FastFLIM™

For Olympus Laser Scanning
Microscope Systems
(FV1000, FV1200, FV3000, FVMPE-RS)

Add functionality to the Confocal Microscopes by Olympus with the Fluorescence Lifetime Imaging (FLIM) and Fluorescence Fluctuations Spectroscopy (FFS) Upgrades from ISS.

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Why FLIM?

Laser scanning confocal microscopy provides a wealth of information in biological sciences (cell biology, genetics, physiology, neurobiology & developmental biology), tissue engineering, quantum optics and material sciences. Most of the instrumentation uses the emission properties of the fluorophores targeted for the measurement. Yet, the intensity depends heavily upon the concentration of the fluorophores; moreover, if two distinct fluorophores feature a similar emission spectrum, they cannot be resolved and separated. The measurement of fluorescence lifetime addresses these limitations.

What is fluorescence lifetime?
Using the quantum mechanics description of the energy levels of a molecule, a number N of fluorophores, upon absorption of photons, undergoes a transition from the ground level to the excited levels. From there, the fluorophores decay to the lowest of the excited levels; hence, they decay back to the ground level with emission of photons, or fluorescence. The decay is a random process occurring at different times for different molecules. If the process is described by a single exponential decay, the lifetime is the time for the population in the lowest excited level to decrease by about 63% of its original value. Decay times range typically from 50 picoseconds to hundreds of nanoseconds (although there are processes shorter and longer).

The measurement of the decay time offers new potentialities for the understanding and quantification of molecular dynamics. In fact, the lifetime of a fluorophore depends on the environment (pH, presence of ions, temperature, electrical fields, viscosity) and it can be affected by the presence of another fluorophore (a principle used for Förster Resonance Energy Transfer, or FRET). This measurement can be used to determine and quantify these interactions. Also, as the decay time is peculiar to each fluorophore, it can be used to identify the presence of two fluorophores even if they have overlapping emission spectra. Within some wide range, the lifetime values are independent of the fluorophore concentration, a property than can be used more often than ever.

Fluorescence Lifetime Imaging (FLIM) is a technique that allows for the acquisition, at each pixel of the image, of the decay times of the fluorophores present in the pixel.

The Vistavision software analyzes the FLIM images and calculates the lifetime image, where at each pixel a color mask representing the values of the decay times is displayed.

Why FastFLIM?

While the TCSPC has been the technique of choice for several years, it suffers from low duty-cycle (the maximum counts rate should be about 1% of the repetition rate of the laser to avoid pile-up effects), low dynamic range (the signal is limited to about 1 million counts/second) and dead time of the electronics (once a photon reaches the detector, another photon cannot be measured for another 150 ns).

FastFLIM is the new Digital Frequency Domain (DFD) approach to FLIM measurements that overcomes the limitations of TCSPC. With negligible dead time, photons can be counted up to 35 million/second on each of the 4 independent channels, greatly decreasing the acquisition time. The light source is a laser modulated with nanosecond pulsewidth pulses at a repetition rate that is selected by the operator. Alternatively, pulsed lasers can be used too (pulsed laser diodes, supercontinuum laser, multiphoton laser). Decay times from the picosecond range to the second can be measured using the same unit. The operator simply selects the time range of the measurement.

Upon excitation, the fluorescence photons are emitted randomly; the DFD provides a histogram of the phase delay of the individual photons reaching the detector with respect to the phase of the excitation pulse. From the histogram, the characteristic decay times of the system are determined.

FastFLIM provides data directly compatible with the phasor analysis approach; no mathematical transformation is required. Phasor plots are a graphical representation derived from the Fourier transform of the fluorescence decay; unlike the traditional analysis approach in which the pixels in a region of interest are fitted to a “pre-determined” model, the phasor plots display all of the pixels of the image at coordinates separated by their decay times without making any a-priori assumption about the decay.

The presence of multiple decay times is displayed on the same plot, a feature that has allowed the separation of up to seven decay times in tissues. Furthermore, since phasor analysis involves the direct representation of raw lifetime data, lifetime data can be obtained with far fewer photons than would be required for reliable multi-exponential fits.

VistaVision includes graphical tools for the analysis of the decay rates using the phasors, for the superposition of several decay rates and the determination of the FRET efficiency in a direct way.

