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Elliot

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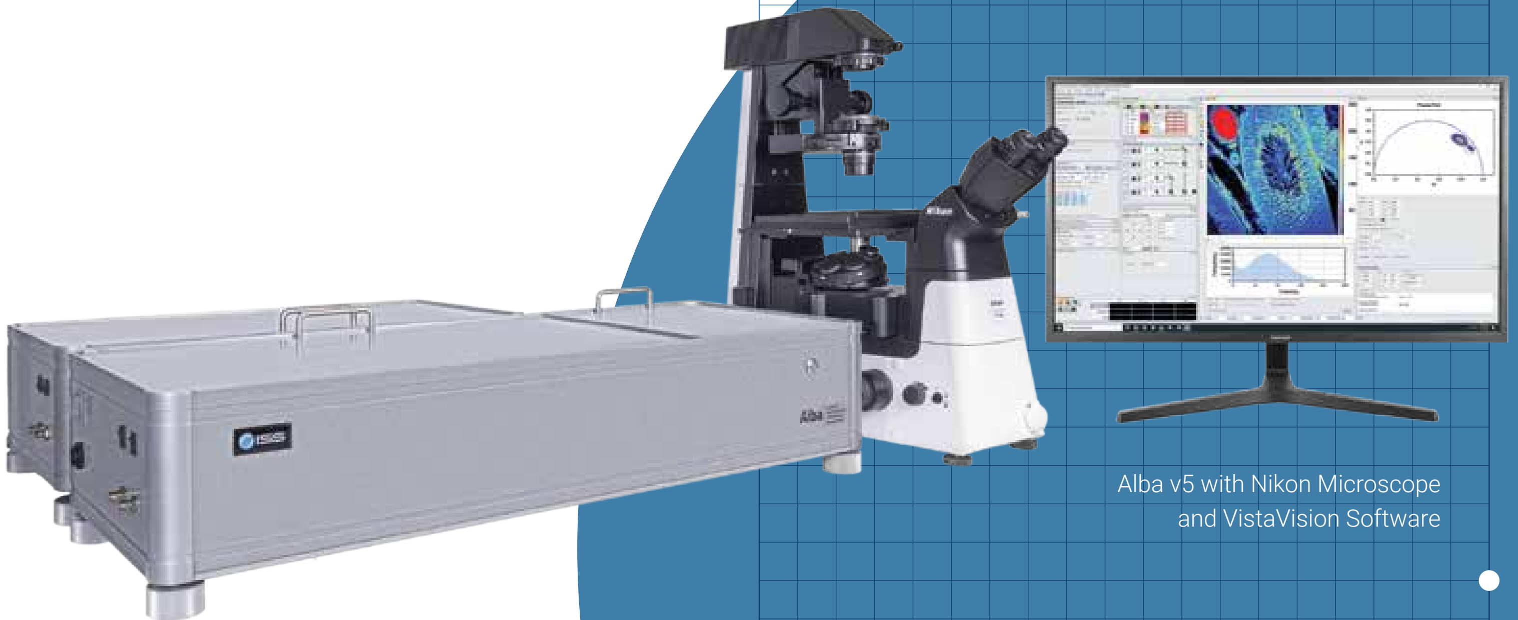
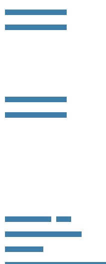
Alba v5

A laser scanning microscope that incorporates several measurement modalities for quantitative biology and material science applications requiring single molecule detection sensitivity.



Alba v5 with Nikon Microscope and VistaVision Software

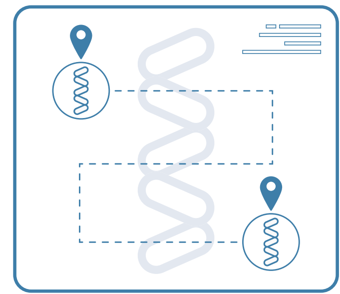


Alba v5

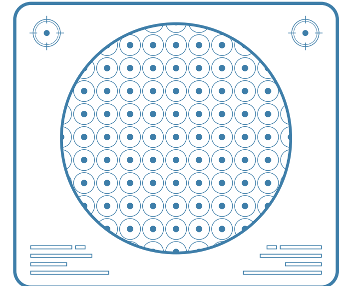
The Ideal Instrument for Quantitative Cell Biology at Single-Molecule Detection Levels

Overview of Alba v5

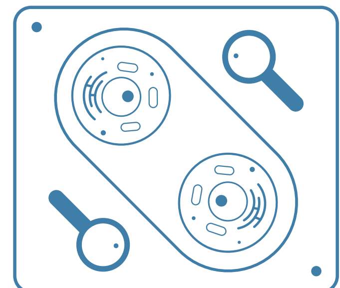
The revolution in quantitative cell biology is here and continues to evolve. Laser Scanning Confocal Microscopy (LSCM) applications are now in place for functional genomics, proteomics and metabolomics, which provide for the analysis of collectives of molecules, the structures they form and their dynamics within the single cell. Alba is a laser scanning microscope that incorporates several measurement modalities for experimental quantitative biology and material sciences applications requiring the single molecule detection sensitivity. Capable of acquisition from the Violet to the NIR region, features two independent laser entry ports. Alba v5 is powered by VistaVision, the comprehensive software package for instrument control, image acquisition and processing.



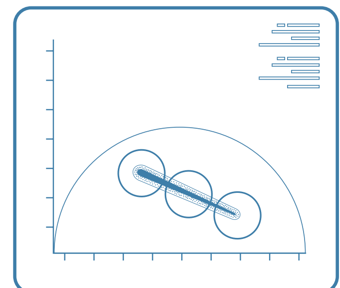
FLIM FRET in Live Cells



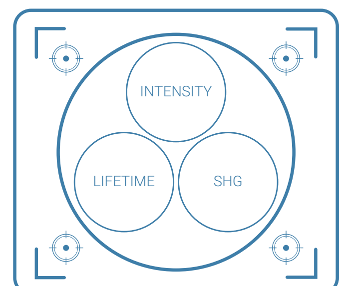
Tissue Imaging



Single Molecule Studies & Detection of Single Emitters by Antibunching



Multiplex Imaging by Time Resolved Unmixing Using the Phasor Plot



Multi-Modality Imaging

Cos7 Anti-Nup-Star635P SPLIT-STED

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The Alba Design

Flexibility

Alba's unique design is based upon an open architecture format, allowing the researcher to accommodate new experimental requirements that arise: at any time, the user can replace or mount filters on the various automated filter wheels without any direct intervention of the factory; and at any time, lasers can be added when new experiments require a different excitation wavelength. An Alba system can be installed with a basic configuration and then grows with changing lab requirements.

Innovation

Alba makes use of innovative technology including the latest light detectors, and the capability of acquiring FLIM data in either the time-domain (TCSPC) or in digital frequency-domain (FastFLIM), as well as data analysis using either standard fitting algorithms or the newer phasor plot approach. Moreover, Alba incorporates innovative data acquisition techniques tailored for quantitative cell biology studies. Alba's efficient optical design, using a minimal number of optical surfaces, provides the researcher with an instrument that is fully optimized for single-molecule detection sensitivity.

Microscope

The Alba is a laser scanning confocal system that can be utilized with most commercial inverted and upright research microscopes, from open frame to fully motorized. The Alba system is fully compatible with both standard and sophisticated microscope accessories, including widefield epifluorescence illumination by a LED and detection by a camera, focus lock, environmental control chambers for precise regulation of temperature / CO2 / humidity, electrophysiology micro-injection devices, patch clamp systems, etc.

Imaging Modalities

Four options are available for image acquisition using the Alba v5 system, namely:

- Laser Scanning using fast galvo-controlled mirrors. The laser beam is scanned over the sample following a predetermined pattern. This is achieved by using galvo-controlled mirrors that move the beam on the XY-plane. The Z-axis control is achieved by moving the objective up and down by a piezo device or an encoded motor, or by moving the sample up and down by a piezo stage insert. Galvo-controlled scanning mirrors offer the best solution when fast imaging acquisition and great precision in the sub-nanometer scale are required. This option is required for scanning FCS, 3D particle tracking, N&B and RICS acquisition.

- Stage Scanning using a piezo-controlled or a linear encoded motorized XY stage. The Z-axis control can be achieved by using a piezo device or an encoded motor mounted on the objective or the stage. When using this option, the beam is set at a position while the sample is moved through the beam path (i.e., the sample stage moves). The piezo stage offers exceptional resolution in the sub-nanometer scale and great stability. However, its speed is much slower than the galvo mirrors and its travel range is much smaller than the linear encoded motorized stage, which can typically travel more than 100 mm in each dimension. The micro-positioning accuracy and repeatability of the motorized state are not as high as the piezo stage and the galvo mirrors. Its scanning speed is very slow, although this can be greatly improved by using the line swapping mode, a feature supported by the ISS VistaVision software.

Alba coupled to an upright microscope (Model Bx53 by Olympus).

- Objective Scanning using a piezo-controlled XYZ stage mounted on the microscope nosepiece. In this modality the objective is mounted on the XYZ stage and moved for scanning, while the sample is immobile.

- Micro and Macro Scanning by using both the galvo-controlled mirrors and the encoded motorized stage. This integration provides the most powerful routines for precise micro-macro positioning and allows automatic acquisition of high-resolution images across a large field of view. This option is required for image tiling and multi-well screening data collection, where accuracy and consistency over extensive areas are crucial. The synergy between the fast, precise movements of the galvo mirrors and the broader range provided by the motorized stage makes this modality a powerful tool for advanced imaging tasks.

Laser Launchers and Accessories

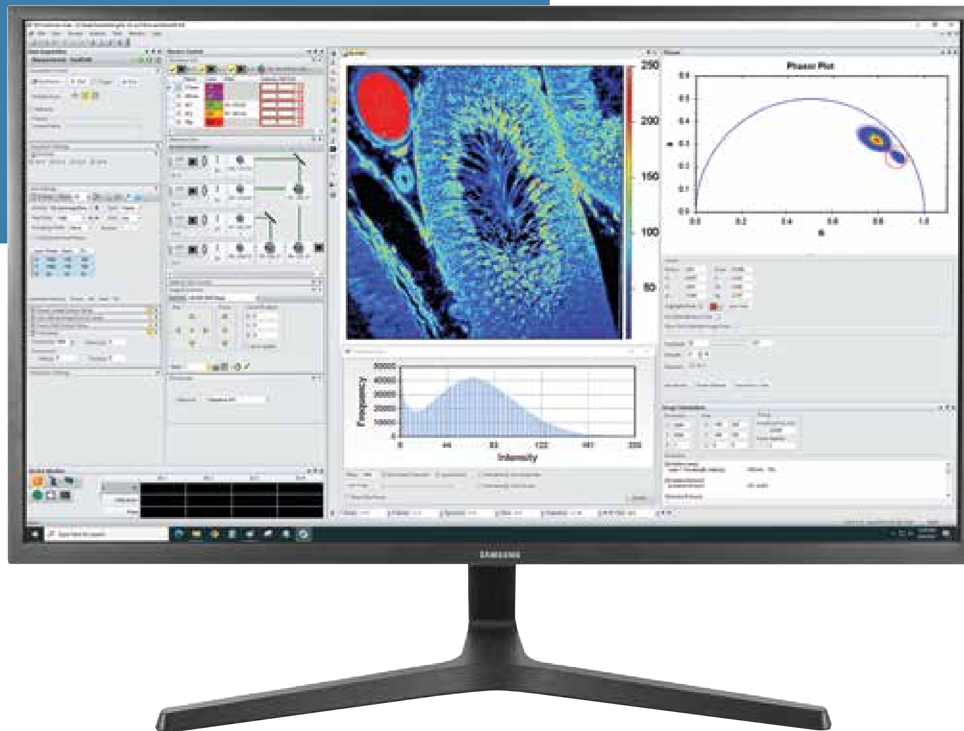
Alba is capable of delivery of excitation wavelengths from deep UV to NIR (266-1300 nm). Various types of lasers, including diode lasers (CW or pulsed), supercontinuum lasers, fiber-based or Ti:Sapphire femtosecond pulsed lasers, etc., can be combined on the Alba system. The single-photon lasers are typically combined by an ISS laser launcher and delivered to the Alba scanner via polarization-maintained fibers. The laser launcher has two exit ports for separating the visible and the NIR wavelengths, to ensure their best laser-to-fiber coupling efficiencies. Each port has a mechanical shutter installed. The laser launcher provides a beam stop and intensity control by using the variable neutral density filter or the Acousto-Optic Tunable Filter (AOTF) for each laser line. A two-photon (2p) laser is typically delivered to the Alba scanner in free space. The beam path is fully enclosed. The Acousto-Optic Modulator (AOM) or the 2p intensity control unit based on a combination of rotating halfwave plate and a fixed polarizer can be used for tuning the 2p laser intensity. The AOM provides fast modulation of the 2p laser intensity and enables phosphorescence lifetime imaging (PLIM) with the FastFLIM or Digital TCSPC. All laser launchers and accessories are fully controlled and operated by the ISS VistaVision software.

The Acquisition Channels and Detectors

Alba is capable of collection of emission wavelengths from Violet to extended NIR (300-1700 nm). The standard detector types include GaAs photomultiplier tubes (PMT), Hybrid PMT and single photon avalanche photodiodes (SPAD). The base system includes 2-channel acquisition which can be upgraded to 4 channels. An additional external port is available, ready for mounting a special detector with a custom optical design, e.g. a spectrograph device or a spectral FLIM module. Each channel can be dedicated to a measurement modality (FFS, FLIM, spectra acquisition) by optimizing the choice of the detectors. Each acquisition channel comprises a filter wheel, a computer-controlled variable-aperture pinhole, the focusing lens and the light detector. The pinhole of each channel can be independently adjusted and optimized for its detecting wavelength – this unique feature ensures the best sensitivity of each channel, critical for precise fluorescence correlation and cross-correlation spectroscopy measurements.

A461: 4 laser launcher pictured.

The VistaVision Software



The Alba system is powered by VistaVision (64 bit, Windows 10/11 Pro), the comprehensive software package for instrument control, image acquisition, processing and analysis.

VistaVision Instrument Control Module

- Alba Unit(s), including the Scanner and the Detection Module
- Laser Launcher Module, including Lasers and Lasers Accessories
- Data Acquistion Cards and Imaging Scan Controllers
- Microscope and Microscope Accessories, including the Motorized Stage

Includes the routines for instrument control (automatic instrument alignment of pinholes and lens positions, shutter control, selection of the light detector gain/bias control, overload protection, etc.); control of the Imaging Devices (galvo-mirrors, piezo-controlled and encoded motorized stages); laser launchers (laser intensity, laser modulation); and control of microscope automation features.

VistaVision Imaging and FLIM Module

- Intensity Imaging
- RICS / N&B and Multi-N&B Analysis
- Digital Frequency Domain FLIM (AKA FastFLIM)
- Time Domain FLIM by Digital TCSPC (Time Correlated Single Photon Counting)
- Multi-image Phasor Analysis (MiPA)
- Steady-state & Time-resolved Polarization Anisotropy Imaging

Includes routines for image acquisition, image processing and image display that allows for the user to acquire single-point data (intensity, kinetics, polarization, FLIM/PLIM); line data; and images. The user interface includes setting/adjusting the acquisition parameters (pixel dwell time, image size, image resolution, etc.) and the selection of measurement type (Imaging, RICS / N&B, Polarization, FLIM). Images and image stacks can be acquired in different spatial directions (XY, XZ, YZ, XYZ), all of which can be combined with time (t) to make time series. FLIM is acquired using either the digital frequency-domain (FastFLIM) or the digital TCSPC technique; both acquisition modalities can be implemented on the same instrument.

FLIM data can be analyzed by both the fitting (Marquardt-Leven berg minimization algorithm) and the phasor plots. Analyzed FLIM results can be exported as lifetime images, or images of pre-exponential factors, or images of fractional contributions. The software includes operations applied to images, namely smoothing, filtering, rotation, zooming, scaling and automatic threshold setting for image contrast enhancement. Images and plots are exported to popular formats (txt, csv, png, jpeg, gif, tif, bmp, etc.) and movies are produced in the avi format, to be opened by third-party software, e.g. ImageJ.

VistaVision Fluorescence Fluctuation Spectroscopy (FFS) Module

- FCS (Fluorescence Correlation Spectroscopy)
- FCCS (Fluorescence Cross Correlation Spectroscopy) with Pulsed Interleave Excitation (PIE)
- Fluorescence Lifetime Correlation Spectroscopy
- Scanning FCS and Carpet Analysis
- PCH (Photon Counting Histogram)
- Single Molecule FRET (smFRET) Bursts Spectroscopy
- Photon Antibunching
- FFS Fitting Plugin

Includes routines for multi-channel data acquisition, processing and analysis for FCS, FCCS, PIE-FCCS, FLCS, Scanning FCS, PCH, Photon Antibunching and smFRET Bursts. Data streams can be acquired in Counts mode, Time Tagged mode, or Time Tagged Time Resolved (TTTR) mode. VistaVision features a real-time display of the auto correlation and cross correlation functions, apart from a nominal delay (less than one second) required for the computation of the function. The data can be acquired from both solution and live cell samples at any spatial coordinates defined by the user. A sequence of multiple data acquisition files can be acquired (for instance, when using a microwell plate on a computer-controlled XY stage) and displayed and stored automatically. VistaVision provides advanced routines for processing FFS data streams, including spike removal and segmentation tools which efficiently clean aggregations; in addition, the gating and lifetime filter unmixing of the TTTR data enable precise separation of photons based their arrival times and decay kinetics.

VistaVision also offers a range of algorithm-based tools to process and analyze smFRET bursts, including background & bleedthrough corrections, FRET efficiency & distance calculations, bursts searching, and more. VistaVision includes a powerful FFS Fitting module for analyzing FCS, FCCS, PCH and antibunching data curves. It has more than 50 fitting models built in, to accommodate various 2D / 3D point spread functions generated by either single-photon or two-photon excitation. Researchers have complete freedom over their data modeling with VistaVision's intuitive text-editor interface, which allows for the creation and entry of custom models.

