Time-Resolved Fluorescence Microscopy and Spectroscopy

Integrated TCSPC for FLIM and FCS in Fluorescence Microscopy

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Fluorescence Lifetime Imaging Microscopy (FLIM) and Fluorescence Correlation Spectroscopy (FCS) are two pivotal techniques for investigating molecular dynamics and photophysical properties in complex biological systems. Both rely on Time-Correlated Single Photon Counting (TCSPC) to capture the arrival times of photons with high speed and precision, enabling the resolution of fast molecular interactions and the detection of subtle environmental changes. [1,2].

Swabian Instruments' Time Taggers provide picosecond-level temporal resolution and support high data rates, fulfilling the demands of both FLIM and FCS. The integration with ISS platforms such as SWISS, Alba, AlbaSTED (Fig. 1b), and Q2 enables highly customizable and user-friendly time-resolved fluorescence microscopy experiments.

EXPERIMENTAL SETUP

Figure 1a illustrates the typical experimental configuration for FLIM and FCS applications, showcasing the seamless integration of Swabian Instruments' Time Tagger 20 or Time Tagger Ultra within the ISS Microscopy platforms.

The setup features a laser source paired with a dedicated focusing system, and single-photon detectors such as hybrid PMTs or SPADs. The precise time stamping by Swabian Instruments' Time Taggers permits the capture of fluorescence intensity fluctuations over time using autocorrelation and cross-correlation functions. The scanning of the images in ISS's microscopes is based on galvo scanners integrated within the setup.

With a data throughput of up to 90 Mtags per second, the system supports high-speed acquisition and real-time phasor analysis without compromising timing accuracy. The system also supports photon counting histogram (PCH) analysis and phasor-based lifetime mapping, a method first commercially realized by ISS using Swabian Time Taggers. This combination offers exceptional flexibility for probing diffusion, binding kinetics, and microenvironmental dynamics in live-cell and single-molecule experiments.

a b

Fig. 1: Experimental setup for FLIM and FCS, with the Time Tagger included. (a) Schematic of the microscope components and their connections to the SWISS digital TCSPC hardware. The galvo pixel scanner provides the trigger for the frame, the line, and the current pixel for FLIM experiments. The scanner itself can also be used for FCS to achieve RICS. (b) Alba microscope setup running VistaVision analysis software during FLIM acquisition with phasor analysis.

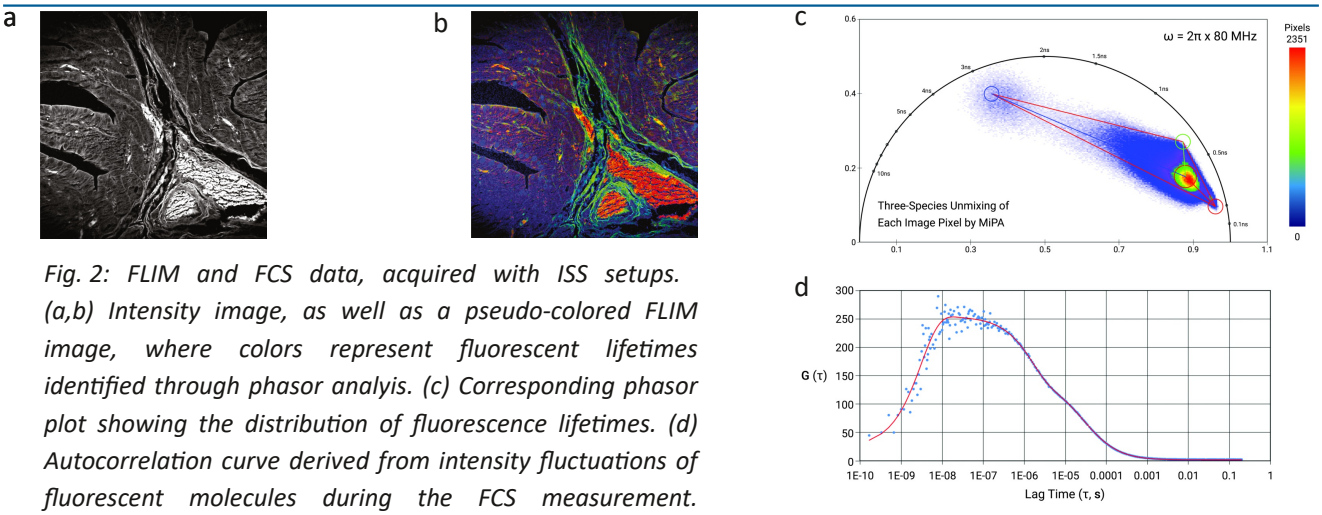

Fig. 2: FLIM and FCS data, acquired with ISS setups. (a,b) Intensity image, as well as a pseudo-colored FLIM image, where colors represent fluorescent lifetimes identified through phasor analyis. (c) Corresponding phasor plot showing the distribution of fluorescence lifetimes. (d) Autocorrelation curve derived from intensity fluctuations of fluorescent molecules during the FCS measurement.

FLIM

During a FLIM measurement, the galvo scanner moves across the sample while a pulsed laser illuminates the defined pixel region. Both the laser pulse and the detected single-photon arrival time are time-stamped by the Swabian Instruments Time Tagger, and the data is streamed into ISS Microscopy's analysis pipeline. The VistaVision software displays the scanned image (Fig. 2a) in real time and, combined with Phasor analysis (Fig. 2c) [3], provides an intuitive, real-time calculation of fluorescence lifetimes, revealing local environmental conditions (Fig. 2b). Regions of interest within the image can be analyzed live during the experiment. ISS Microscopy pioneered real-time Phasor analysis based on the fast Time Tagger data stream, marking a milestone in commercial FLIM technology.

FCS

During an FCS measurement, the confocal microscope acquires photons generated by a detection volume of approximately 1 µm³, typically containing between 0.1 and 200 fluorescent particles. Fluorophores attached to these particles emit photons, which are detected by single-photon detectors and time-stamped by the Time Tagger. As particles enter and exit the volume, the photon count fluctuates. These intensity fluctuations are analyzed in VistaVision using the autocorrelation function (Fig. 2d) to precisely determine diffusion coefficients and particle concentrations.

The setup can be extended for multi-channel detection, enabling analysis of molecular interactions and co-diffusion using dual-channel Cross Correlation Spectroscopy (FCCS) and Pulsed Interleaved Excitation (PIE). When combined with the galvo scanner from the FLIM setup, Raster Image Correlation Spectroscopy (RICS) becomes possible as well, allowing spatial mapping of diffusion dynamics across the sample.

CONCLUSION

The integration of Swabian Instruments' Time Tagger hardware into ISS's quantitative microscopy platforms enables precise, flexible, and high-throughput time-resolved fluorescence experiments. Supporting a wide range of modalities from FLIM and FCS to single-molecule techniques and phasor analysis, the ISS - Swabian collaboration provides researchers with a unified and powerful toolkit for exploring biological complexity at high temporal resolution.

REFERENCES

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