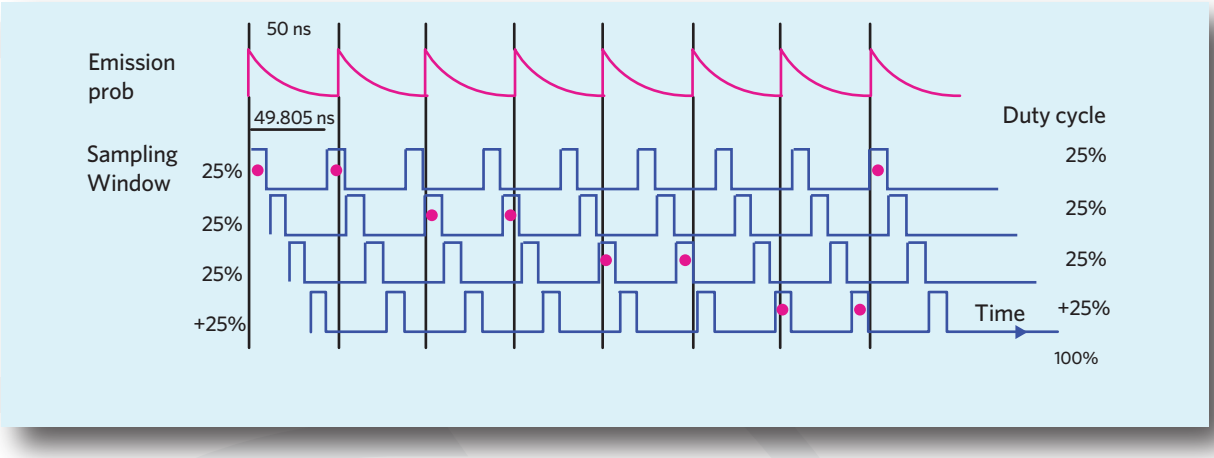


Figure 1. Graphical illustration of the Digital Frequency Domain principle for a light source modulated at 20 MHz. The cross-correlation frequency is 1/8 the sampling frequency (in practice it is 1/256). The fluorescence photons (red dots) are recorded into any of the four sampling windows according to their arrival times. The sampling windows slide through the entire period of the emission response in 256 steps because of the slight difference in the frequency.

Direct calculation of FRET efficiency in cells using the phasors

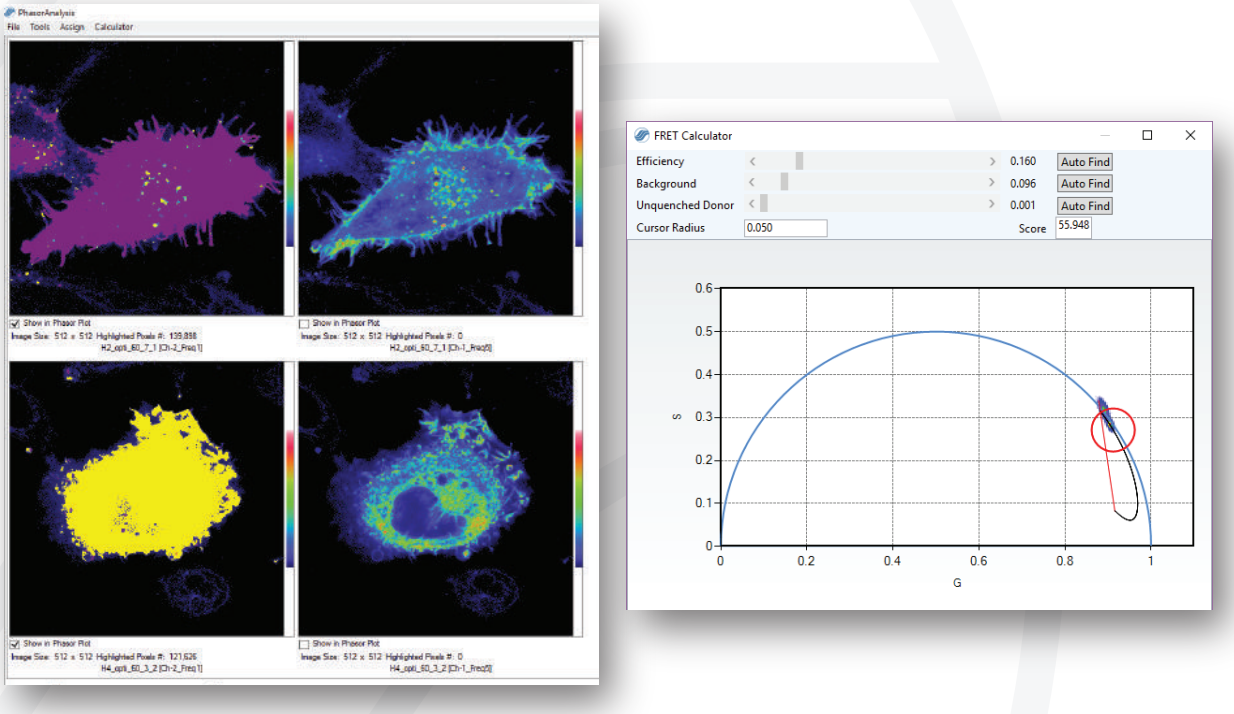


Figure 2. Cells were expressed with EGFP only (left top) and EGFP-mCherry pair (left bottom). The purple area (top left) represents the pixel selected by the purple circle (cursor) on the phasor plot (right). The yellow area (bottom left) represents the pixel selected by the yellow circle (cursor) on the phasor plot (right). In order to calculate the FRET efficiency, the decay times of EGFP only cells (2.8 ns) and of EGFP-mCherry cells (2.4 ns) are measured. A more convenient approach is offered by the FRET calculator using the phasors (right): pixels are assigned to the background and to EGFP only locations; the trajectory in the phasor plot outlines the locations of the pixels undergoing FRET. In the specific case, the efficiency of 16% is determined. Data acquired using ISS Q2 system coupled to an Olympus microscope ; excitation is 488 nm and emission is collected through 500nm-550nm filter. *(Courtesy of Dr. Hsiao Jye-Chian, Institute of Chemistry, Academia Sinica, Taiwan).*

