the valley in both green and red channels, using TCSPC-PIE at 20MHz. Ch1 (Green): Ex1, 488-nm, Em1, 525/30-nm; Ch2 (Red): Ex2, 561-nm, Em2, 624/40-nm; Nikon CFI 60X / 1.2NA water objective. Both excitation wavelengths were selected from the Fianium SC-400-4-pp supercontinuum laser source (provided by the customer) using our hybrid laser launcher (see Figure 7).

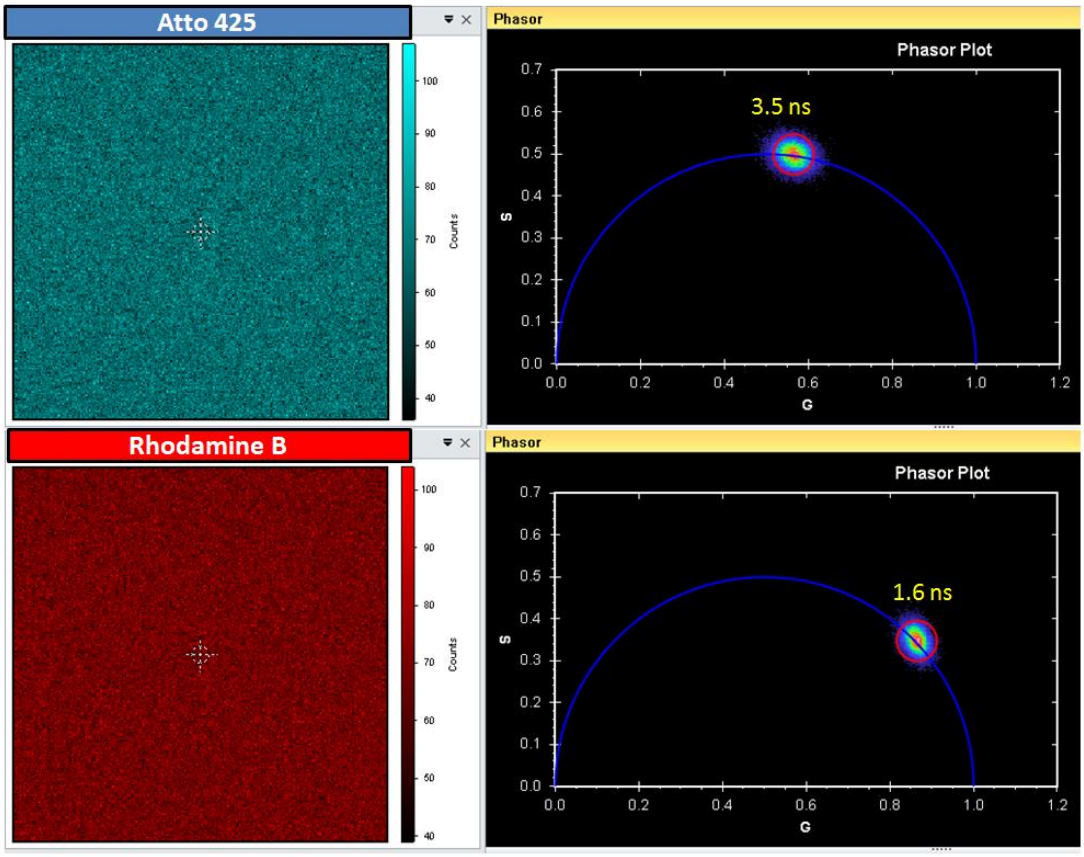

Figure 9: FastFLIM-PIE allows simultaneously measuring the lifetimes of the Atto425 and RhodamineB dyes mixed in water.