VistaVision 3D Particle Tracking Module

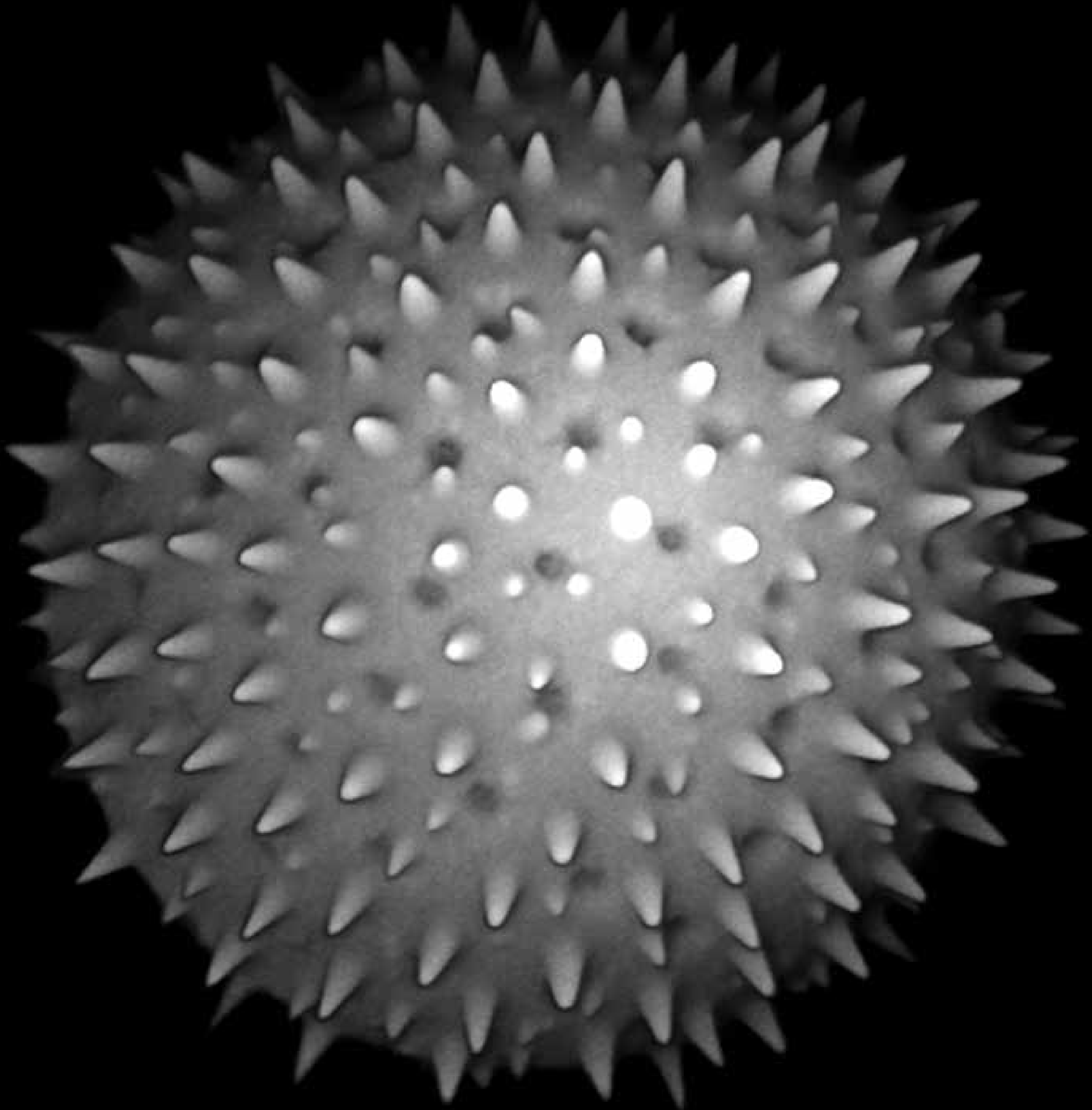
Includes routines for tracking movements of a single particle / molecule within a living cell in 3D, offering a high temporal resolution down to 32 ms and a high localization precision down to 20 nm. VistaVision displays live feedback as well as XZY positions during tracking. It calculates the Mean Square Displacement (MSD) as a function of a lag time to estimate the particle motion parameters such as velocity and diffusion rate.

All Measurements for Quantitative Biology in One Integrated Unit

Alba v5 Measurements:

- Multi-dimension (x, y, z, λ, t) confocal Imaging
- Image tiling and multi-well screening
- Ratiometric imaging
- FLIM and Phasor Plots
- FRET (Förster Resonance Energy Transfer) by FLIM
- Time Resolved Emission Spectra (TRES) and Spectral FLIM
- Steady-state and Time-resolved Polarization Anisotropy Imaging
- RICS (Raster Image Correlation Spectroscopy)
- N&B (Number & Brightness)
- FCS (Auto- and Cross-Correlation)
- Two-color FCCS by Pulsed Interleave Excitation (PIE)
- FLCS (Fluorescence Lifetime Correlation Spectroscopy)
- PCH (Photon Counting Histogram)
- Scanning FCS
- Single fluorophore imaging and photon antibunching
- PIE-smFRET (single molecule FRET) bursts spectroscopy
- 3D Particle Tracking
- SPLIT-STED

Figure 1: 3D volume rendering of the Z-stack of images acquired from a pollen grain using the 2p excitation wavelength at 780 nm.



Cell Imaging

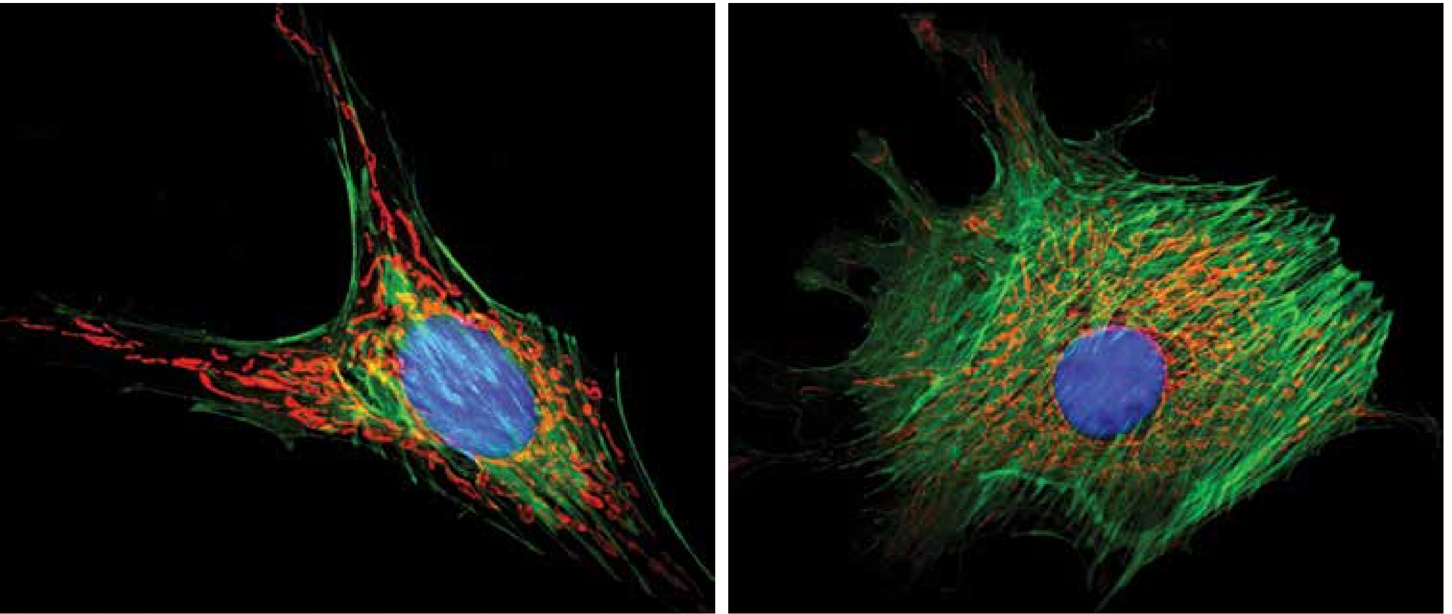


Figure 2 & 3: Confocal 3-color imaging of FluoCellsTM (Thermo Fisher): Blue = Nucleus labeled with DAPI; Green = Phalloidin labeled with Alexa FluorTM 488 Phalloidin; Red = Mitochondria labeled with MitoTrackerTM Red.

Tissue Imaging

Figure 4: Time-resolved confocal autofluorescence imaging of a tissue section using the 375-nm laser excitation wavelength (courtesy of Dr. Aneesh Alex, GSK).

Tile Imaging

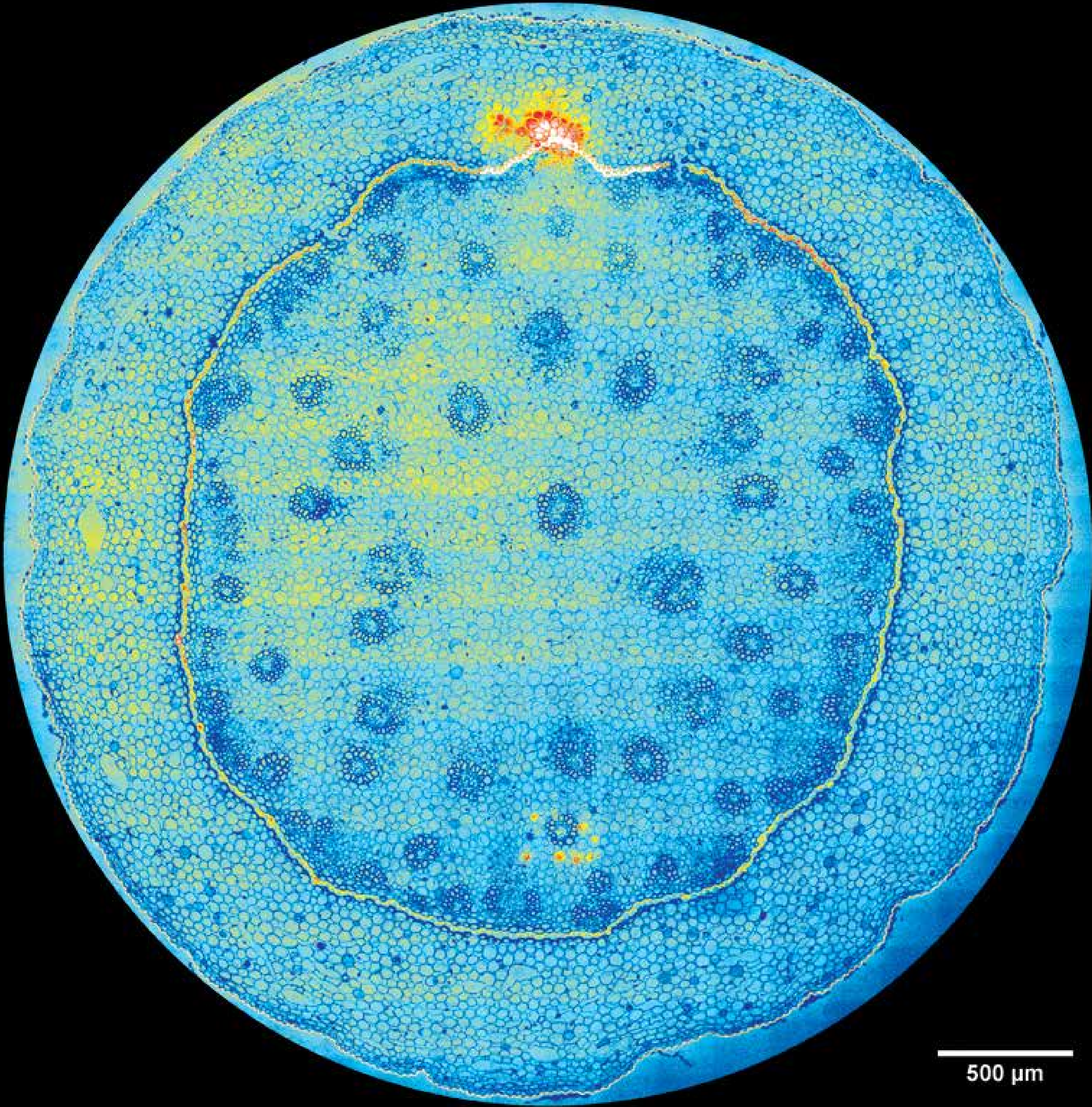


Figure 5: Stitching of 400 tile images acquired from a Convallaria sample slide, using both the galvo mirrors and the motorized XY stage to scan the entire sample.

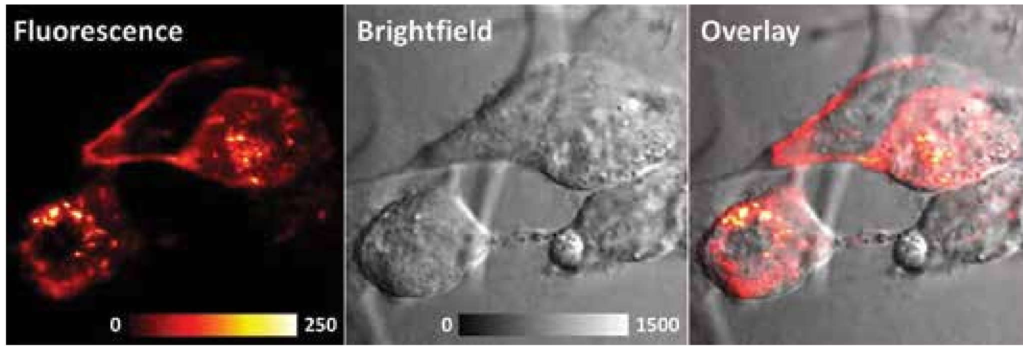


Figure 6: Merge of fluorescence and brightfield images of live cells, acquired simultaneously with both fluorescence and transmission PMT detectors.

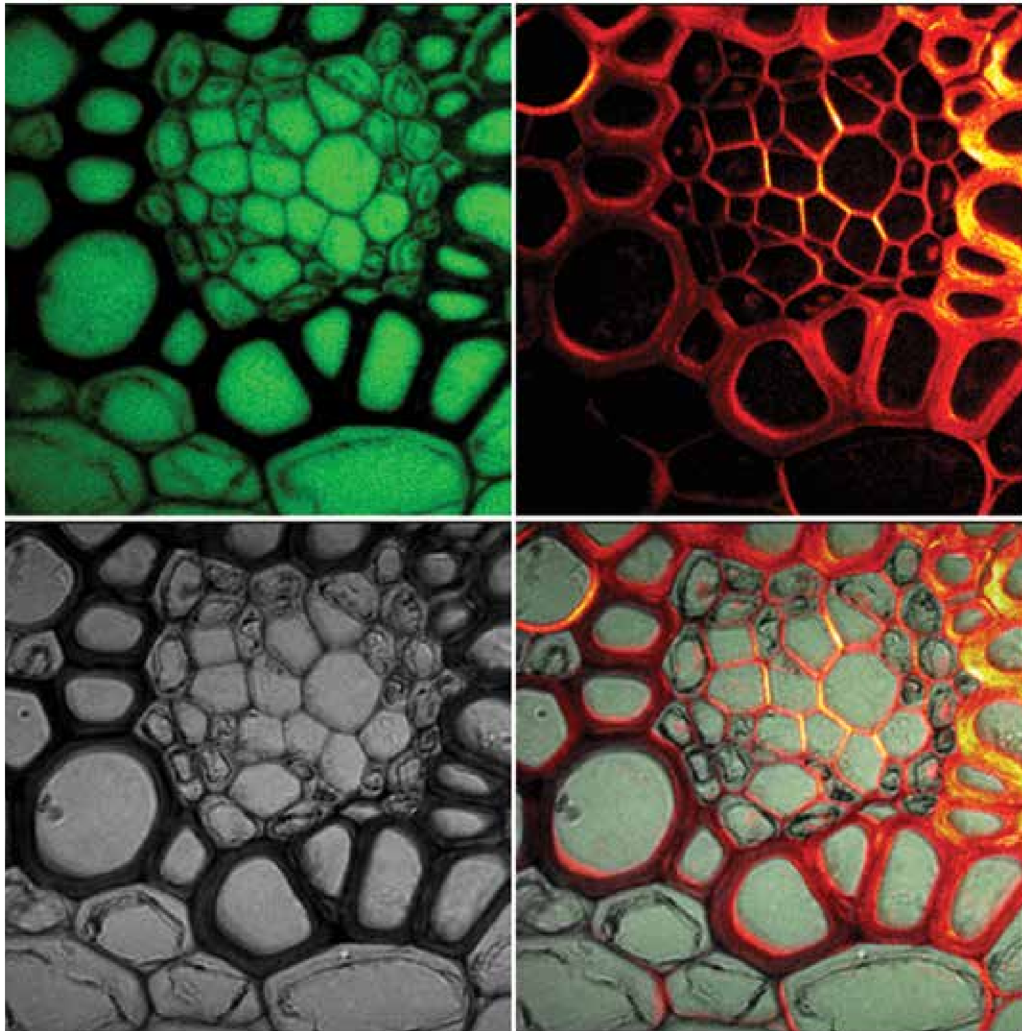


Figure 7: Confocal 2-color + transmission imaging of Convallaria, acquired using the 488-nm excitation wavelength: Green = 525/50-nm emission channel; Red Hot = 630/92-nm emission channel; Grey – transmission channel; Merge of the three channels.