Metabolic State of Kidney Physiology by monitoring NADH and FAD

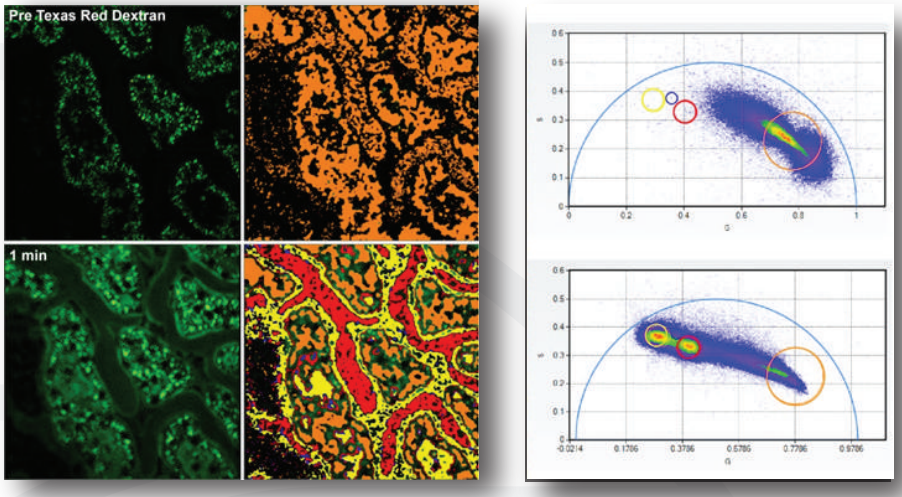


Figure 3 – Fluorescence images (left) and FLIM images (middle) for a region of the living rat kidney prior to (top) and 1 minute after injection (bottom) of a small, freely-filtered Texas Red dextran. FLIM image colors derived from regions of interest on phasor plots (right). Top FLIM images and phasor reflect tissue autofluorescence, with lifetimes along the FAD-NADH axis (orange). Bottom FLIM images and phasor plots displays lifetime data for tissue autofluorescence (orange) as well as two populations of Texas Red dextran, a short-lifetime form in the microvasculature (3.3 ns, red) and a long-lifetime form in the interstitial fluid and tubular lumen (3.9 ns, yellow). Measurements acquired on a multiphoton FV1000 system. *(Courtesy of Dr. Kenneth Dunn and Dr. Bruce Molitoris; Indiana Center for Biological Medicine, Indianapolis, IN).*

Using a sensor to monitor oxygen consumption in breast cancer cells spheroids FastFLIM measures decay times of fluorescence ranging from the sub-nanosecond to the second.

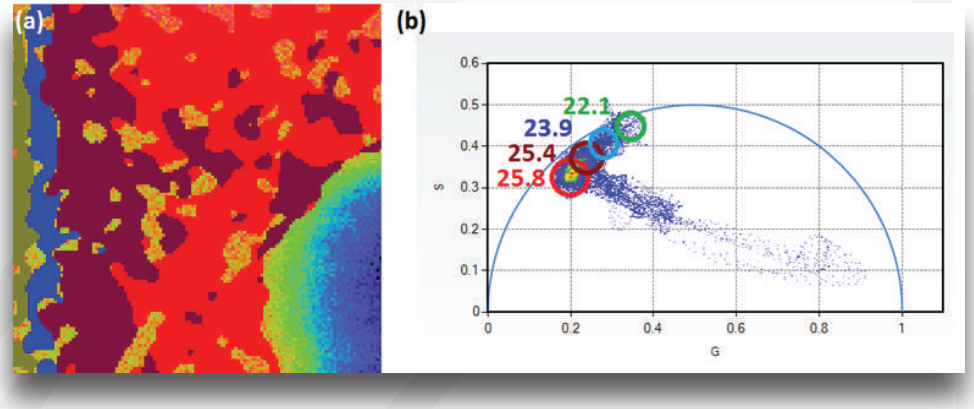


Figure 4 –Lifetime map of oxygen consumption in breast cancer cell spheroids (MDA-MB-231 cell line) embedded in a fibrin gel. Oxyphor G4 dye, introduced in the media lines, is used to monitor the local oxygen concentration by measuring the decay times, which are sensitive to the local concentration of oxygen. Measurements were acquired on an Olympus FV1200 equipped with a 60X water objective; excitation was at 635 nm using a modulated laser diode (by ISS) and emission was collected thorugh a 80nm-bandpass filter centered at 809 nm by the NIR detectors (ISS). (Left) lifetime image, 159x159 µm, taken 7 days after the initial load of the dye cancer cell spheroid. The different color masks outline regions of different values of the decay times that translate in different oxygen concentrations. The spheroid is located on the bottom right side (blue color) surrounded by a narrow ring (green); the remaining of the picture displays the dye in the fibrin gel support. (Right) phasor plot of the lifetime image. The decay times are binoexponential as the pixels do not lie on the semicircle and the plot number report the average lifetimes. The colors of the cursors correspond to the colors in the lifetime image, from left to right, 22.1 µs (green area), 23.9 µs (blue area), 25.4 µs (magenta area) and 25.8 µs (red area); the decay times become longer and reflect the reduction in the oxygen concentration in the areas closer to the cancer cell spheroid (blue area in the lower right side of the image). (*Courtesy of Dr. Steven C. George, Washington University in St. Louis, St. Louis, MO).*

Why FFS?

