FastFLIM STED for Alba v5

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1. Introduction

Stimulated Emission Depletion (STED) is a powerful microscopy technique that allows for the observation of macromolecular fluorescence structures with spatial resolution below the diffraction limit. The ISS module, developed for Alba v5, uses the pulsed excitation and pulsed depletion approach (pSTED) in combination with the digital frequency domain fluorescence lifetime imaging (FastFLIM) to record the time-resolved photons which allows for an increase in the image resolution and the separation of two labels with a single pair of excitation / depletion lasers.

2. Instrument Set Up

Figure 1 is a schematic of the typical instrument setup used for pSTED. The excitation laser source is a pulsed laser

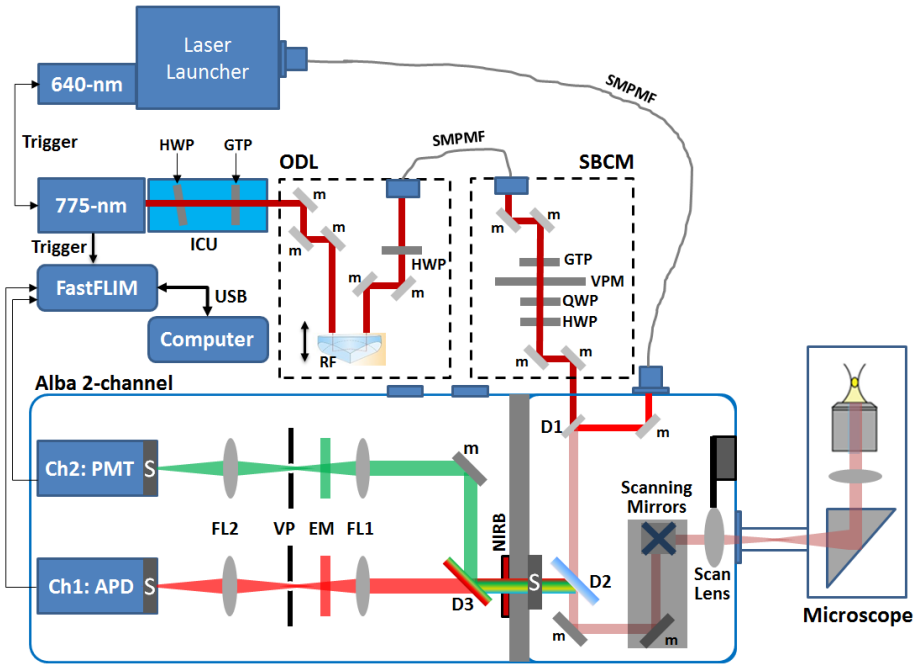

Figure 1: Schematic of the pulsed FastFLIM-STED. (ICU: intensity control unit; HWP: halfwave plate; GTP: Glan–Thompson polarizer; ODL: optical delay line; RF: Retroreflector; SMPMF: single mode polarization maintained fiber; SBCM: STED beam conditioning module; QWP: quarter wave plate; VPM: vortex phase mask; m: mirror; D (D1, D2, D3): dichroic; S: shutter; NIRB: NIR light blocking filter; FL (FL1, FL2): focusing lens; EM: Emission Filter; VP: Variable Pinhole).

diode by B&H emitting at 640 nm (pulsewidth = 120 ps; rep rate=20/50/80 MHz); the laser is mounted in the ISS laser launcher and it is delivered to the Alba through a polarization maintained fiber. The depletion source is a fiber laser by OneFive emitting at 775 nm (pulsewidth about 600ps; rep rate: 20~80 MHz). The intensity control unit (ICU) allows for control of the laser intensity prior to entering the Optical Delay Line (ODL) module. The beam goes through the single mode polarization maintained fiber (SMPMF) before entering the STED Beam Conditioning Unit (SBCM), where it goes through the vortex phase mask (VPM) to generate the "donut-like" point spread function (PSF) after the objective lens.

In the excitation area of the Alba, the two beams are combined at dichroic D1 and sent to the scanning mirrors module and hence to the sample.

3. FastFLIM for the time-resolved pSTED acquisition

FastFLIM (www.iss.com/microscopy/components/FastFLIM.html) is the data acquisition card for FLIM acquisition when acquisition speed is of the essence. The card is based upon the Digital Frequency Domain (DFD) technique that allows for the acquisition of Time-Tagged-Time-Resolved data without the dead time typical of TCSPC approach. The card features an extremely high dynamic range: signals of up to 13 million counts/sec when using the appropriate detector can be recorded (versus the about 100,000 counts/sec typical of TCSPC). The 4 independent input channels can be configured for accepting signals from PMTs, APDs with TTL output, or a combination of the two types of detectors. Decay times from 1 ms to 50 ps can be resolved. The card is supported by Windows 10 operating system and the connection to the computer is through USB2.