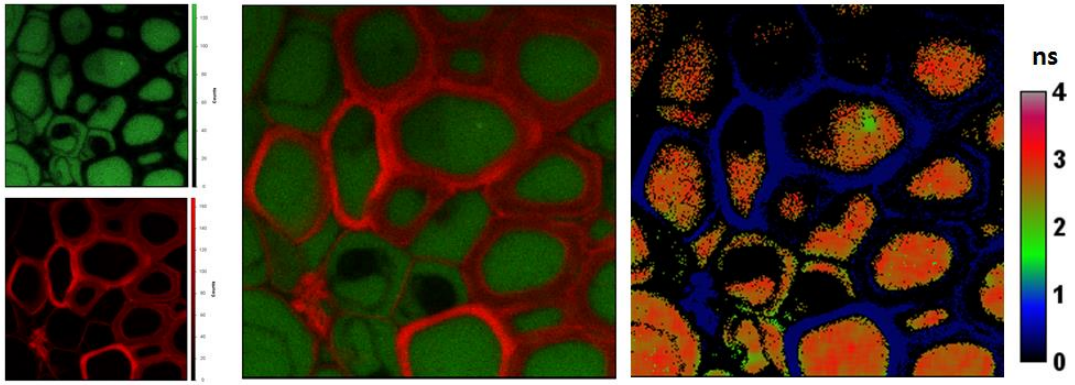

Figure 10: Measuring the lifetimes of the Convallaria in both green and red channels by TCSPC-PIE with the supercontinuum laser source.

3.3 PIE improves the accuracy of the smFRET measurements

Single molecule imaging techniques allow tracking dynamic behaviors of individual molecules, providing insight information of molecular processes that could be hidden by the ensemble average. Measuring FRET of individual molecules has become most commonly used in single molecule techniques, since it answers many fundamental questions (5-7). Using PIE both the donor and the acceptor fluorescent probes on the same individual molecule complex can be accurately quantified. This makes the FRET efficiency calculation more robust. PIE is useful in

cases where multiple sample populations are present, especially when the corresponding FRET efficiencies are roughly the same. PIE also allows low-FRET-efficiency populations be resolved from the zero peak that results from molecules that are donor-only labeled or have photo-bleached acceptor dye. In the smFRET experiments shown in Figure 11, purified α-synuclein protein modified to include two cysteine residues were fluorescently labeled with Alexa488 and Alexa594 maleimide dyes as FRET donor and acceptor probes, respectively. The detergent sodium dodecyl sulfate (SDS) was used to mimic lipid-binding of the protein and employed to modulate protein conformation. Single-cysteine proteins labeled with donor or acceptor dyes were used as control samples. Bursts in both donor (Ex1, 488-nm, Em1, 525/50-nm) and acceptor (Ex2, 594-nm, Em2, 641/75-nm) channels (Figure 11A) were acquired simultaneously using FastFLIM-PIE at 20MHz; however both channels were not gated in order to measure the acceptor sensitized emission (FRET) signal in Em2 due to Ex1. A bleedthrough-correction routine provided in VistaVision was applied to quantify the FRET signal and efficiency, subject to thresholding the burst events (8).