Fluorescence Fluctuations Spectroscopy (FFS) encompasses a group of measurements for the study of molecular dynamics at the single-molecule sensitivity, based upon the local perturbation induced by the thermodynamic status of the system. The Fluorescence Correlation Spectroscopy (FCS), that describes molecule subjected to the Brownian motion, is but one of these measurements; it studies the correlation in time of the detected signal . Photon Counting Histogram (PCH) retrieves the information contained in the distribution of the amplitudes of the signals. The FFS measurements are acquired at a single location by directing the laser beam of the microscope to the XYZ position of interest or, they are acquired in an area of interest scanned by the laser beam. Single point measurements include the following information:

- Number of fluorescent molecules in the volume of observation
- Brightness of the molecules
- Diffusion coefficients (or diffusion times)
- Binding constants
- Conformational dynamics
- Rotational Motion information
- Protein Folding

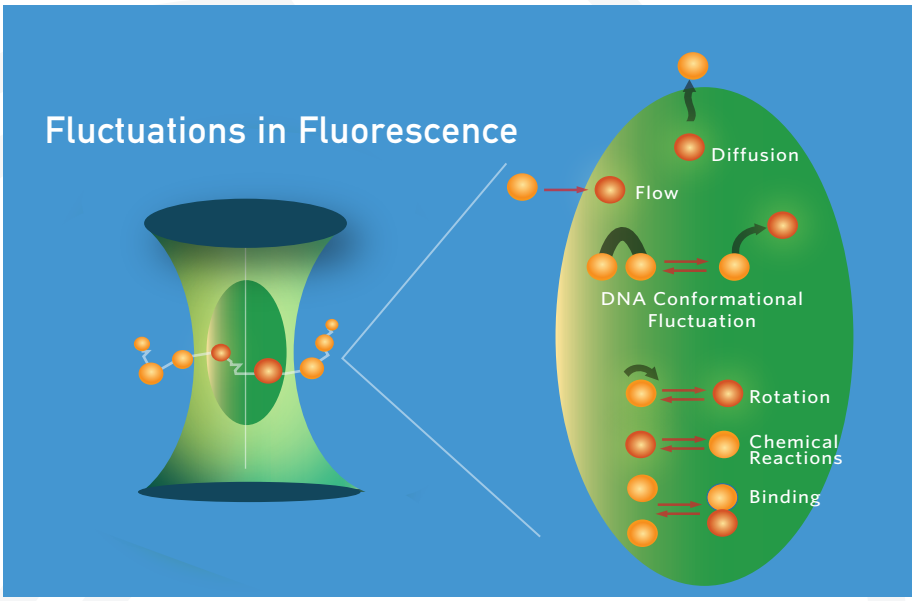
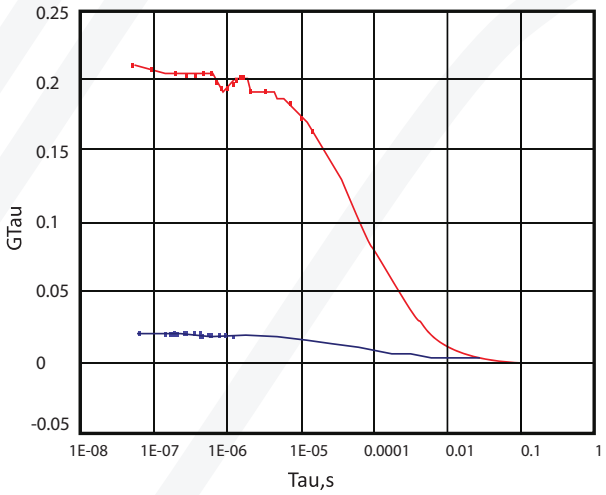
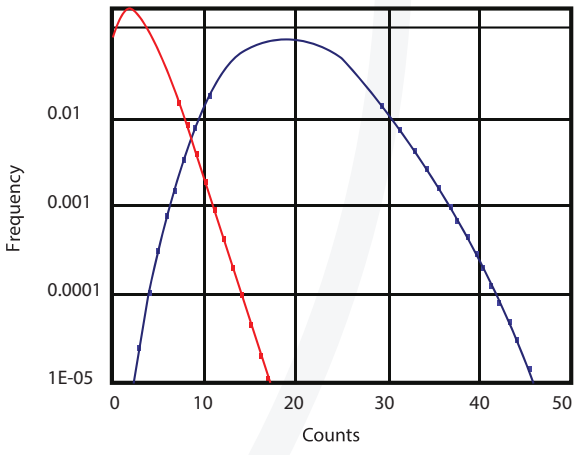


Figure 5. As a particle enters into the observation volume, a burst of photons is emitted and collected. The temporal correlation provides the FCS curve (left); the distribution of the amplitudes provides the PCH (right). Measurements were acquired on two solutions of Rhodamine 110 at 3 nM (red curves) and 24 nM (blue curves) using an Olympus IX73 microscope equipped with a water 60X objective; excitation was from a laser emitting at 473 nm and emission was acquired through a high pass filter with 50% transmission at 530 nm.