Fluorescence Lifetime Imaging (FLIM)

Data Acquisition: Digital Frequency Domain (FastFLIM) and/or Digital TCSPC. Alba can acquire FLIM data using the Digital Frequency Domain (FastFLIM) or the digital TCSPC (Time Correlated Single Photon Counting), the user selects the modality of preference for the instrument, or may decide to have both approaches on the same instrument. Either data acquisition card is capable of measuring lifetimes in a wide range from below 100 ps to beyond 100 ms.

When acquiring a FLIM image, the image includes the steady state information as well. Also, if the dwell time is selected properly, the image includes the information for RICS analysis and N&B analysis. Note: all of this information is available in the same image!

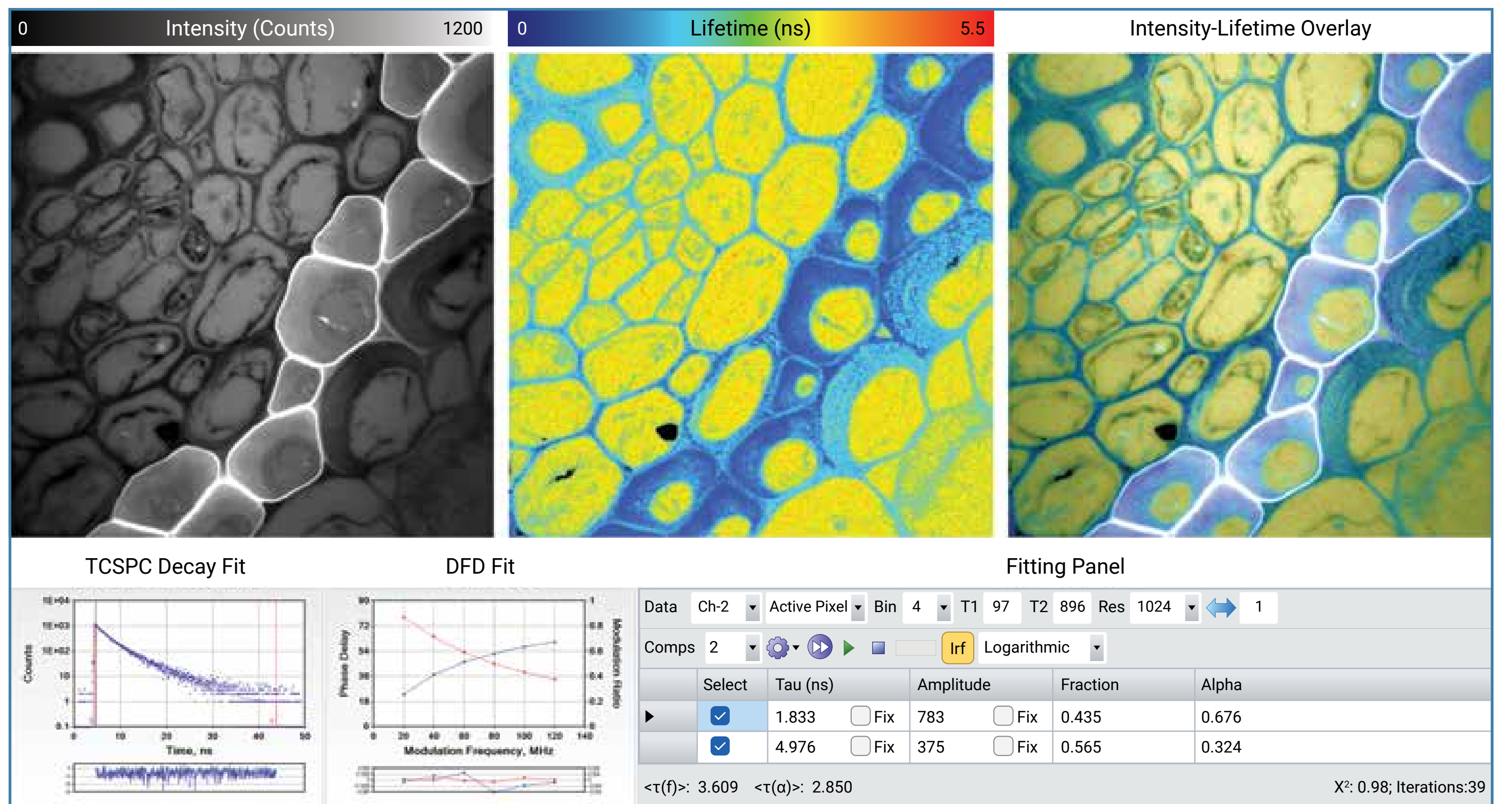


Figure 8: shows an example of confocal FLIM of Convallaria and the data analyses by both time-domain (TCSPC) and digital frequency domain (FastFLIM) fittings. VistaVision allows users to specify up to 5 exponential components for tail or deconvolution fitting. For deconvolution fitting the software can estimate the instrument response function (IRF) or allow users import the measured IRF. The fitting routines can be applied to each image pixel or a free-hand drawn ROI (region of interest) which is defined by user and for all the pixels in the whole image to produce the lifetime map.

Phasor Plots and MiPA

(Multi-Image Phasor Analysis)

The phasor plot is a graphical representation of raw FLIM data and a powerful tool for FLIM data analysis. VistaVision provides the phasor plots analysis routines including the Multi-image Phasor Analysis (MiPA) module, for using both FastFLIM and TCSPC. When using FastFLIM, VistaVision can display the phasor plots in real time, providing immediate visual feedback on the fluorescence lifetime image being acquired.

MiPA allows users to apply multiple harmonic frequencies to generate the phasor plots and load many images into the same phasor plots graph for comparison and analysis.

In MiPA, users can activate various cursors of different colors and shapes, such as circle, oval, square, and rectangle, with adjustable sizes to select populations of distinct lifetime signatures shown by the phasor plots. MiPA offers unmixing routines for separating two and three lifetime species and produces the lifetime-unmixed images. MiPA also provides the FRET calculator to generate the FRET efficiency map. In addition, many denoising filters (Median, Mean, Gaussian and their combinations) along with the intensity thresholding are included by MiPA to ensure robust results.

References for MiPA: Biophotonics paper; SPIE paper. (doi.org/10.1117/12.2626879).

Two-Species Unmixing

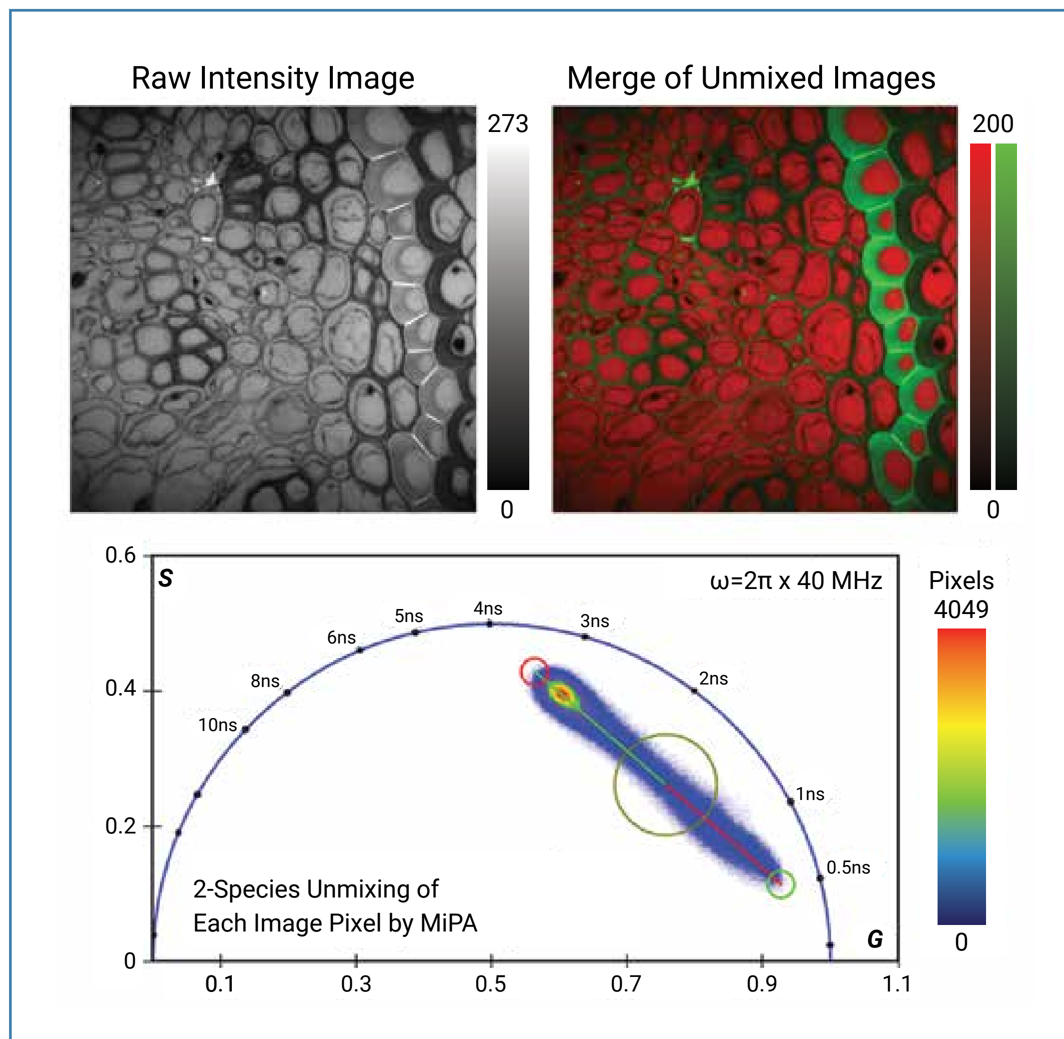
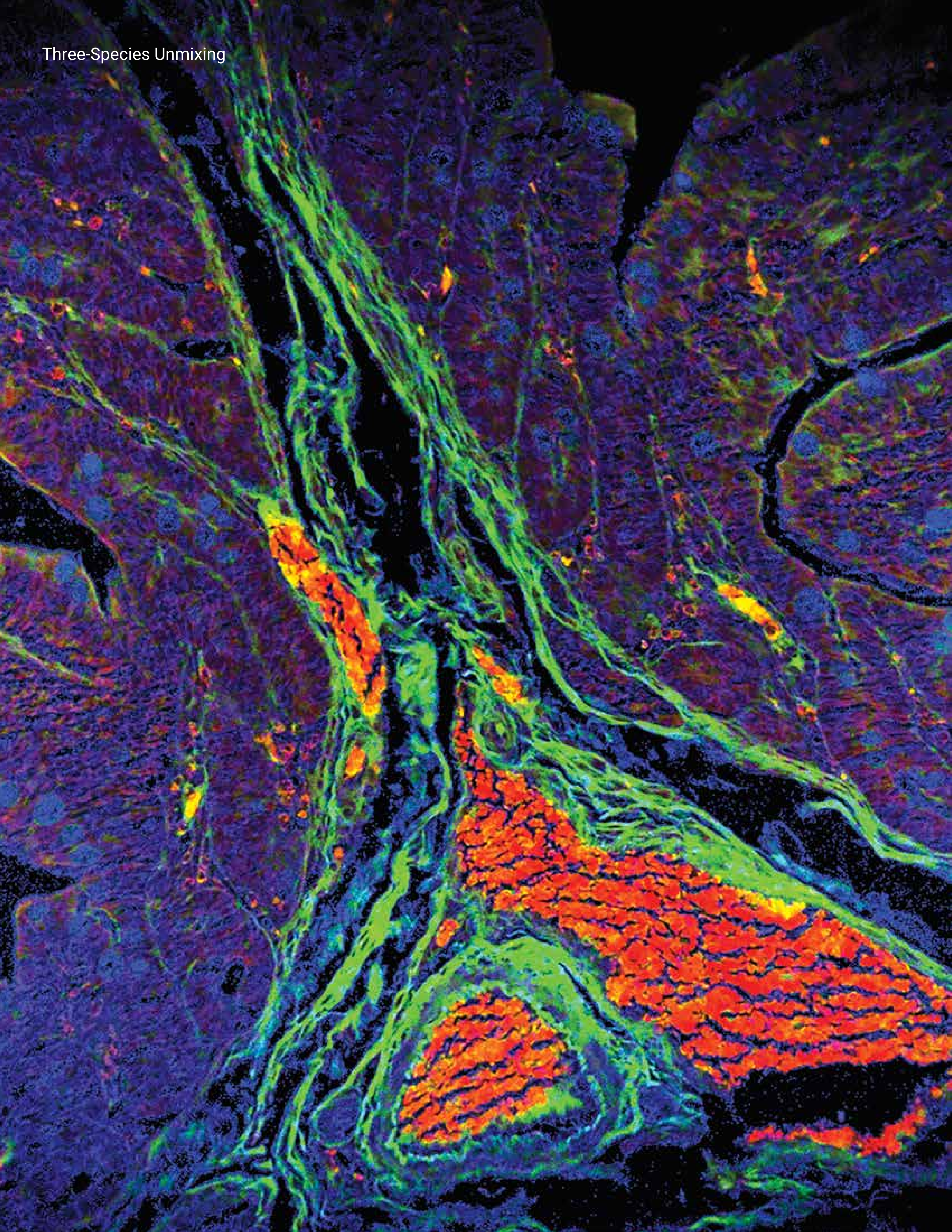
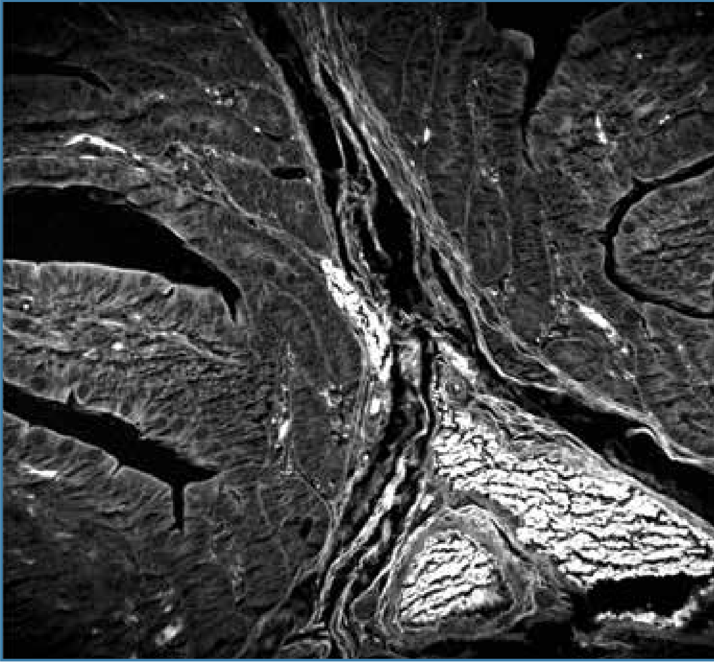
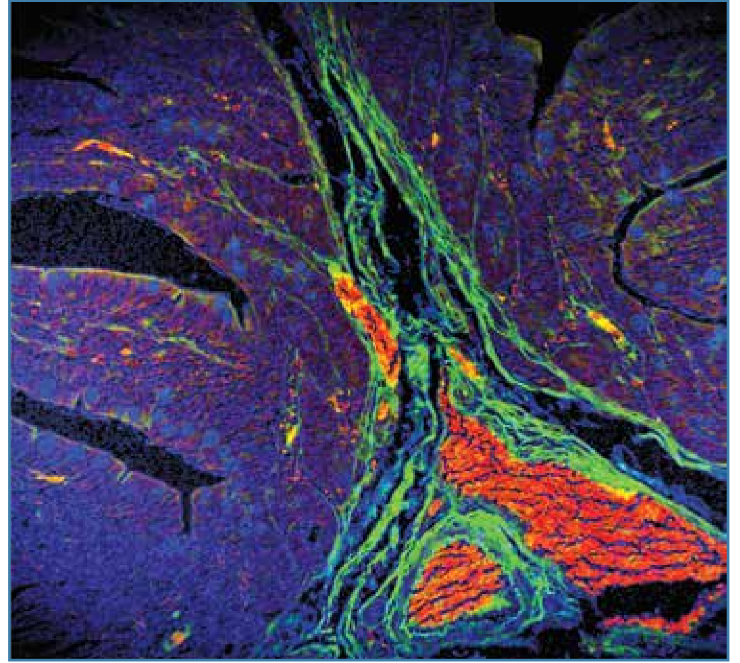


Figure 9: Two-species Lifetime Unmixing. The FLIM image's phasor plots from a Convallaria sample reveal a linear distribution, suggesting the contributions of two distinct lifetime species at each pixel. The longer and shorter lifetime species are marked by positioning red and green cursors at the ends of the line. MiPA calculates the fractions of both species at each pixel, to produce unmixed images as well as a merged one utilizing false colors.