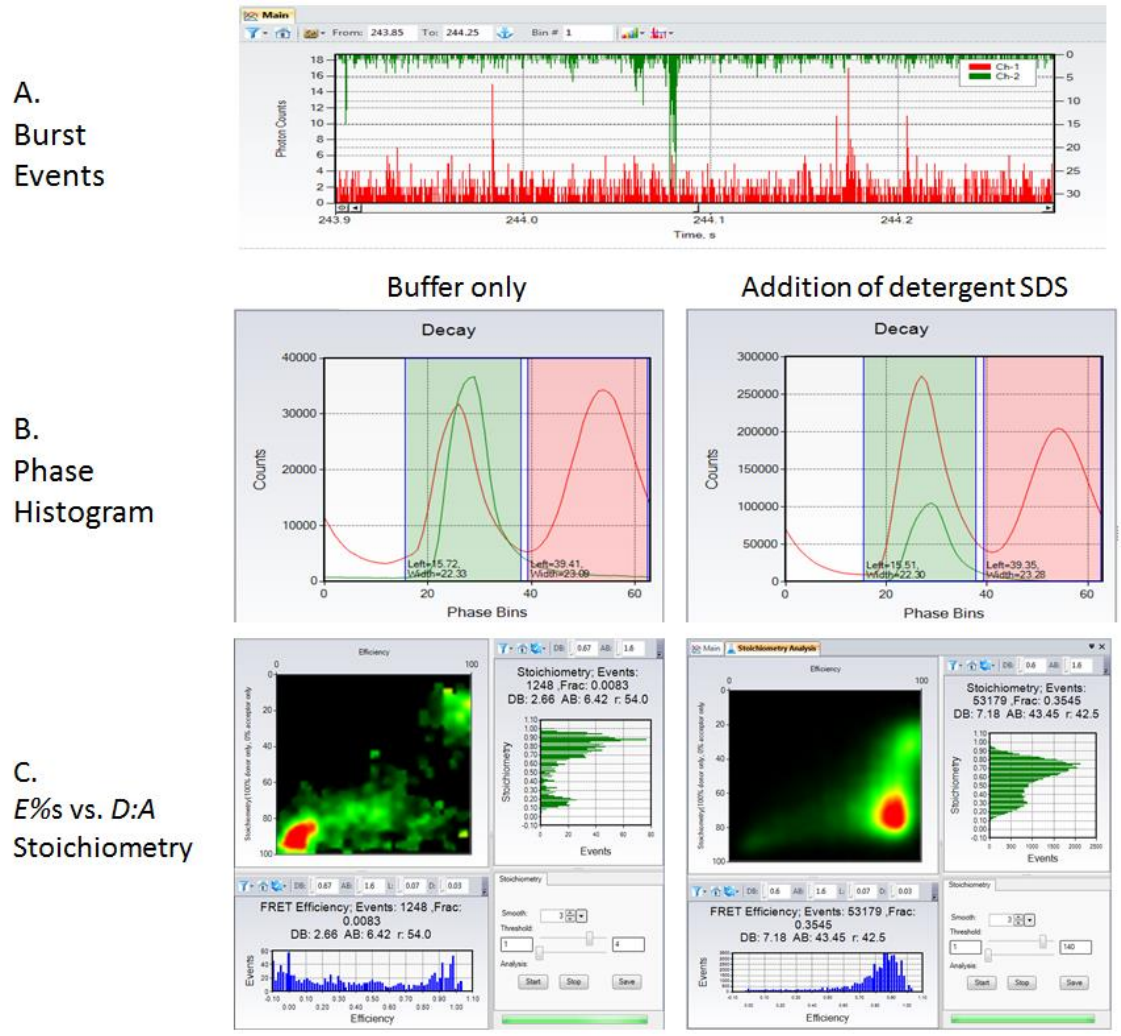

Figure 11: FastFLIM-PIE resolved the α-synuclein folding and conformational switching at different environment: (left) buffer only, (right) with SDS.

As shown in Figure 11B, PIE photon events show in different time-resolved window of α-synuclein folding and conformational switching at different environment: (left) buffer only and (right) with SDS – with SDS, the acceptor counts (the red curve, sensitized) are much higher compared to the donor counts (the green curve, quenched) in green window (due to Ex1, the donor excitation wavelength). Using the smFRET analysis routines in VistaVision, the

"*FRET efficiency*" histograms and the "*FRET efficiencies (x) vs. D:A stoichiometry (y)*" maps were generated (Figure 11C) – the histograms clearly shows the difference between buffer and the SDS; the maps that the events happened mostly in high FRET efficiency when the acceptor is more abundant.

4 Conclusion

PIE further pushes the limit of the alternating laser excitation to the nanosecond regime and provides unique benefits to many advanced biophysical applications, especially in FCCS and smFRET experiments. PIE is also suitable for regular multi-color imaging for co-localization analysis in laser scanning microscopy. In the regular scanning time scale (typically milliseconds per line), PIE would be just as "being simultaneous" and only requires a single scan per line, while two scans on the same line are typically required by using the AOTF to switch two lasers. It is demonstrated that PIE of using various laser sources can be set up by both TCSPC and FastFLIM with or without gating on the ISS Alba or Q2 laser scanning microscopy system, powered by the ISS 64-bit VistaVision software.

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