Particle Tracking & Nanoimaging

Most of the methods used so far for particle tracking in fluorescence microscopy rely on ultrafast video cameras that can detect single particles with accuracy of 10–100 nm in the range of ms. The main limitation of these methods is that they can only detect particles moving within the focal plane, and thus they are generally limited to applications such as studying molecular processes in membranes.

To localize and track fluorescent particles in three dimensions, most techniques are based on imaging the sample with a camera at different z-positions and then analyzing the resulting image z-stacks as a function of time. These methods have a time resolution in the range of seconds and can only be applied to slow moving particles. In addition, as they require repetitive illumination of large sample volumes, their use in biological applications is limited because of photodamage and bleaching.

The single molecule tracking upgrade for FV1000 uses the internal detectors of the system. Molecules are tracked in the XY plane using the galvo-controlled mirrors of the microscope and in the z-axis by a piezo-controlled stage.

Nanoimaging is an extension of the particle tracking. With the Nanoimaging approach to super-resolution, the laser beam does not scan the sample following a predetermined pattern as is the case in raster images ; instead, the laser scanning imaging is based upon a feedback algorithm where the path followed by the laser beam is continuously adjusted during the scan according to the shape of the object to be imaged. The algorithm moves the laser spot at fixed distance from the object's surface; as the position of the laser spot and the distance from the surface are known parameter they are utilized to reconstruct the shape of the object. 3D cellular structures can be resolved down to 20-40 nm with a precision of 2 nm.

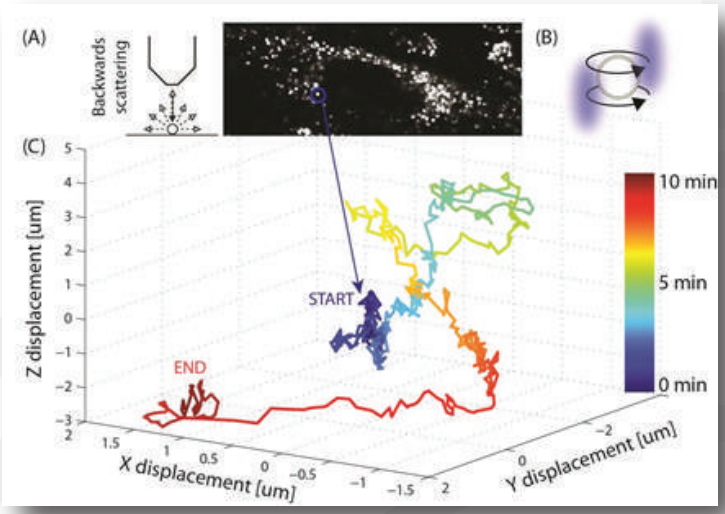


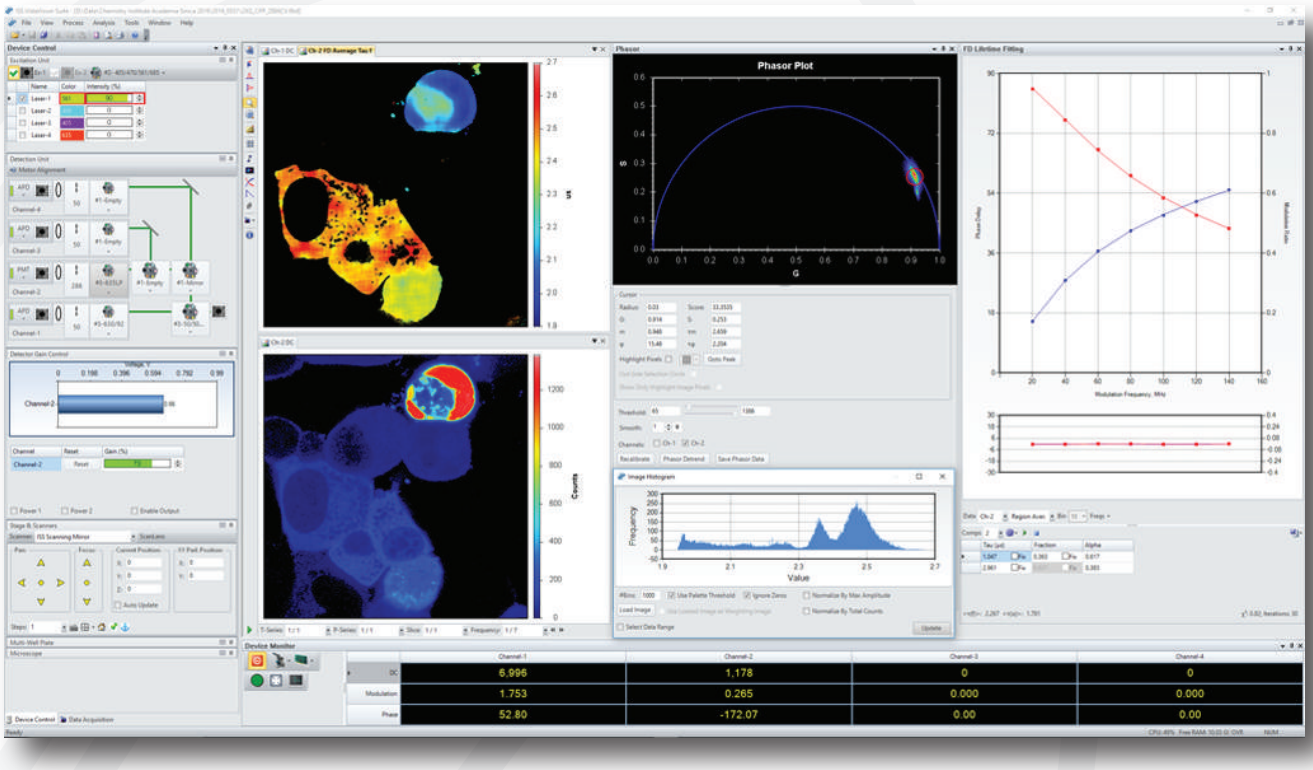
Figure 6- Given their dense core and size in the 200-400 nm range, which is similar to the wavelength of light, insulin granules are highly reflective due to Mie scattering of visible light. Such a robust, imperishable signal is used here to track granule position with high accuracy. Here a single granule 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. Measurements acquired on a FV1000 equipped with multiphoton excitation. *(Courtesy of Dr. Francesco Cardarelli, Scuola Normale Superiore, Pisa)*