Three-Species Unmixing





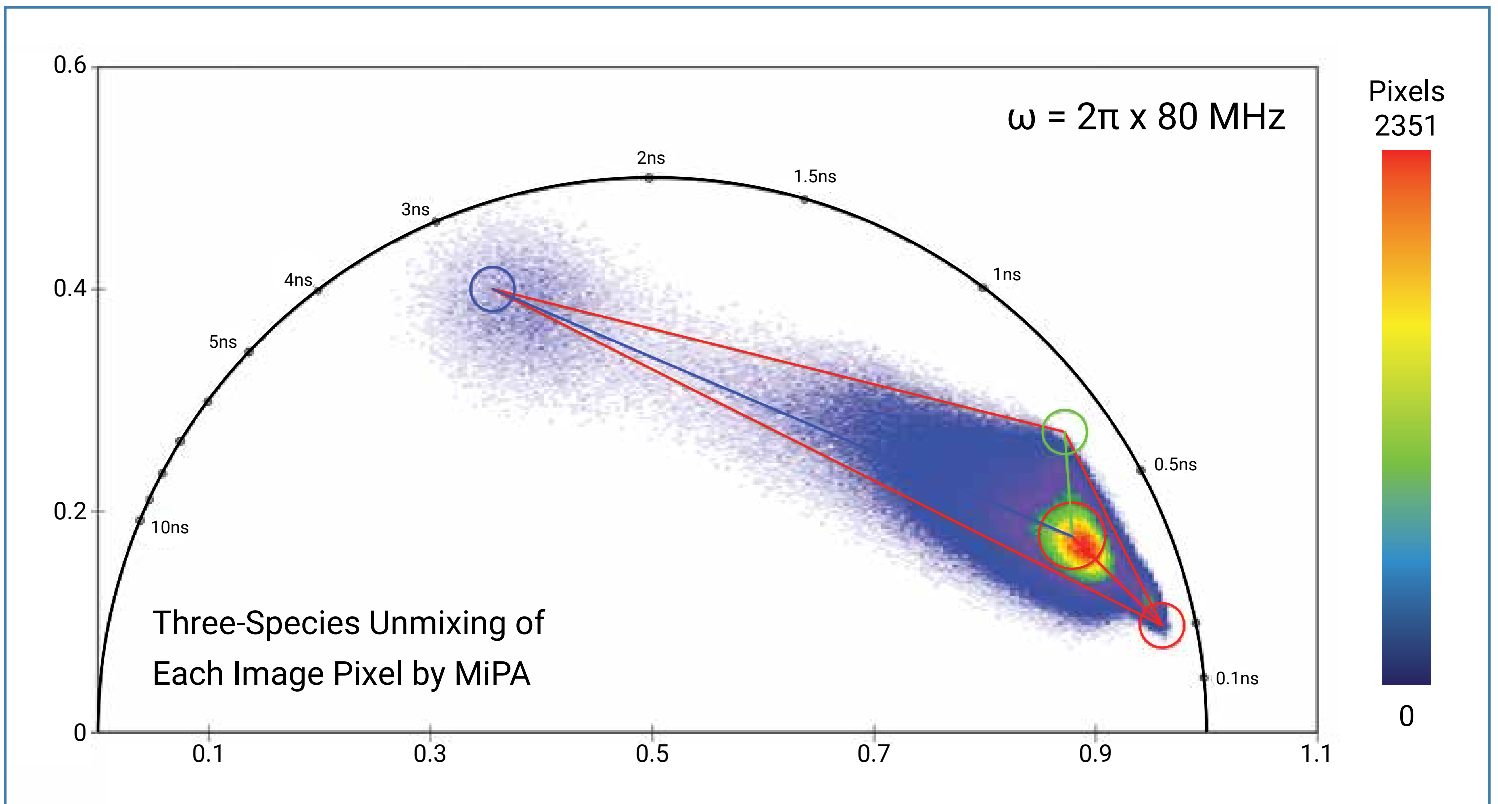


Figure 10: Three-Species Lifetime Unmixing. The phasor plot of the FLIM image acquired from a HE-stained tissue sample reveals a triangle distribution, suggesting the presence of three distinct lifetime species. The shortest, intermediate and longest lifetime species are marked by positioning red, green and blue cursors at the three extreme corners of the triangle-shaped distribution, respectively. MiPA calculates the fractions of all three species at each pixel, to produce unmixed images as well as a merged image utilizing false colors.

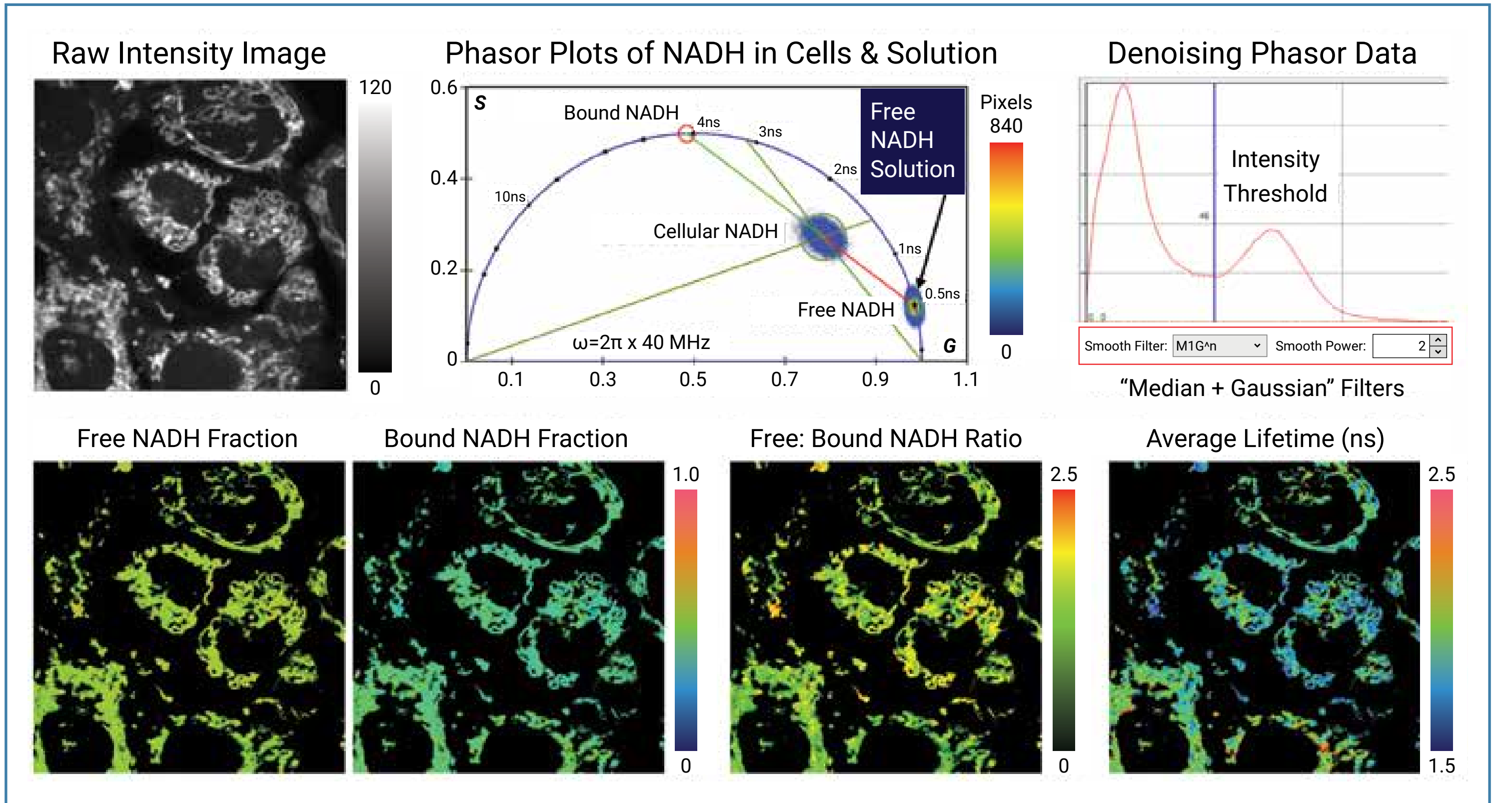


Figure 11: Two-Component Lifetime Unmixing. Using the phasor plots of the FLIM images acquired from NADH in live cells and solution which only contains the free form, MiPA derives the phasor coordinate of the bound form of the cellular NADH. MiPA computes the fractions of both free and bound NADH for each pixel in the cellular NADH image, producing a cellular map depicting the ratios of free-to-bound NADH. Given the free and bound NADH lifetimes, it also generates the cellular NADH average lifetime map.

FLIM-FRET & FRET Calculator

FLIM provides one of the most robust methods for measuring FRET in live specimens (Nat Protoc, Vol. 6, No. 9, pp. 1324-1340). Changes in FRET can be easily identified from the phasor plots of raw FLIM data. The FRET Calculator in MiPA, developed based on the FRET trajectory method (Biophys J, Vol. 94, No. 2, pp. L14-L16), can accurately determine the FRET efficiency per pixel, enabling quantification and localization of FRET within biological samples. This is an effective way to track protein-protein interactions in live cells.

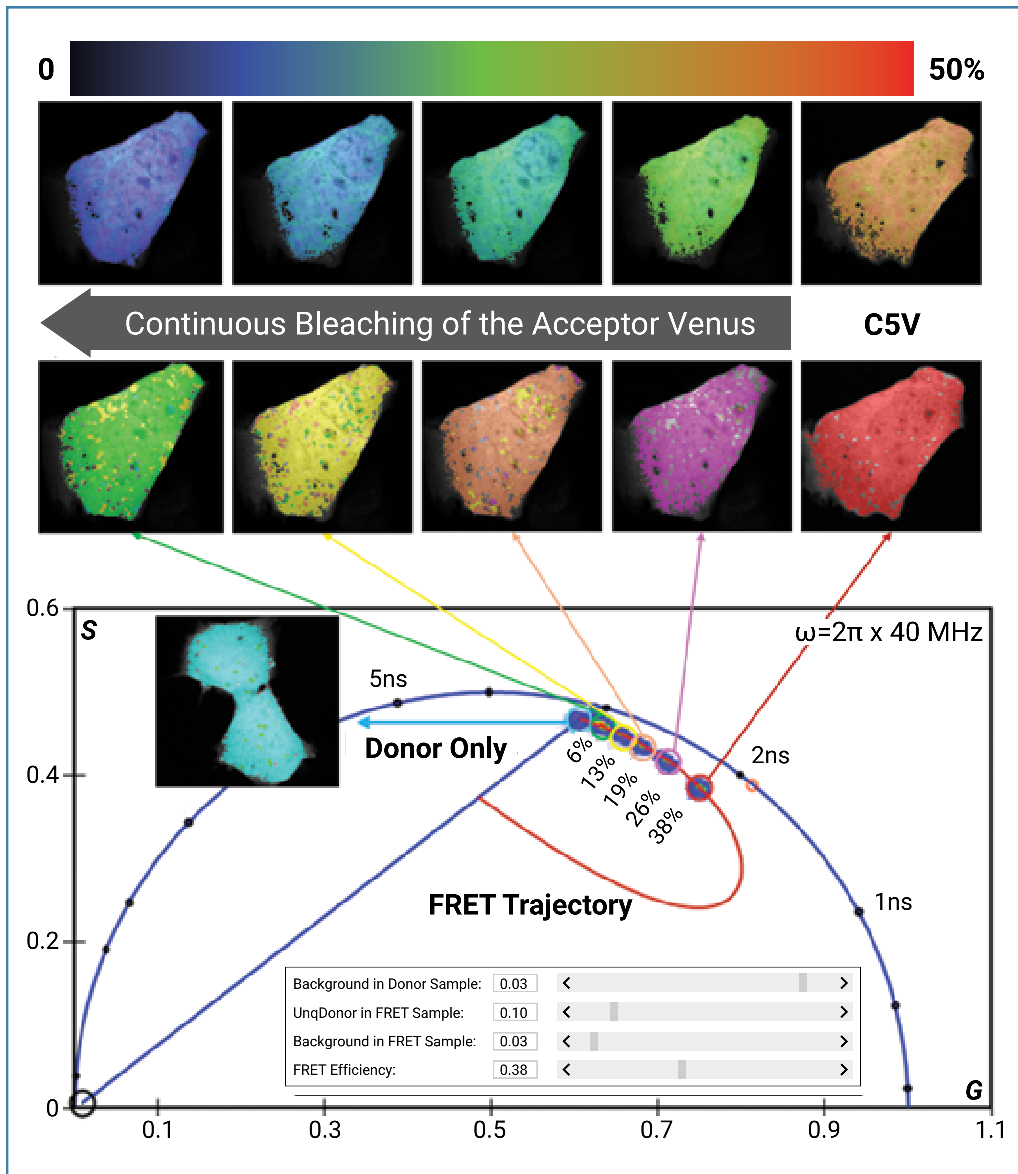


Figure 12: FRET Calculator. The FRET trajectory curve is drawn for the phasor plots of the FLIM images, measuring the donor (Cerulean) in live cells expressing the Cerulean-5aa-Venus (C5V) FRET standard construct, while continuously bleaching the acceptor (Venus). The unquenched donor location is obtained from the phasor plot of cells expressing only the donor (Cerulean) and the background is determined from the background pixels. The phasor plots of the C5V FLIM images obtained at five time points during bleaching are well separated and marked by different colors to highlight the corresponding images; their FRET efficiencies (averaged at 38%, 26%, 19%, 13%, 6%) are calculated by the FRET Calculator, which also generates the false-colored FRET efficiency map for each C5V image.

Steady-state and Time-resolved Polarization Anisotropy Imaging

For polarization anisotropy measurements, the laser excitation is linearly polarized and further cleaned up by a Glan-Thompson polarizer; the emission light goes through a polarization beamsplitter and is then split to two light paths, which are parallel and perpendicular to the excitation, respectively. Both are detected simultaneously in two separate channels. VistaVision provides routines to monitor the steady-state anisotropy in kinetic measurements, calculate the steady-state anisotropy map from a pair of parallel and perpendicular images, and fit the time-resolved parallel and perpendicular decays to obtain both fluorescence lifetimes and rotation times.

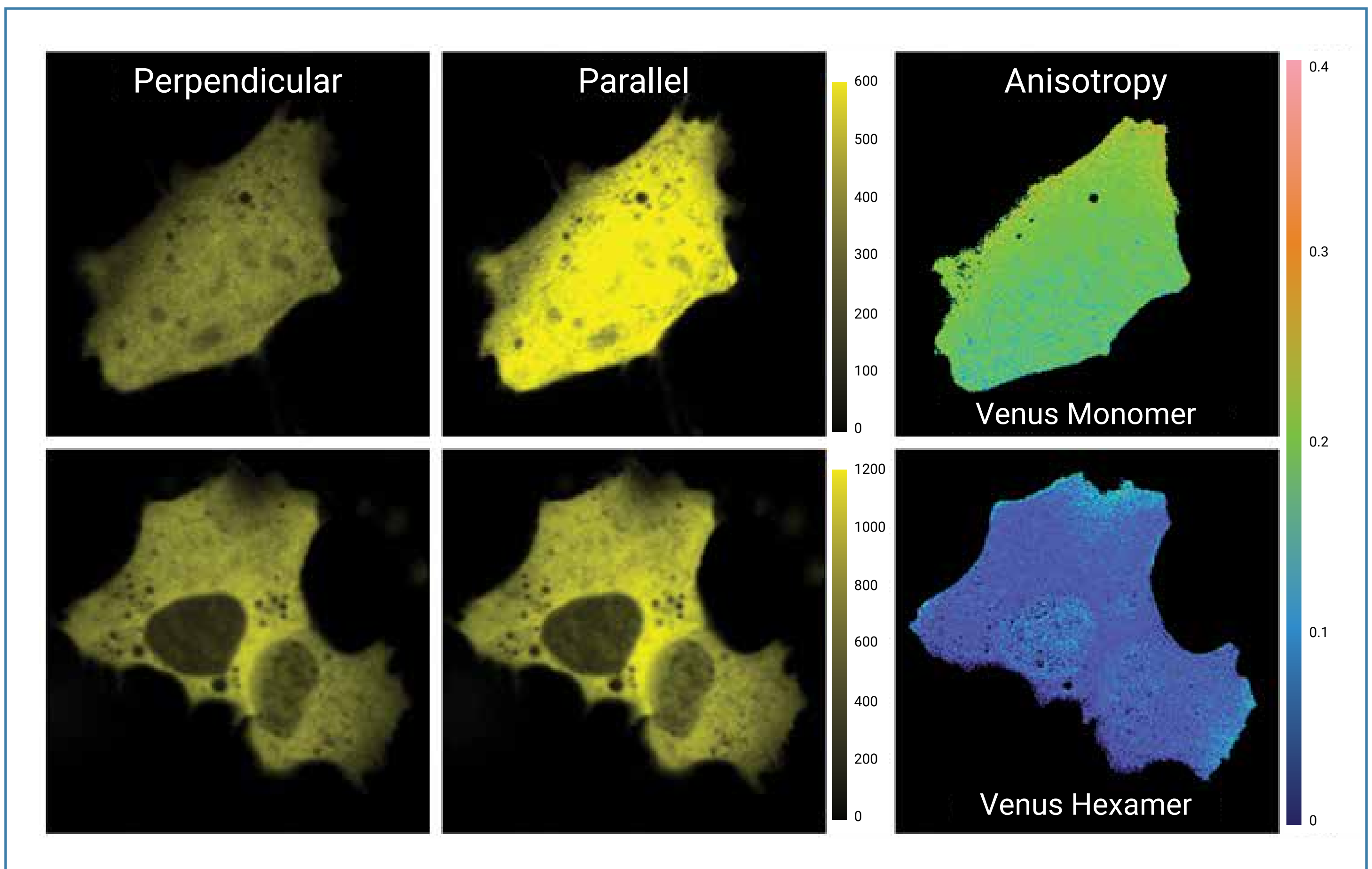


Figure 13: Steady-state polarization anisotropy imaging of live cells expressing Venus Monomer vs. Venus Hexamer. The steady-state anisotropy map is calculated in VistaVision using “(I// - G•⊥)/(I// +2G•I⊥)”, where G is the correction factor compensating different detection efficiencies between the two channels. Due to HomoFRET in the Venus Hexamer, its anisotropy is much lower than that of Venus Monomer.