4. The Phasor Plot

FastFLIM provides the phase (ϕ) and the modulation (m) measurements at each modulation frequency as well as the total intensity at each pixel. These data are ready for analysis using the Phasor Plot (http://www.iss.com/resources/pdf/technotes/FLIM_Using_Phasor_Plots.pdf), a powerful technique for graphically separating complex decays times without the assumption of *ad-hoc* decay models. The phase (ϕ) and the modulation (m) measurements at each pixel of the image are transformed to the phasor space (G, S), as $G_\omega = m_\omega \cdot \cos(\phi_\omega)$ and $S_\omega = m_\omega \cdot \sin(\phi_\omega)$, where ω is the modulation frequency. Thus, the raw FLIM data measured at each pixel can be directly located on a 2D phasor plot.

The phasor plot approach provides a linear way of analyzing a complex mixture (G_ω, S_ω) composed of multiple (e.g. N) species, each of which is represented by a unique phasor ($G_{\omega,i}$, $S_{\omega,i}$) and can behave as a single-exponential (on the semicircle) or multi-exponential (inside the semicircle) decay kinetics:

$$G_\omega = \sum_{i=0}^{N}(f_i \cdot G_{\omega,i}) \; ; \; S_\omega = \sum_{i=0}^{N}(f_i \cdot S_{\omega,i}) \; ; \; \sum_{i=0}^{N}(f_i) = 1 \; ;$$

where f_i denotes the contribution of the i^{th} species to the mixture. When the phasor signature of each species ($G_{\omega,i}$, $S_{\omega,i}$) is known and given the measurements of enough harmonics (ω), decomposing the mixture will yield the contribution of each species (f_i).

Ideally, only the molecules excited by the excitation laser in the donut center should be detected in order to acquire an image with high resolution; the background fluorescence given from the depletion (donut) area should be rejected increasing the deplection laser power is an effective way to reduce this incomplete depletion; however it will also cause more photo-toxicity. The use of the phasor plot to the time-resolved pSTED images acquired by FastFLIM allows for the separation of the decay times of completely and partially depleted molecules thus increasing the image resolution.

5. Results

Figure 2 shows a comparison between the confocal and the pSTED images of the 60-nm fluorescent beads – the resolution of the bead size is achieved.

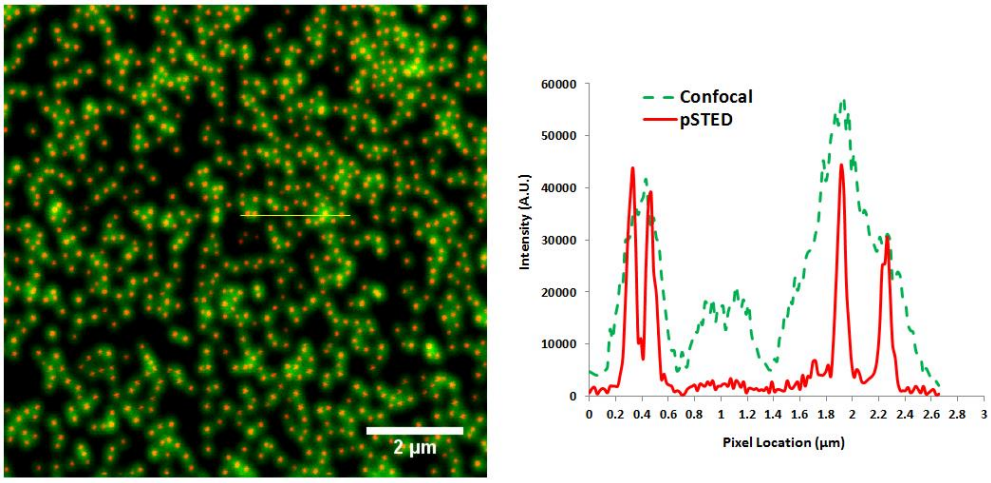

Figure 2: Confocal (green) vs. pSTED (red) images of 60-nm fluorescent beads, acquired by FastFLIM.

Figure 3 displays the Confocal image (left) and pSTED image (right) of the actin labeled with the SiR dye in fixed glia cells, acquired by FastFLIM.