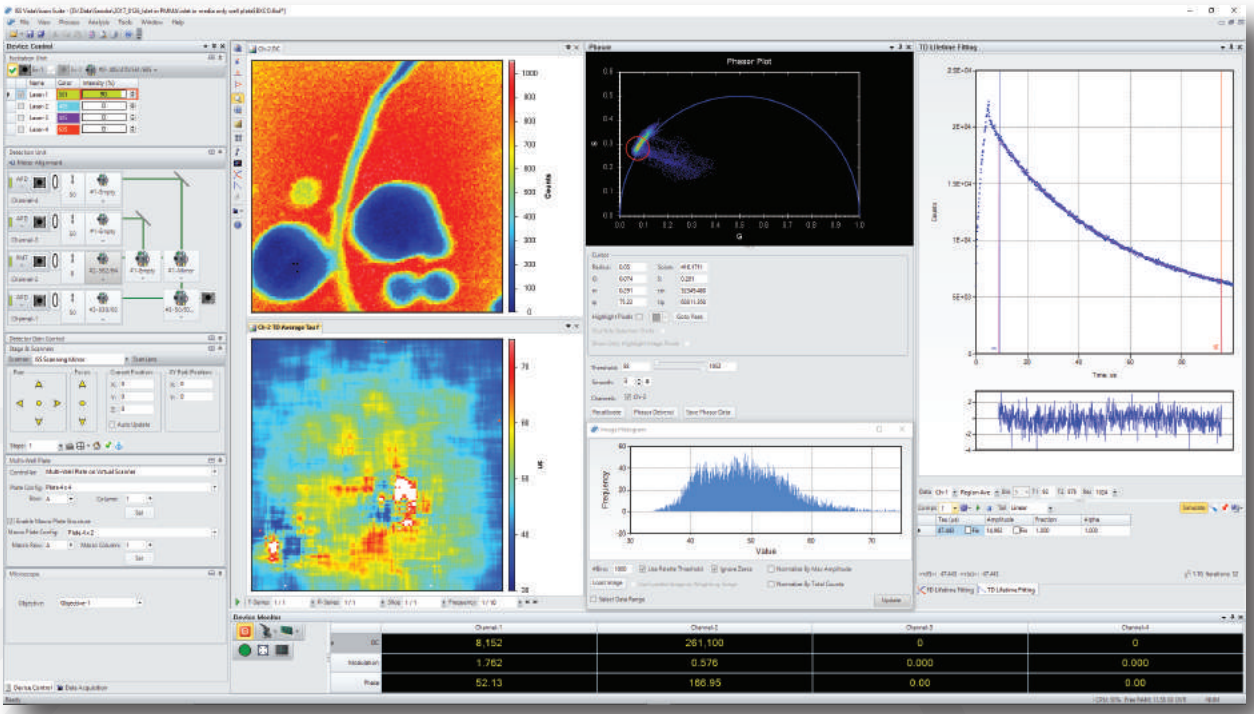
Vistavision Software

VistaVision (Windows 10, 64-bit), is a user friendly software that interfaces to the software running the Olympus microscope system for a smooth and easy operation.

1.The VistaVision Imaging and FLIM module is for image acquisition, processing and image display. The acquisition parameters entered in the Olympus system (image size, number of pixels, dwell time) are mirrored in the software for synchronized acquisition of images, time lapses, z-stack images. The processing includes operations between images, smoothing, filtering and automatic threshold setting for image contrast enhancement. FLIM analysis is carried out using the non-linear least-square fitting algorithm and the phasor analysis. Images can be exported to *avi format for movies, and to ImageJ for 3D rendering. Plots are exported to popular formats (png, jpeg, gif, tiff, bitmap).

2.The VistaVision FFS module includes routines for FFS data acquisition (FCS, FCCS, PCH, RICS, N&B). Analysis is performed using autocorrelation function (FCS), cross-correlation function (FCCS) or photon counting histogram (PCH, proprietary technology). Custom model functions can be input by the user and fitted by the software for single point FCS. Multiple files can be analyzed using global analysis with either autocorrelation functions or photon counting histograms. Plots are exported to popular formats (png, jpeg, gif, tiff, bitmap).