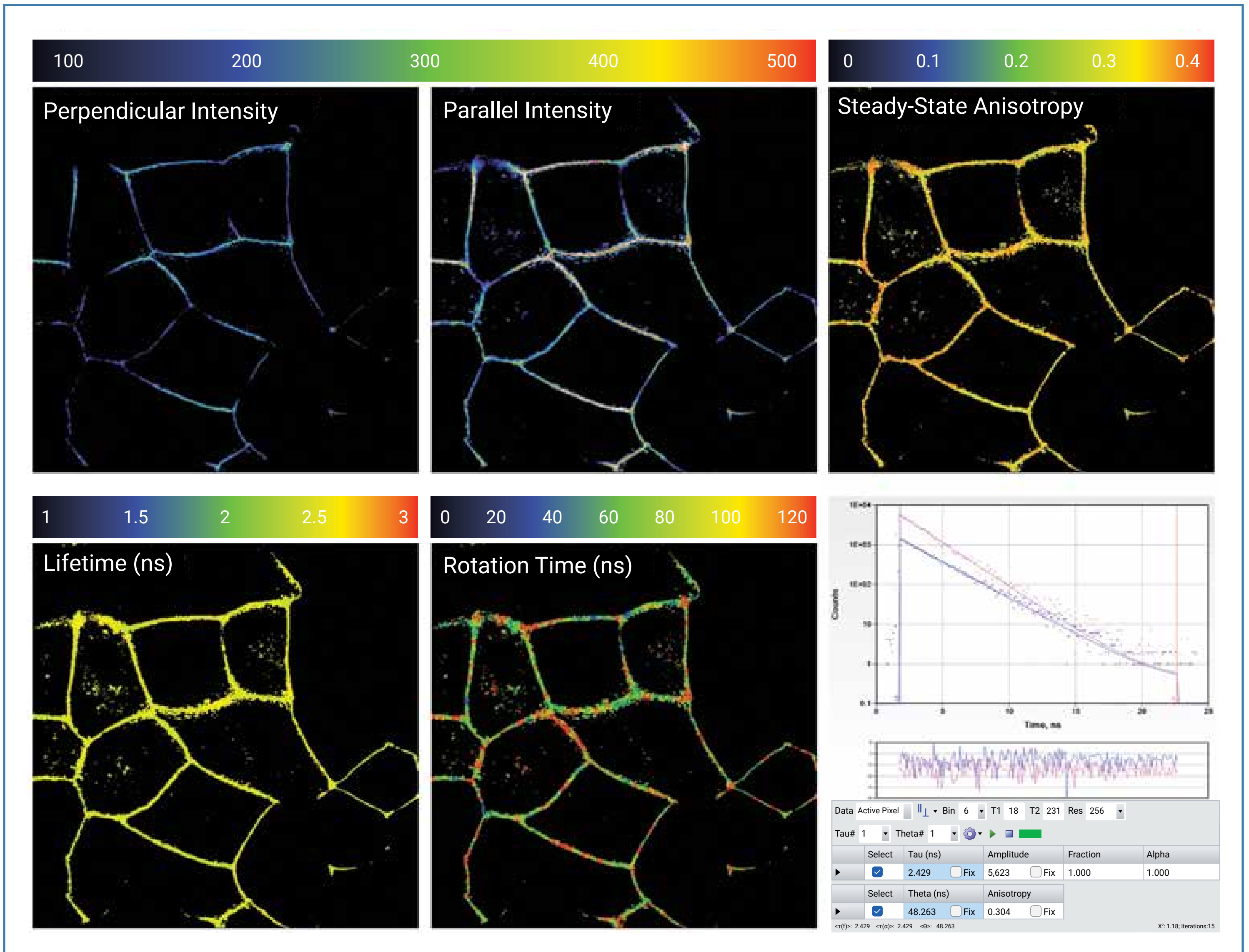


Figure 14: Time-resolved polarization anisotropy imaging of live cells expressing GFP on the membranes. Other than the steady-state anisotropy map, VistaVision can produce lifetime and rotation time maps by fitting both the parallel and the perpendicular decays globally.

3D Particle Tracking

The 3D Particle Tracking method utilizes the galvo mirrors (XY) and the Z-piezo stage (Z) to trace the movement trajectory of a single particle within the 3D space. The Galvo mirrors are driven to move the excitation beam in a circular orbit around a structure of interest such that the radius of the orbit is comparable to the radius of the confocal volume. The intensity of the fluorescence emission along the orbit is analyzed with a Fast Fourier Transform (FFT) based algorithm. The modulation and the phase obtained from the FFT provide feedback to the size and the direction of the particle drift, which is used to update the center of the new circular orbit.

The piezoelectric stage can be used to move the image plane relative to the sample to collect signals from two spatially separated orbits along the axial direction. The difference in the average intensity of the two orbits is used to localize the structure along the axial direction. Generally, the temporal resolution of this technique is around 32 ms with a spatial resolution of 20 nm in each direction.

The EMBO Journal 2019 (38), e100809; doi.org/10.15252/embj.2018100809; iss.com/media/Particle_Tracking_2_Photon_Excitation.pdf.

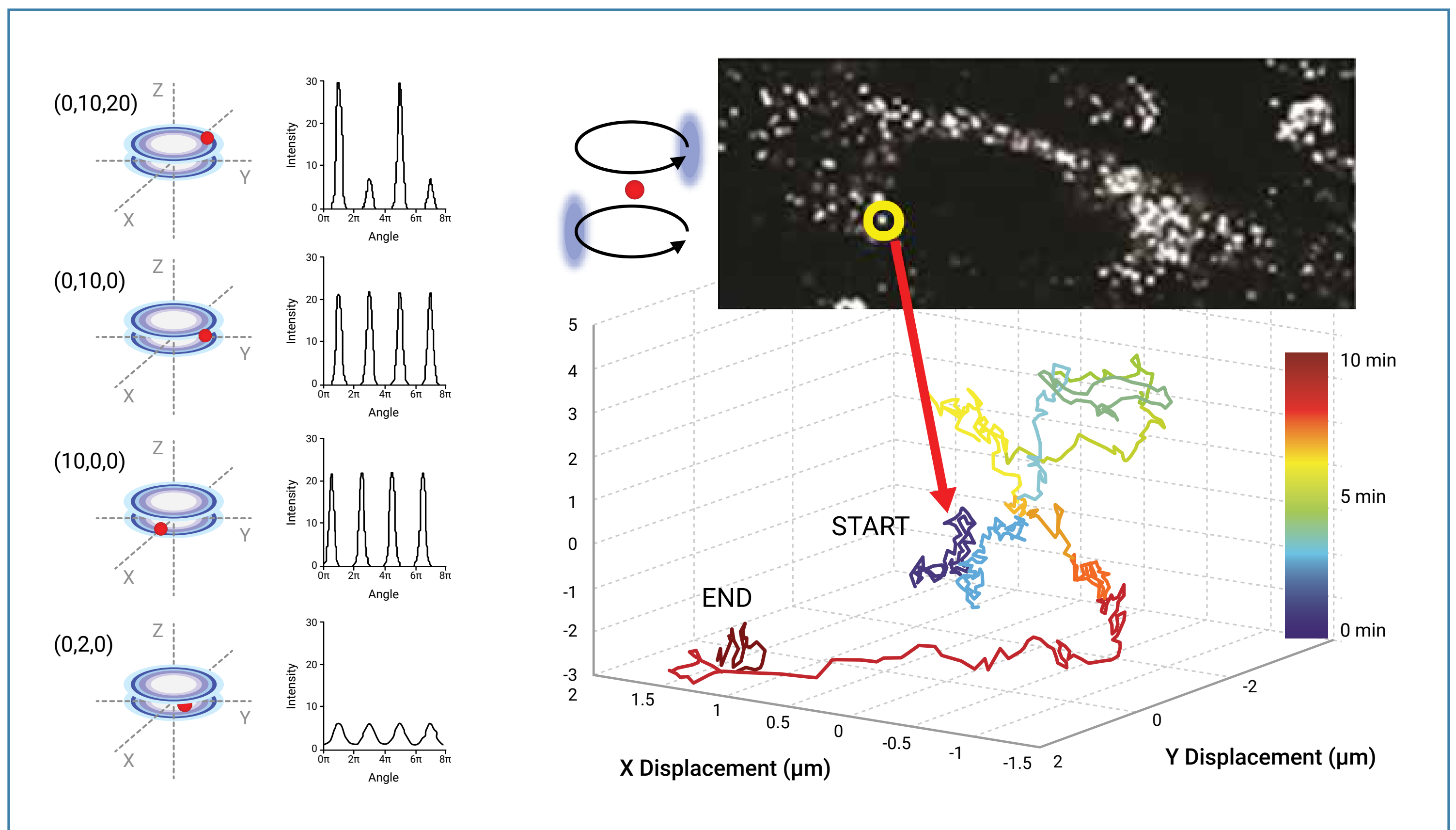


Figure 15: Given their dense core and size in the 200-400 nm range, which is similar to the wavelength of light, insulin granules are highly reflective due to Mie scattering of visible light. Such a robust, imperishable signal is used here to track granule position with high accuracy. Here a single granule is followed in 3D, in a live cell, for several minutes by using the light scattered at 633 nm. Measured trajectories can be used to describe granule mode of motion over the entire spatial scale of the secretory process with millisecond time resolution. (Courtesy of Francesco Cardarelli, Scuola Normale Superiore, Pisa)

RICS and N&B

The imaging counterparts of the FFS measurements include:

- RICS, raster image correlation spectroscopy
- N&B, number and brightness

Both modalities are achievable with the Alba and provide the researcher with an unparalleled amount of information about the dynamic cellular environment. N&B is a fluorescence fluctuation spectroscopy technique based on fast laser scanning microscopy and sensitive photon counting detection, that enables quantification of absolute protein counts and oligomeric state in each image pixel. VistaVision provides the N&B analysis module, which allows users to process and analyze multiple N&B image series in parallel.

It can show three different types of density plots (Apparent Brightness vs. Intensity, Apparent Brightness vs. Apparent Number, True Brightness vs. True Number) to visualize the N&B results. Various denoising filters (Median, Gaussian, Mean, Median + Gaussian) and intensity threshold settings are offered to smooth the density plots. The N&B density plots are connected to the cellular images in two interactive ways: first, a binary mask can be loaded as a ".TIF" file and applied as a segmentation tool to the cellular images, to select the pixels processed and shown on the N&B density plots; second, regions of interest can be defined on the N&B density plots using colored cursors (circle, oval, square, rectangular) of tunable sizes, to select populations of pixels based on their brightness and/or number signatures and highlight them on the cellular images.

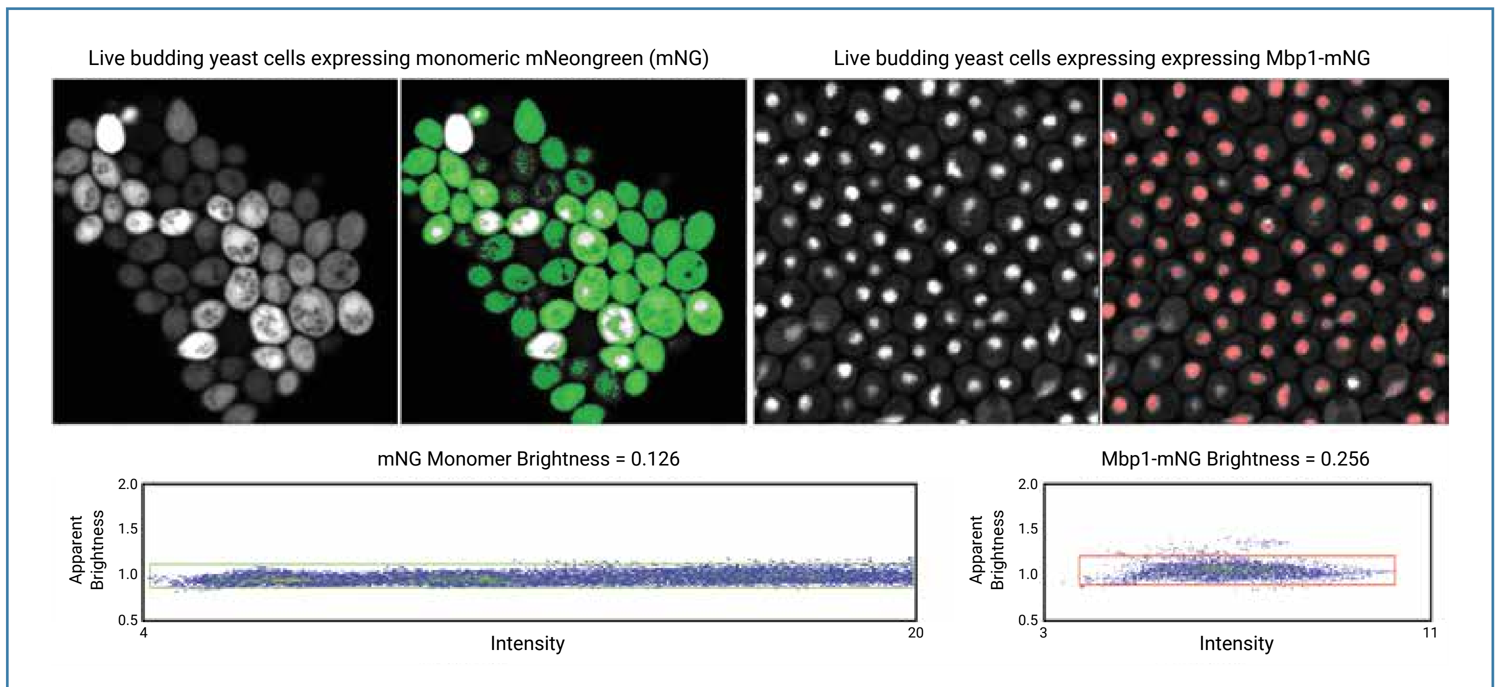


Figure 16: Quantification of the oligomeric states of Mbp1 in live budding yeast cells by N&B. The live cells expressing monomeric mNeonGreen (mNG) were first measured by N&B to characterize the brightness of the monomeric form of mNG – the average is 0.126. In the same imaging conditions, the N&B measurements of the live cells expressing Mbp1-mNG yielded the average brightness of 0.256, suggesting Mbp1 in these live cells being present on average as a dimer (doi.org/10.1117/12.3002810).

Fluorescence Fluctuation Spectroscopy (FFS)

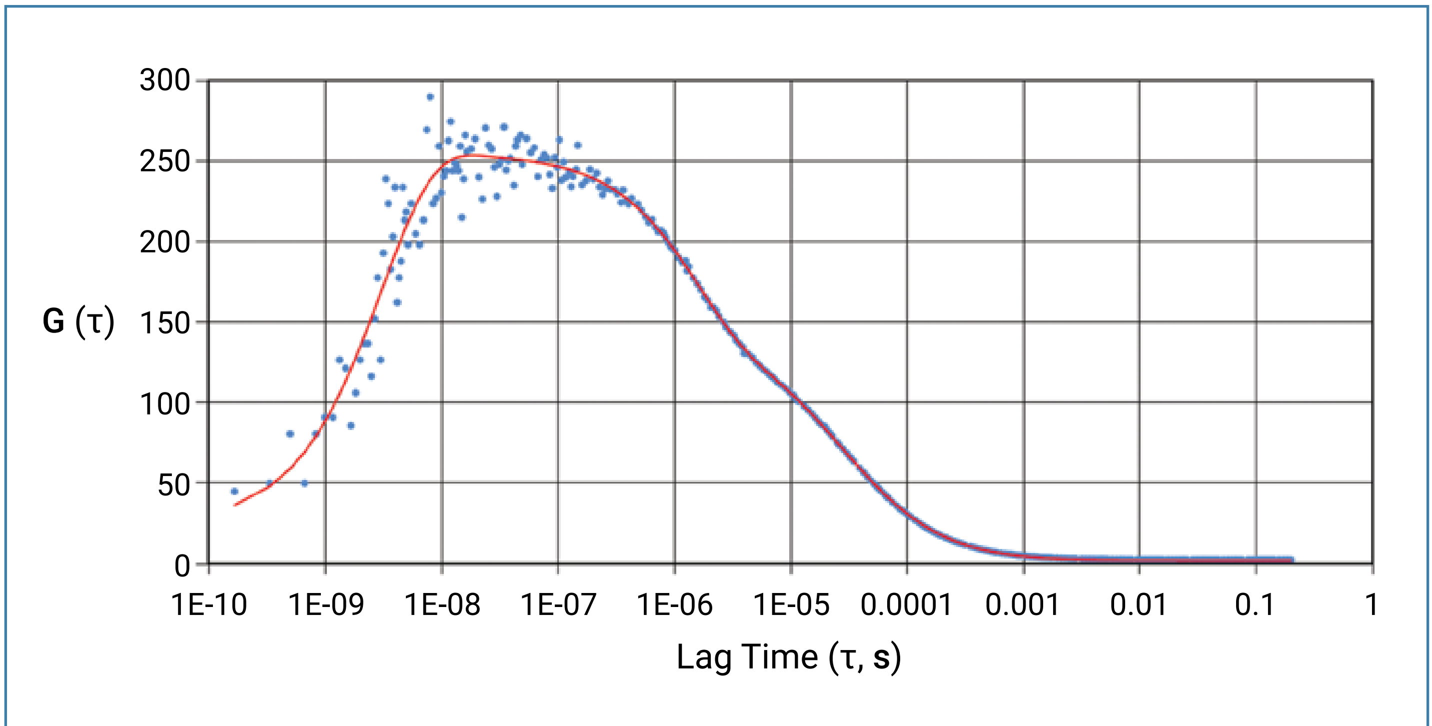
FFS is used to measure translational and rotational diffusion coefficients, kinetic rate constants, molecular aggregation, polydispersity, and molecular weights. Measurements can be acquired in solution or inside living cells. In a cellular environment, the technique allows for the measurements of molecular dynamics parameters in different compartments of a cell (cytoplasm, nucleus, membrane). A variety of applications result from the measurements of molecular dynamics parameters:

- Kinetics Rate Constants
- Antibody-Antigen Interactions
- Receptor-Ligand Interactions
- DNA/Protein Hybridization
- Nucleic Acid/Nucleic Acid Interactions
- Enzymes Activity
- Protein-Protein Interactions
- Molecular Aggregation, Polydispersity & Molecular Weights
- Properties of Viruses

FFS comprises a whole family of methods which can reveal the inner molecular dynamics upon the detection of fluctuations of molecules due to thermal motion. They include:

- FCS, Fluorescence Correlation Spectroscopy
- FCCS, Fluorescence Cross-Correlation Spectroscop with PIE, Pulsed Interleave Excitation
- Scanning FCS
- FLCS, Fluorescence Lifetime Correlation Spectroscopy
- PCH, Photon Counting Histogram

FCS, from sub nanoseconds to sub seconds



$$G(\tau) = \frac{\left(1 - f_A e^{-\tau/\tau_A}\right)\left(1 + f_T e^{-\tau/\tau_T}\right)}{N\left(1 + \frac{\tau}{\tau_D}\right)\sqrt{1 + \frac{\tau}{s^2 \tau_D}}} + b$$

$\tau_D = 25.8\,\mu s\,(diffusion\ time)$
$\tau_A = 4\,ns\,(fluorophore\ lifetime)$
$\tau_T = 1.33\,\mu s\,(triplet\ state\ lifetime)$
$f_T = 0.7619\,(amplitude\ of\ triplet)$
$f_A = 0.81\,(amplitude\ of\ antibunching)$

Figure 17: FCS measurement and fitting of the Rhodamine 110 molecules freely diffused in water in the time range from ~200 ps to ~200 ms reveal several dynamics of the molecules including its fluorescence lifetime, triplet time and diffusion time.