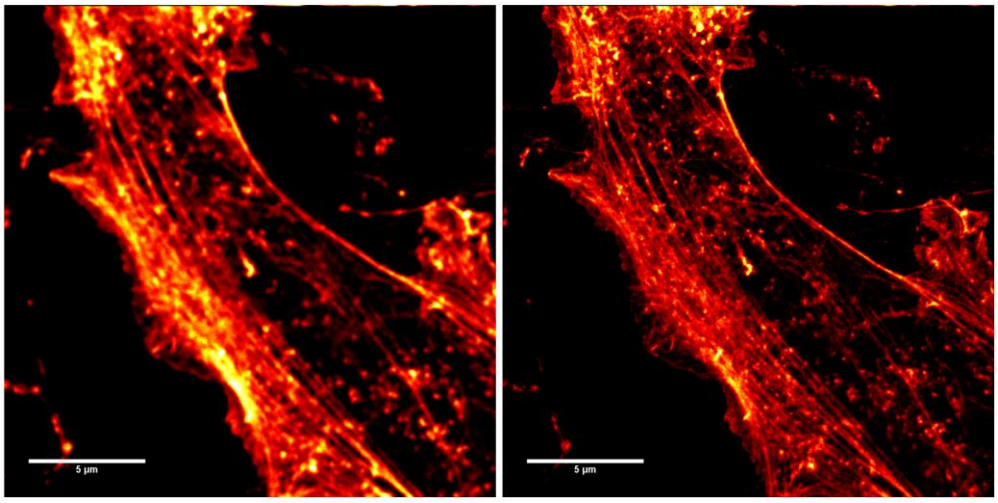

Figure 3:. Confocal image (left) and pSTED image (right).

The acquisition of time-resolved pSTED image allows for the separation of completely and partially depleted molecules for an increased resolution without increasing the STED laser power. The separation is visualized in the Phasor Plot because of the different decay times of the molecules. A software filter is implemented in order to select the molecules excited by the excitation laser only; the final result is an image featuring a larger resolution that the standard STED image (Figure 4.).