Phasor Plot
Frequency
Value
Device Control
Device Data Control
Multi-Well Plate
Microscope
Device Monitor
Channel-1
Channel-2
Channel-3
Channel-4
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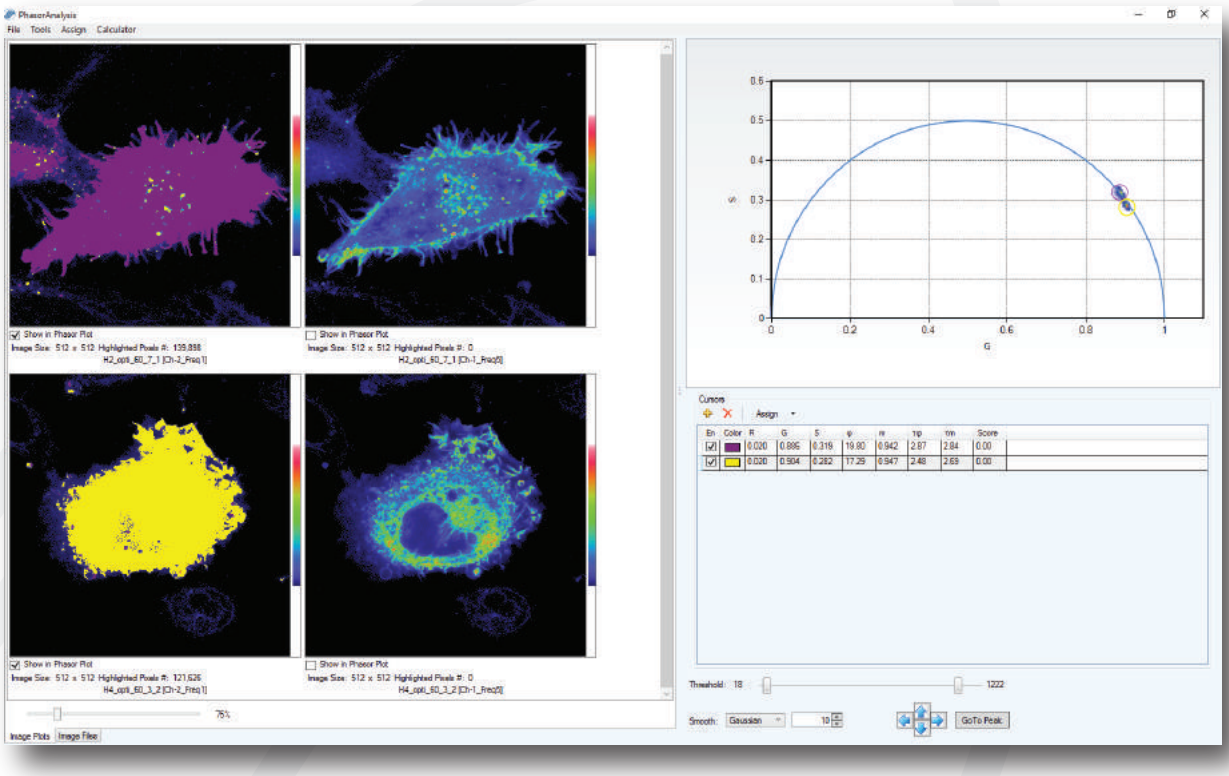
PhasorAnalysis
File Tools Assign Calculator
Show in Phasor Plot
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Show in Phasor Plot
Image Size: 512 x 512 Highlighted Pixels #: 0
H2_opti_60_7_1 [Ch-1_Freq0]
Show in Phasor Plot
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H4_opti_60_3_2 [Ch-2_Freq1]
Show in Phasor Plot
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H4_opti_60_3_2 [Ch-1_Freq0]
Image Plots
Image Files
Cursors
Assign
Threshold: 18
1222
Smooth: Gaussian
GoTo Peak

What functionality is added by the upgrade package?

With the addition of our upgrade package, the microscope retains all of the original capabilities. In addition to the standard measurements provided by the Olympus confocal microscope, the upgrade package adds the following measurements capabilities to the system:

Fluorescence Lifetime Imaging Measurements, can be carried out in a combination of X,Y, Z, and t dimensions.

Digital frequency-domain (DFD) FLIM	Acquired by FastFLIM.
Time-domain FLIM	Acquired in time-correlated single photon counting (TCSPC).

Fluorescence Fluctuation Spectroscopy utilities:
Data can be acquired in the counts, the time tagged or the time tagged time resolved (TTTR) mode.