FCS in Live Cells

VistaVision provides routines linking the imaging / FLIM and the FFS data acquisition modules, allowing one-click FFS data acquisition from an image pixel or multiple image pixels selected by the user. These features make it very easy to carry out FCS experiments in live specimens.

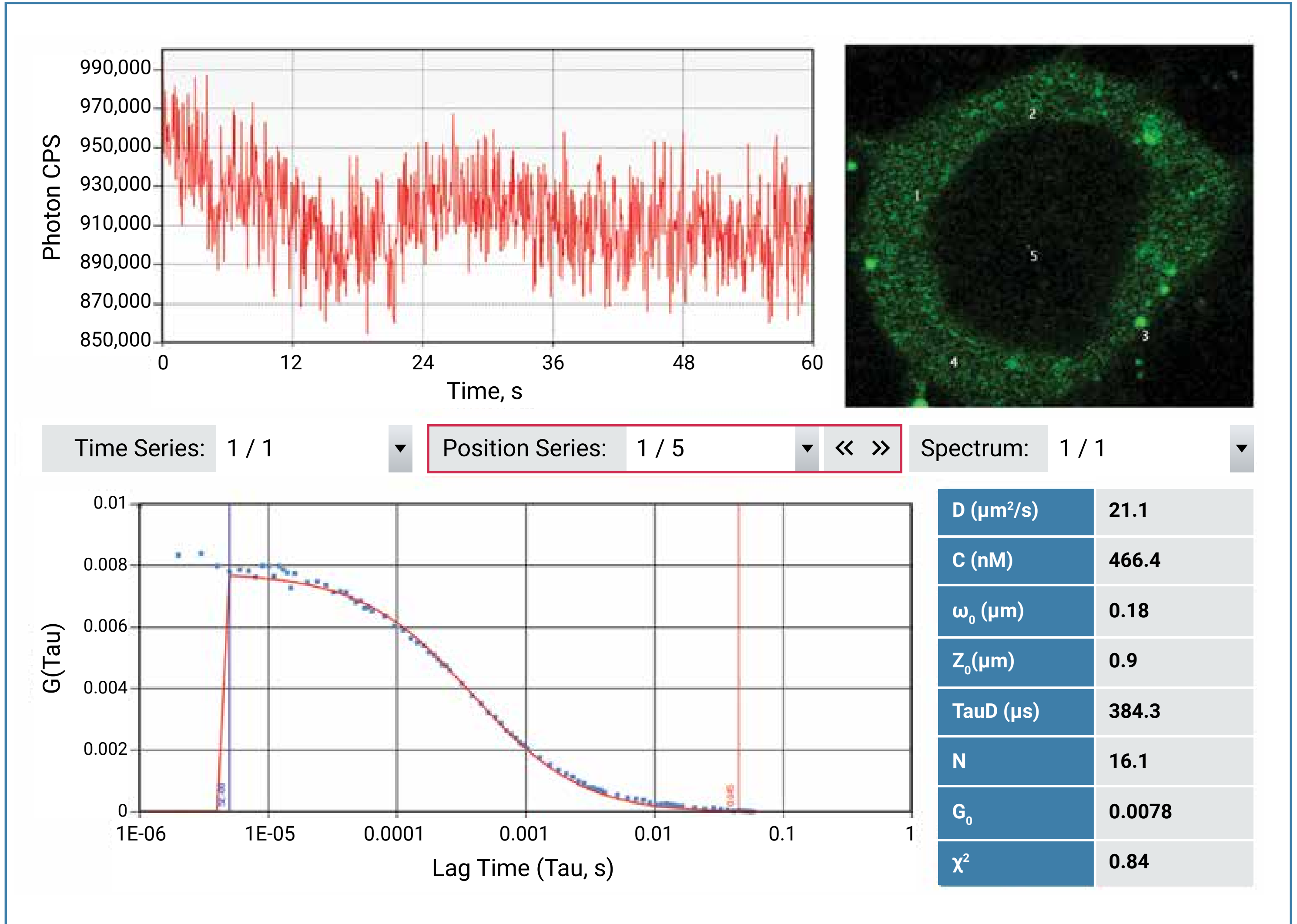
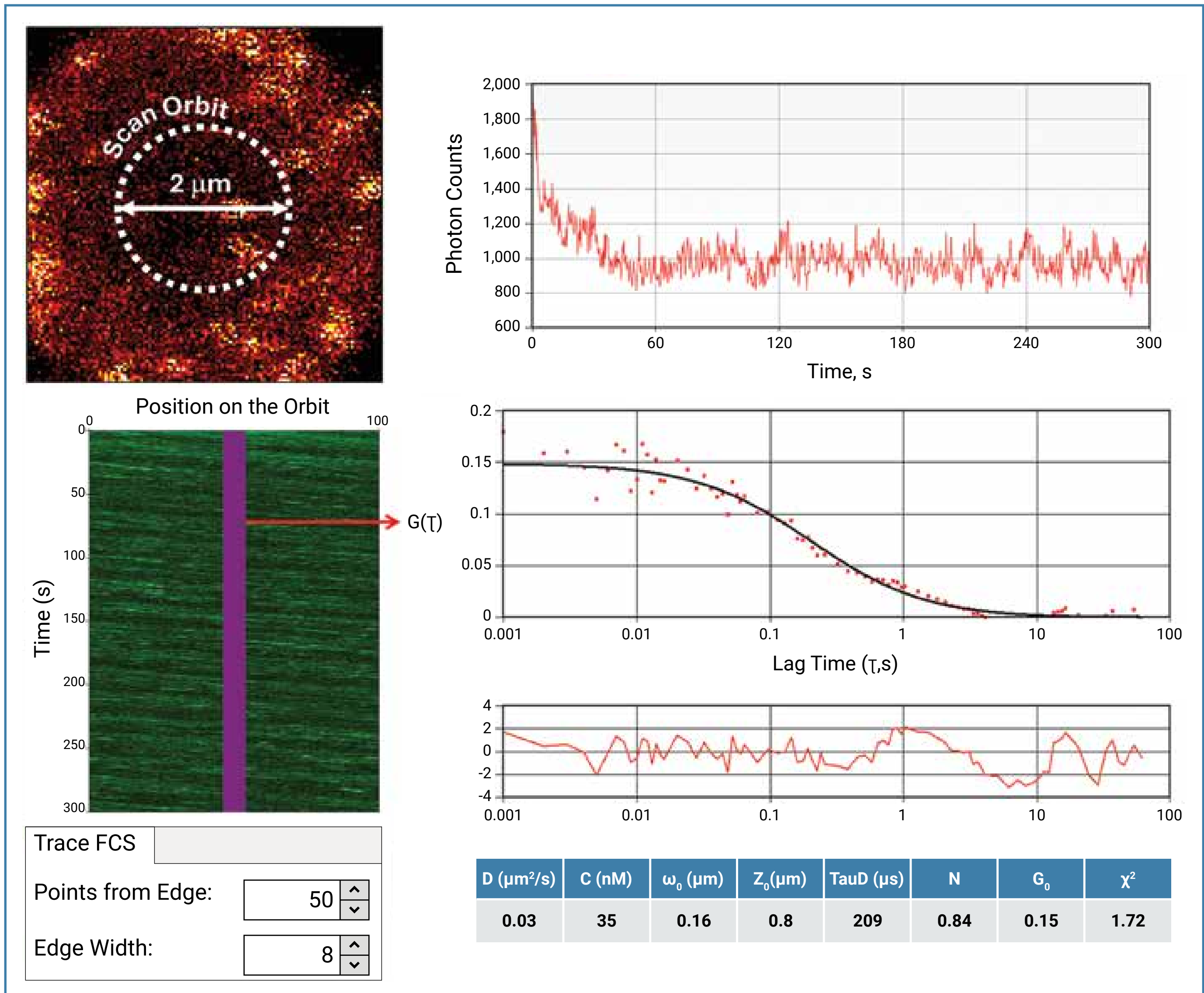


Figure 18: FCS measurements and analysis of live cells expressing GFP. The cellular image was first acquired in the imaging mode; five spots on the image were selected by using a pen cursor; the imaging mode was switched to the FFS mode given a right click by the user, which automatically started collecting the FCS data at each spot sequentially; all five FCS data streams were saved in a position series, along with the image and the XYZ coordinates of the selected spots (shown as the white markers on the cellular image). The protein diffusion rate at each position was obtained by fitting the corresponding diffusion curve in the VistaVision FFS Fitting module.

Scanning FCS and Carpet Analysis

By circulating the laser beam on many points along an orbit, Scanning FCS not only collects the FCS data on these locations simultaneously, but also significantly reduces photobleaching – this is crucial for FCS experiments of live cells. VistaVision provides an intuitive interface for conducting Scanning FCS measurements. More importantly, the Carpet Analysis routine integrated into VistaVision enables users to fully leverage the advantages of the Scanning FCS technique.



D (µm²/s)	C (nM)	ω_0 (µm)	Z_0(µm)	TauD (µs)	N	G_0	χ^2
0.03	35	0.16	0.8	209	0.84	0.15	1.72

Figure 19: Scanning FCS measurement and Carpet Analysis of the protein diffusion in a protein-formed droplet. The FCS data was collected for 300 seconds on 100 points evenly distributed along the orbit of the 2-µm diameter. The Carpet was constructed by plotting the photons at each point (X axis) over time (Y axis). The FCS data collected from 8 points (50 ~ 57, selected from the Carpet) was binned together to calculate the diffusion curve, which was then fitted in the VistaVision FFS Fitting module to estimate the diffusion rate of the Alexa 488 labeled proteins in the protein -formed droplet.

PIE-FCCS

Two-color FCCS is an extension of FCS - it measures the autocorrelation functions of two fluorescent species simultaneously, and in addition their cross-correlation function, which can be evaluated to study molecular interactions. One major issue in two-color FCCS experiments is that the spectral crosstalk between two fluorophores likely causes a false positive cross correlation. By utilizing two Pulsed Interleave Excitation wavelengths and collecting FCCS data in the TTTR mode by FastFLIM or digital TCSPC on Alba, users can apply the gating FCCS process in VistaVision to eliminate the false cross correlation caused by the spectral crosstalk.

The Alba system offers several ways to implement the Pulsed Interleave Excitation scheme for various types of lasers. FastFLIM provides tunable PIE setups and synchronized gating detections, which can be specifically tailored and optimized for applications such as FLIM, FCCS, and smFRET.

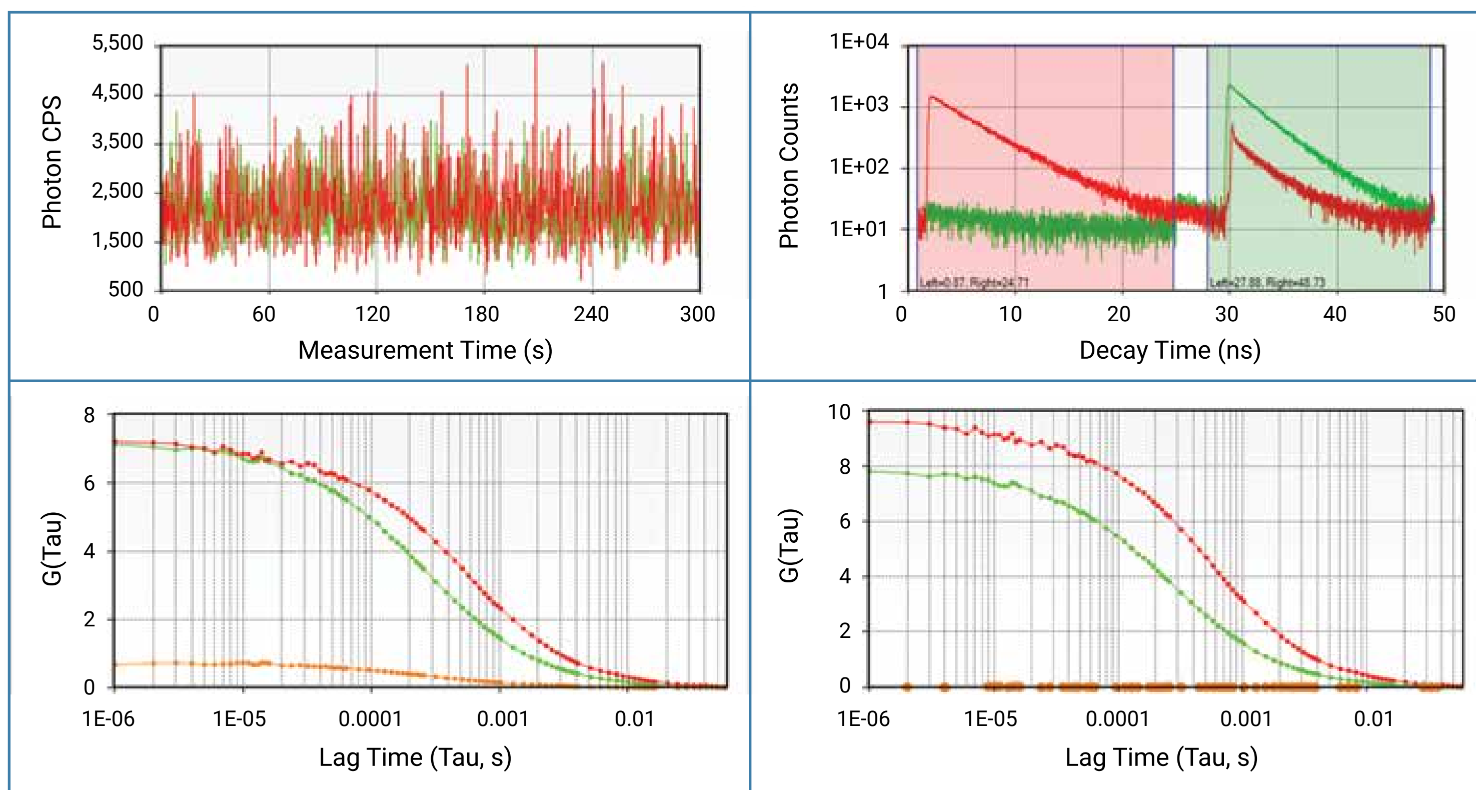
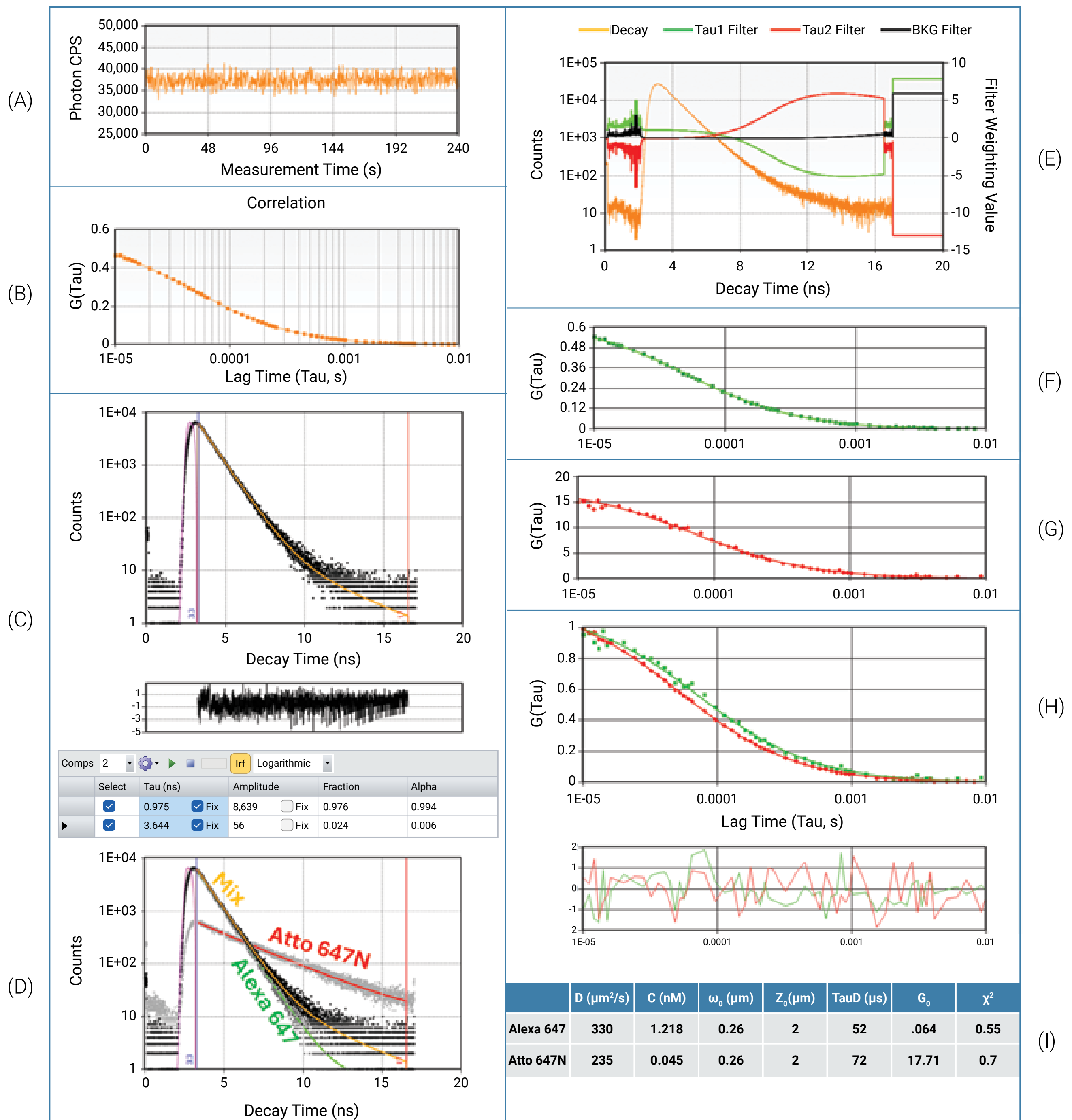


Figure 20: PIE-FCCS measurement and gating-FCCS process of the mixture of Cy3B and Atto 647N dyes freely diffused in water. A noticeable false cross correlation was observed due to the bleedthrough of Cy3B into the Atto647N detection channel. The decays of the two fluorophores were shifted by PIE, allowing separation of the Cy3B bleethrough photons from the Atto 647N photons with the gating process. The false cross correlation was completely removed by the cross correlation of the Cy3B and the Atto 647N photons selected in the two gating windows. *Courtesy of Dr. Priya Banerjee, University at Buffalo.*

Fluorescence Lifetime Correlation Spectroscopy (FLCS)

By collecting FCS data in the TTTR mode by FastFLIM or Digital TCSPC on Alba, users can leverage the powerful FLCS routine provided by VistaVision, to separate two diffusion species based on their different fluorescence lifetimes, generating two separate diffusion curves. They can then be analyzed in the VistaVision FFS Fitting module to determine the diffusion dynamics of each species.