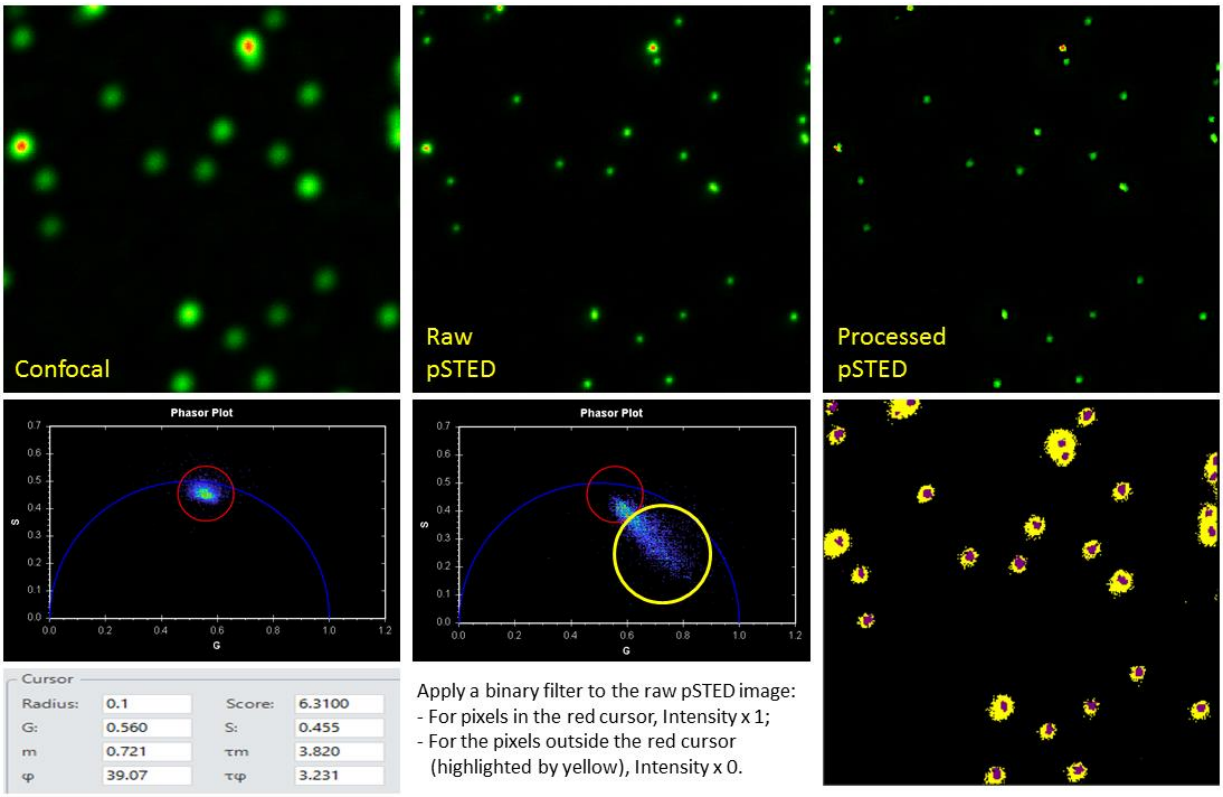

Figure 4. Left: Confocal image of 60nm beads (top) and phasor plot (bottom) displaying the location of the phasor plot. Middle: pSTED image (top) and position of phasor plot (bottom).
Right: Processed pSTED image (top) and display of

Two labels that can be excited with the same combination of wavelengths for pSTED can then be separated using the time-resolved information provided by FastFLIM. Figure 5 shows an example of the dual-label (Atto 647N and Atto 655) pSTED image acquired by FastFLIM. The two dyes are first separated from the phasor plots, and then assigned with two different false colors (Atto 647N – yellow, Atto 655 - purple) to produce the processed and merged pSTED image of the two labels.

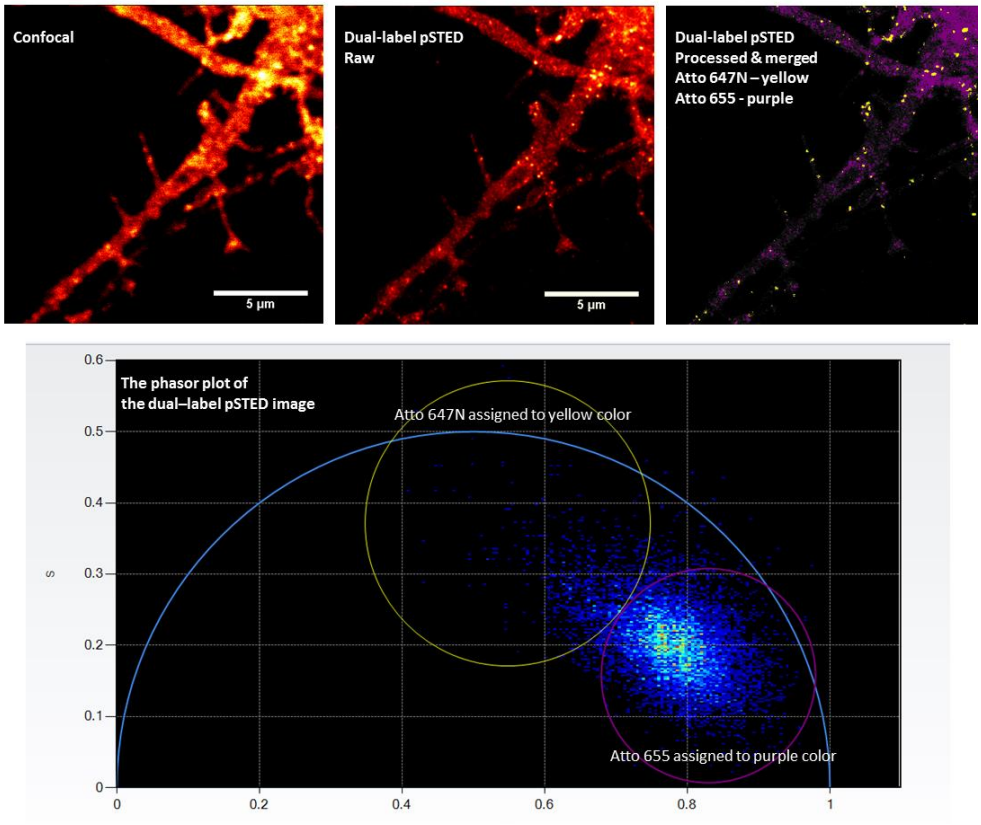

Figure 5. Dual-label (Atto 647N and Atto 655) pSTED image acquired by FastFLIM. The two dyes are first separated from the phasor plots, and then assigned with two different false colors (Atto 647N – yellow, Atto 655 - purple) to produce the processed and merged pSTED image of the two labels.

6. Conclusion

The FastFLIM pSTED introduces several features:

- Lower STED power laser to reduce photo-toxicity.
- Improvement of the image resolution by using time-resolved information
- Capability to do dual-label STED by a single pair of excitation and depletion lasers.

References

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