Autocorrelation (FCS)	The FCS function provides the temporal correlation of the fluctuations. It provides the number of molecules in the observation volume and their diffusion coefficient.
Cross-correlation (FCCS)	The FCCS function provides the temporal correlation of the fluctuations related to events occurring simultaneously on two or three channels.
Photon Counting Histogram (PCH)	The PCH function plots the distribution of photon counts at the specified time interval. It provides the number of molecules in the observation volume and their brightness.
FFS measurement at target XYZ locations in an image	The user selects the XYZ locations by moving the cursor or entering the values in the software. The laser beam moves sequentially to each location to acquire FFS data that are then analyzed.
Fluorescence Lifetime Correlation Spectroscopy (FLCS)	The knowledge of the fluorescence lifetime allows for the correction of detector artifacts, scattering and the recovery of dual species diffusion coefficients when the diffusion coefficients are similar.
Raster Image Correlation Spectroscopy (RICS)	It retrieves the diffusion coefficients at each pixel of a confocal image.
Number & Brightness (N&B)	Raster images are acquired at each pixel with a dwell time that is much less than the decay time of the fluctuations. The measurement separates areas with mobile and immobile molecules; for clusters, it distinguishes between monomers, dimers and higher aggregation orders.

Particle Tracking and Nanoimaging (optional upgrade)

Particle Tracking	• Tracks isolated particles • Tracks particles with nanometer resolution • Analyzes the dynamics of the system like motion characteristics (ballistic, diffusion, trapped, etc), lifetime, brightness along the path.
Edge Tracking	• Studies dynamics on interface surfaces like cell membrane.
Nanoimaging	• Provides image for a micrometer size extrusions with nanometer diameter like microtubules.

Components of the upgrade package

Three components are required to assemble the FastFLIM Upgrade Package and their selection is straightforward: the data acquisition unit, the detectors and the lasers. All of ISS units are controlled through the USB port of the computer by the ISS VistaVision software .

Data acquisition

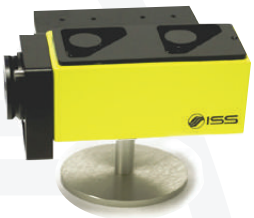
FastFLIM Unit

FastFLIM is the data acquisition card to use when time is of essence. Developed using Digital Frequency Domain (DFD) technique, it allows for the acquisition of Time-Tagged-Time-Resolved data without the dead time typical of TCSPC approach.

Detectors Units

Two On-line Detector Unit

The unit connects directly to the confocal head. Filters and dichroic are replaced manually.



Two Channel Detector Unit

The unit connects to the microscope via fiber optics and features automatic dichroic wheel, filterwheels and shutters.

Laser launchers & lasers

3-, 4-, 6- Lasers Launcher

ISS laser launchers can accommodate 3-, 4- and 6-lasers made by ISS, B&H, Coherent and other manufacturers. Each laser beam can be turned ON/OFF and its intensity can be regulated independently through a computer-controlled variable filter.
The lasers beams are superimposed in the laser launcher using a combination of dichroics and mirrors and coupled into a single-mode fiber that delivers the light to the microscope.

Specifications

Laser Sources	1p Excitation - ISS laser launcher (models for 3, 4 and 6 laser diodes), wavelengths available from 300 nm to 1000 nm; adjustable output power. 2p Excitation - Ultra fast femtosecond pulse Ti:Sapphire lasers - Ultra fast femtosecond pulse fiber lasers	
Data Acquisition Unit FastFLIM (Frequency Domain FLIM)	- Input Channels: 4 simultaneous - Phase resolution: 0.1 degrees - Count rate: › 60 million counts per second (CPS) at each channel - Dead time: 2 ns - Computer interface: USB	
Data Acquisition unit TCSPC (Time-domain FLIM)	- Input Channels: 1 CH on the card, up to 4 using a router - Time resolution: 4 ps - Count rate: ‹ 1 million counts per second (CPS) for a 80 MHz repetition rate laser - Dead time: 100 ~ 150 ns depending upon the TAC range - Computer interface: PCI bus	
Detectors	- GaAs PMT (Hamamatsu H7421 and H7422P models) - Hybrid PMTs (Hamamatsu R10467U models) - SPADs	
Software	- VistaVision (Windows 10 OS, 64 bit)	
Computer & Monitor	- 3 GHz Processor, 64GB RAM, 64-bit VistaVision - 27” 25556 x 1440 resolution monitor - Windows 10 64 bit professional	
Power Requirements	- Universal power input: 110-240 V, 50/60 Hz, 400 VAC	
FLIM Processing	- Lifetime fitting (Marquardt- Levenberg minimization algorithm) - Phasor plots	
FLIM Display	- Lifetime image - Fractional contributions image - Pre-exponential factors image	
Image/Plots export format	- Gif, tiff, jpeg, png, bitmap	
Movies export format	- avi	
Statistical function utilized for FFS data analysis Autocorrelation (FCS) Photon Counting Histogram (PCH) Cross-correlation (FCCS)	Single set and global fitting models available in the FFS module - One or two species using: ◦ 2D- or 3D-Gaussian PSF ◦ 3D-Gaussian-Lorentzian PSF ◦ One-photon excitation ◦ Two-photon excitation ◦ Presence of flow - Global analysis fitting	Parameters determined by the FFS module - One or two species using: ◦ Diffusion coefficient ◦ Concentration ◦ Triplet state decay time constant ◦ Triplet function ◦ Flow rate ◦ Size of excitation volume
User Defined Equation	- More than 50 different user defined equations - Simple interface for user to generate custom equations - Global analysis fitting	- Up to 30 parameters allowed in the equation

FastFLIM is covered by US Patent 8,330,123; other patents are pending.

For more information, please call (217) 359-8681
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