Figure 21: FLCS measurement and analysis of the mixture of Alexa 647 (free) and Atto 647N (attached to a small functional unit) dyes in water. The FCS data stream collected in the TTTR mode (A) generates both the correlation (B) and the decay (C) curves. The fluorescent lifetimes of Alexa 647 (0.975 ns) and Atto 647N (3.644 ns), obtained from the double exponential fitting of the decay curve (D), are used to construct the lifetime filters (E)- Tau1 Filter, Tau2 Filter and BKG Filter are the weighting factors for separating the photons from Alexa 647, Atto 647N and Background, respectively. The separated Alexa 647 and Atto 647N photons are then used to calculate their correlation curves (F – Alexa 647, G – Atto 647N, H - both), which are then fitted to obtain their diffusion rates (I).



Select	Tau (ns)		Amplitude		Fraction	Alpha
☑	0.975	☑ Fix	8,639	☐ Fix	0.976	0.994
☑	3.644	☑ Fix	56	☐ Fix	0.024	0.006

	D (μm²/s)	C (nM)	ω_0 (μm)	Z_0(μm)	TauD (μs)	G_0	χ^2
Alexa 647	330	1.218	0.26	2	52	.064	0.55
Atto 647N	235	0.045	0.26	2	72	17.71	0.7

PCH

The same data collected in FCS measurements can be used to generate the Photon Counting Histogram (PCH) curve within the VistaVision FFS module, which can then be analyzed in the VistaVision FFS Fitting module for comprehensive PCH analysis.

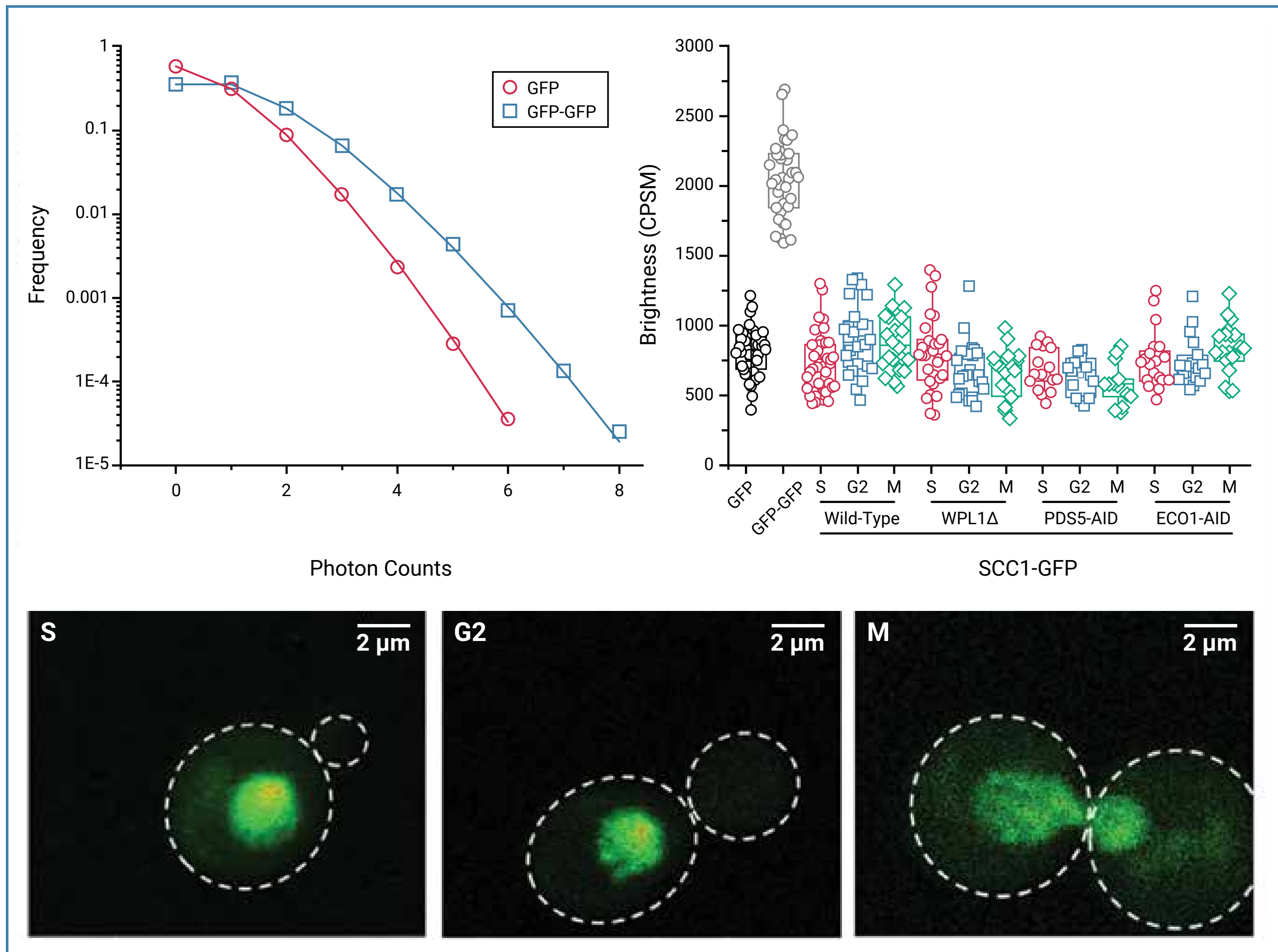


Figure 22: PCH reveals a monomer form of cohesin. The top-left graph shows typical fitted photon counting histograms of GFP monomer and dimer in live yeast cells. The top-right graph reports the molecular brightness of different GFP constructs using the GFP monomer and dimer as the brightness standards. Cells of strain yI0664 (SCC1-GFP) were analyzed throughout the cell cycle by PCH. To examine the effects of Wpl1, Pds5, and Eco1 on cohesin dimerization, the PCH analysis was repeated in strain yEB005 cells (SCC1-GFP WPL1D), strain yEB002 cells (SCC1-GFP PDS5-AID), and yAM946 (SCC1-GFP ECO1-AID) in which Pds5-AID/Eco1-AID was depleted with auxin. The bottom row shows representative images of SCC1-GFP cells at S, G2, and M phases of the cell cycle. Scale bar 2 μm. (EMBO rep 2019 21: e48211, doi.org/10.15252/embr.201948211)

smFRET Bursts Measurement and Analysis

smFRET bursts, acquired over a long period of time, can be analyzed to count and identify single-molecule events, determine individual FRET efficiency and labeling stoichiometry, and calculate the distance between the FRET pair. VistaVision implements a well-established algorithm for smFRET data processing (Nature Methods 2018 (15): 669–676; doi.org/10.1038/s41592-018-0085-0). This includes corrections for background, bleedthrough, efficiency and stoichiometry. VistaVision also incorporates several bursts searching algorithms including the one described in J. Phys. Chem. B 2009, 113, 10965–10973 (doi.org/10.1021/jp903671p).

VistaVision can display histogram plots showing distributions of FRET efficiency / distance and stoichiometry, as well as the 2D plots mapping FRET efficiency (or distances) vs. stoichiometry. VistaVision offers a fitting routine to reveal two FRET states and their transition rates using the Maximum Likelihood Estimator algorithm. It also allows users to define custom equations for fitting the FRET efficiency distribution curve.

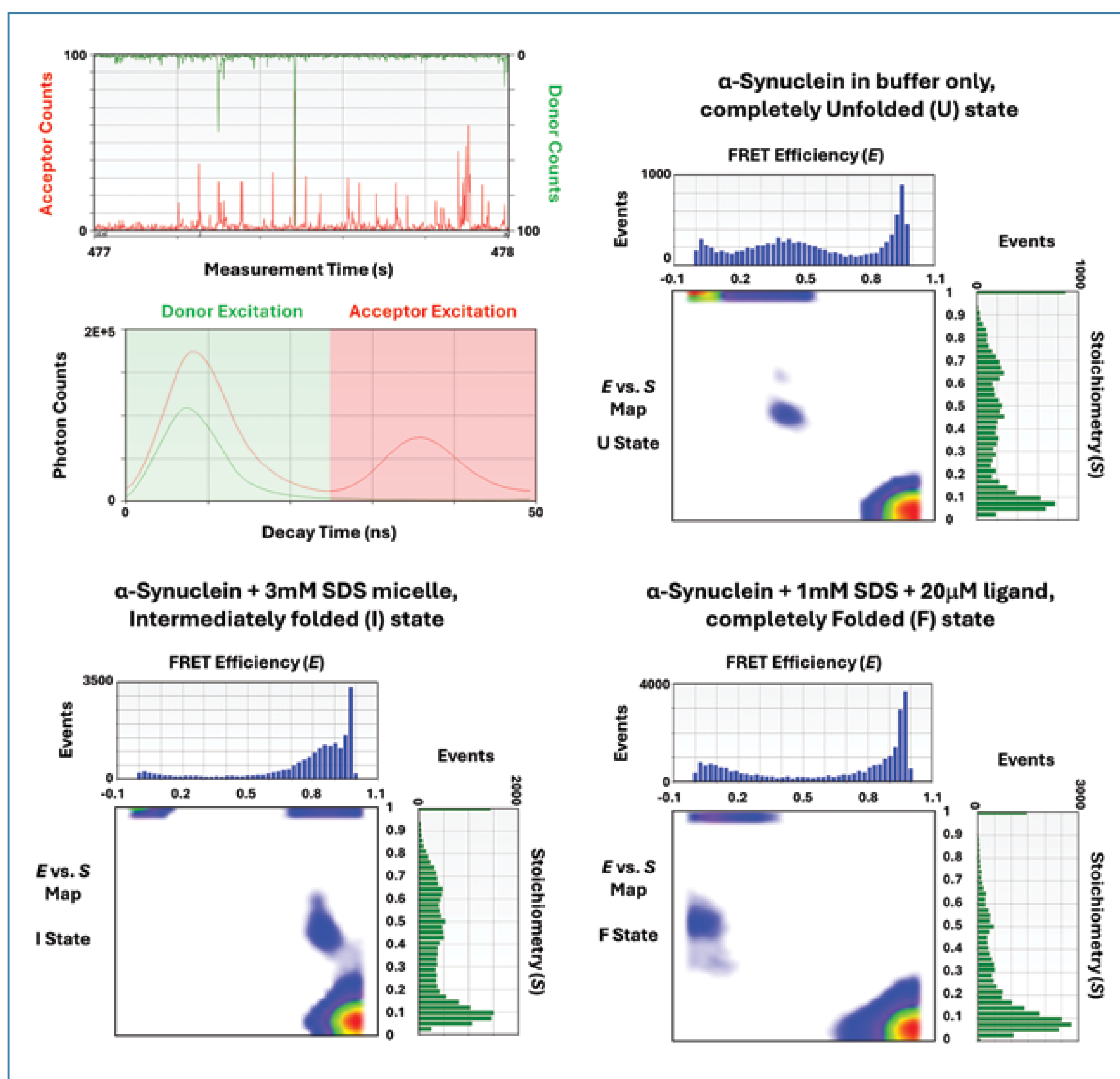


Figure 23: smFRET by PIE-FastFLIM reveals folding and binding dynamics of α-Synuclein, an intrinsically disordered protein (IDP) linked to Parkinson's disease. *Courtesy of Dr. Allan Chris Ferreon, Baylor College of Medicine.*

The fluorescence signal emitted upon excitation by an ensemble of fluorophores is made by a stream of photons emitted at random times. This is not the case for a single emitter where, following the excitation-emission processes, photons are emitted and detected at more regular time intervals, a phenomenon called antibunching. Using the Hanbury Brown and Twiss interferometry (Figure 24), antibunching is measured by splitting the emission signal into two beams and directing each to a separate detector in Alba. The ultrafast correlator used by Alba generates a histogram of the time difference between arrival of the photons at the detectors. The histogram shows a dip at the coincidence point: the depth of the dip indicates the number of independent emitters within the observation volume, while the shape of the dip depends on the excited state lifetime. Both continuous-wave and pulsed lasers can be used for antibunching measurements on Alba. VistaVision provides fitting routines for analyzing 2nd-order correlation antibunching curves.

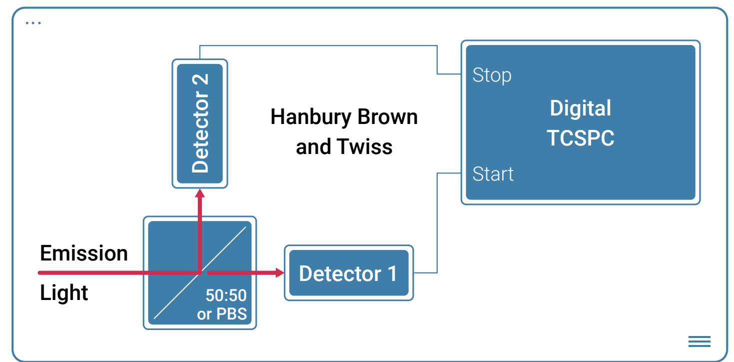


Figure 24: Hanbury Brown and Twiss interferometry scheme.

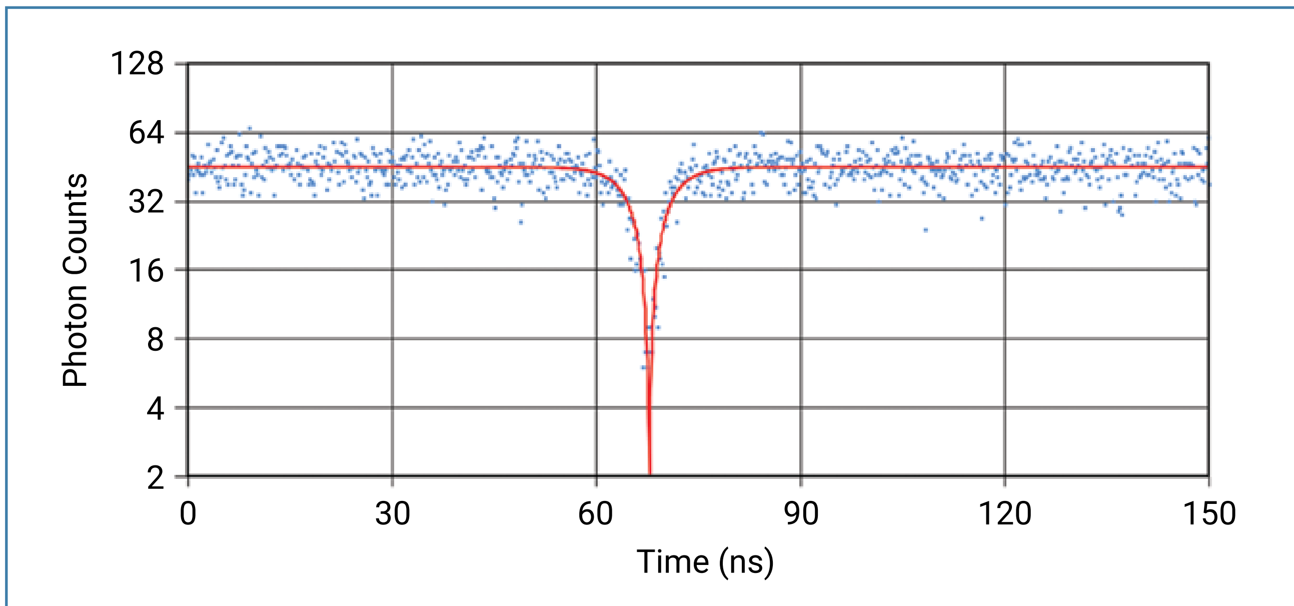


Figure 25: Photon Antibunching measurement and fitting analysis of Rhodamine 110 molecules freely diffused in water (~ 10 pM).

$$G(\tau) = A\left(1 - \frac{e^{-\frac{|t-c|}{\tau}}}{N}\right)$$

τ = xns is the decay time of the fluorophore

$c =$

$N = 1.027$ is the number of molecules in the observation volume

A = multiplicative factor

SPLIT-STED

Stimulated emission depletion (STED) microscopy is a powerful super-resolution microscopy technique which enables the observation of macromolecular complexes and sub-cellular structures, with spatial resolution well below the diffraction limit; however, obtaining high resolution is generally possible only with the use of a high intensity depletion laser. Doing this comes at the cost of increased photo-bleaching and phototoxicity, along with a higher anti-stokes emission background that degrades the signal-to-noise ratio, both of which significantly limits STED applications in living specimens.

To overcome these limitations, ISS has developed an efficient, multi-color STED microscopy method that combines digital frequency domain FLIM (FastFLIM) with phasor plots. This development was done in collaboration with several research groups in the US and Italy (Nanoscale 2019 (11):1754 1761; doi.org/10.1039/C8NR07485B; Nanoscale 2023 (15): 9449-9456; doi.org/10.1039/D3NR00305A).

The SPLIT-STED Approach

Utilizing both pulsed excitation and pulsed depletion lasers, the Abla-STED acquires time-resolved STED images. ISS VistaVision software employs the separation by lifetime tuning (SPLIT) technique, in which phasor analysis is used to efficiently distinguish photons emitted from the center and from the periphery of the effective fluorescent region. This approach improves the resolution without increasing the STED beam intensity.

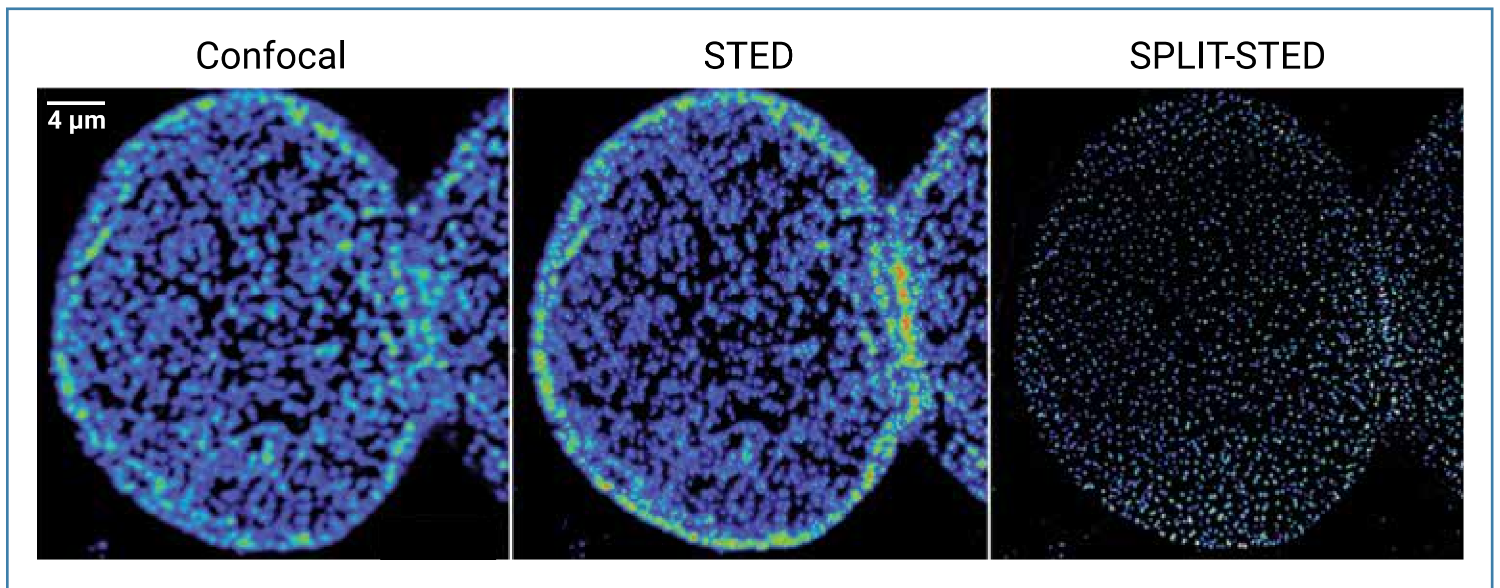


Figure 26: Comparison of confocal, pSTED and pSTED-SPLIT images of nuclear pore complex (NPC) labeled with Star635 dyes in fixed Cos7 cells. *Courtesy of Dr. Paul R. Selvin, University of Illinois Urbana-Champaign.*

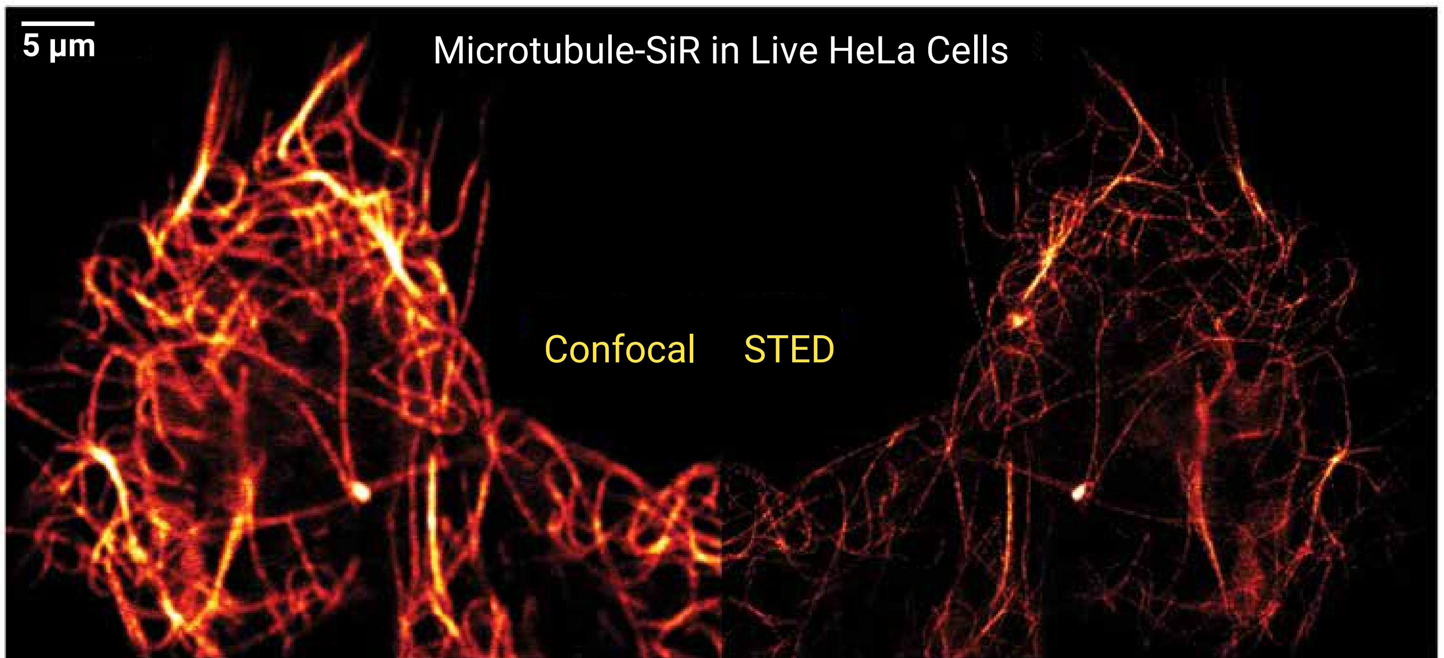


Figure 27: Comparison of confocal and pSTED images of microtubules labeled by SiR dyes in live Hela cells.

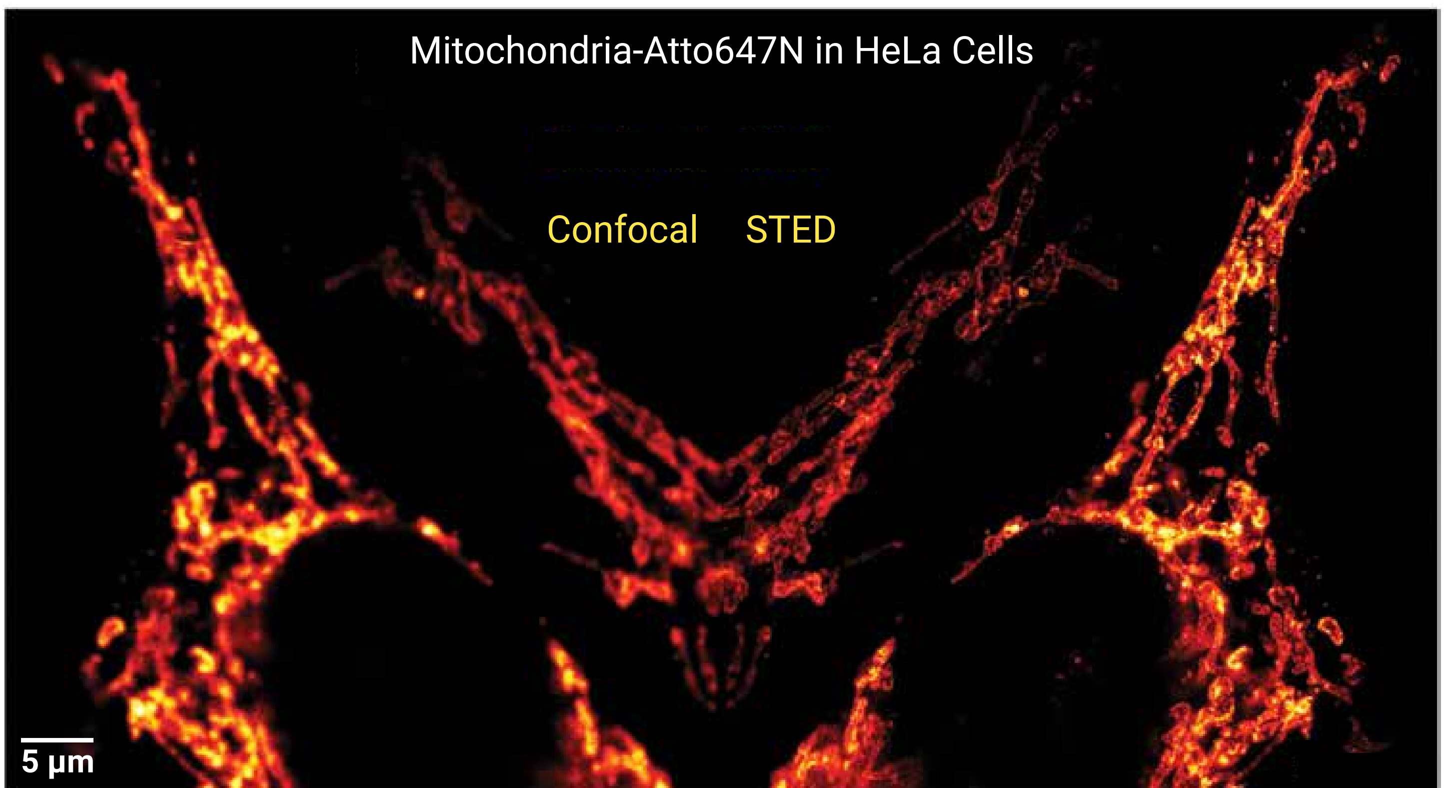


Figure 28: Comparison of confocal and pSTED images of mitochondria labeled by Atto 647N dyes in Hela cells. *Courtesy of Dr. Paul R. Selvin, University of Illinois Urbana-Champaign.*

Technical Specifications

Hardware

Instrument Features

- Individual pinholes on each acquisition channel
- Computer-controlled setting of the pinhole variable aperture
- Computer-controlled positioning of the pinhole in the imaging plane
- Single-photon or multi-photon excitation
- Up to 4 channels data acquisition
- Auxiliary port for camera

Image Parameters Acquired by Alba:

- Pixels numbers: user selectable from 20 to 4096
- Max line frequency: 4 KHz (on 20 points)
- XY and XZ sections

Optical Unit

- Light Sources:
  - Single photon lasers housed in a laser launcher w/ control of each laser intensity and shutter; or,
  - Multi-photon excitation with laser intensity control and shutter
- Optics:
  - Microscopes: Inverted and upright microscopes (Evident (Olympus), Nikon, Zeiss)
  - Objectives:
    - Air objectives with 20X, 40X, 60X magnification and 1.5-8.1 working distances
    - Oil immersion objectives, 1.4 NA and 60X (standard); other apertures available
    - Water immersion objectives,1.2 NA 60X (standard), with coverslip correction (for 0.15-0.18 coverslip); other apertures available
  - Dichroic Filters:
    - 25mm-diameter
    - For single-photon excitation: 1-, 2-, 3-band filters
    - For multi-photon excitation
  - Polarizer:
    - Cube beam splitter, wavelength range: 450-1100; extinction ratio: 10,000:1 at ±3 degrees
  - Variable Pinhole:
    - Motorized and tunable from 20 µm to 1 mm
  - Internal Magnification: 2
- Galvo-Mirrors:
  - 2 silver-coated galvanometer scanning mirrors
  - Clear optical surface: 3 mm
  - Maximum scan rate: 5 KHz for 3 mm
  - Scanning resolution: 64x64 to 4096x4096 pixels
  - Scanning mode: Pt, Xt, XZ, XY, XZt, XYt, XYZ
  - ROI scanning: rectangle, ellipse, polygon, line
- Detectors:
  - Cooled Fast photomultiplier tubes (PMTs)
  - Cooled Hybrid PMTs
  - SPADS

Data Acquisition

- FastFLIM (Digital Frequency Domain FLIM)
- SWISS TCSPC card (Time Domain FLIM)

- Computer
  - High-performance Processor, 64GB RAM, Windows 10/11, 64-bit
  - 32” monitor, 3440 x 1440 resolution

- Power Requirements
  - Universal power input: 110-240 V, 50/60 Hz, 400 VAC

- Dimensions
  - 538 mm (L) x 563 mm (W) x 205 mm (H)

- **Weight:** 40Kg

Software

- VistaVision
- Operating System
  - Windows10/11, 64-bit
- Image format
  - Export to ImageJ
  - Plots export to png, gif, jpeg, bitmap formats

Measurement Modules:

FFS Module

- Fluorescence Correlation Spectroscopy (Single Channel & Cross-Correlation)
- Photon Counting Histogram (PCH) (ISS Patent)
- Fluorescence Lifetime Correlation Spectroscopy (FLCS)

Confocal Imaging Module

- Confocal Images
- Fluorescence Lifetime Images (FLIM/PLIM)

Polarization Module: Polarization Measurements

Measurement Requiring Imaging & FFS Modules

- Scanning FCS
- Raster Imaging Correlation Spectroscopy (RICS)

Fluorescence Fluctuations Spectroscopy (FFS) Module:

Parameters Determined by the FFS Software Module

- When using autocorrelation & cross-correlation functions:
  - One or Two Species Using:
    - Diffusion coefficient
    - Diffusion time
    - Concentration
    - Triplet state decay time constant
    - Triplet function
    - Flow rate
    - Size of excitation volume
    - Number of molecules
- When using photon counting histogram (PCH):
  - One or Two Species Using:
    - Number of Molecules
    - Molecular Brightness

Data Acquisition Modes

- Time Mode
- Photon Mode

Number of Channels Acquired Simultaneously: Up to 4

Modeling of Laser Beam PSF

- Single Photon
- Milti-Photon

Statistical Functions Utilized for Data Analysis

- Autocorrelation Function (FCS)
- Cross-Correlation
- Photon Counting Histogram (PCH)

Single Set & Global Fitting Models Available in the FCS Software

- When using autocorrelation & cross-correlation functions
  - One or two species, w/ 1- or 2-photon excitation using:
    - 2D- or 3D Gaussian PSF
    - 2D- or 3D-Gaussian PSF Triple State
    - 3D-Gaussian-Lorentzian PSF
    - Presence or Flow
- Input or user-defined equation
- When using photon counting photon counting histogram (PCH):
  - One or two species, w/ 1- or 2-photon excitation, using:
    - 2D- or 3D-Uniform
    - 2D- or 3D-Uniform
    - 3D-Gaussian-Lorentzian PSF
- Input of user-defined equation

Minimization Routine

- Marquardt-Levenberg Algorithm
- Report of Fitted Curves

Scanning FCS: User Defined Area

Raster Image Correlation Spectroscopy: Up 30 3 KHz

Confocal Imaging Module:

FLIM Modality

- Frequency Domain
- Time Domain

FLIM Time-Resolution: 100 ps - 100 ms

Raster Scan

- Resolution: up to 1.5 nm
- Pixels number: user selectable from 2 to 8192
- Max line frequency: 4 KHz (on 20 points)
- Min line frequency: 0.01 Hz
- Max frame rate 512x512: 3 sec
- Max frame rate 512x16: 25 Hz
- Beam park
- Panning
- Field rotation: 200o optical
- Field diameter: 18 mm

Scan Modes

- Kinetic Studies:
  - t, Xt, XYt, XZ, XYZ and XZt
- Optical Sectioning:
  - XZ, XYZ of specimens

Image Formats

- Export to: ImageJ
- Plots can be saved and exported to:
  - GIF, TIFF, JPEG, PNG, Bitmap & Metafile formats

2D Visualization & Operations

- Rotation
- Histogram based colocalization
- Zooming
- Scaling
- Arithmetic
- Smoothing

Input / Output

- 2 Channels Input
- 8 Channels Output

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Elliot Scientific Limited
Unit 11 Sandridge Park, Porters Wood,
St Albans, AL3 6PH, United Kingdom
Tel: +44 (0)1582 766300
Fax: +44 (0)1582 766340
www.elliotscientific.com
sales@elliotscientific.com