

FLIM & FFS UPGRADE FOR EVIDENT SYSTEMS

Evident (Olympus) Confocal Microscope Systems (FV1000, FV1200, FV3000, FV4000, FV5000 & FVMPE-RS) can be easily expanded to Fluorescence Lifetime Imaging (FLIM) and Fluorescence Correlation Spectroscopy (FCS) capability using the ISS Upgrade Kits.



FLIM/FFS Upgrade
for the Evident
(Olympus) FV4000
Photo Credit / Evident



Why FLIM?

Laser scanning confocal microscopy provides a wealth of information in the life sciences (cell biology, genetics, physiology, neurobiology, and 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 states. 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 that 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 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 pulse-width 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.

Phasor Plots

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 "predetermined" model, the phasor plots display all of the pixels of the image at coordinates separated by their decay times without making 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.

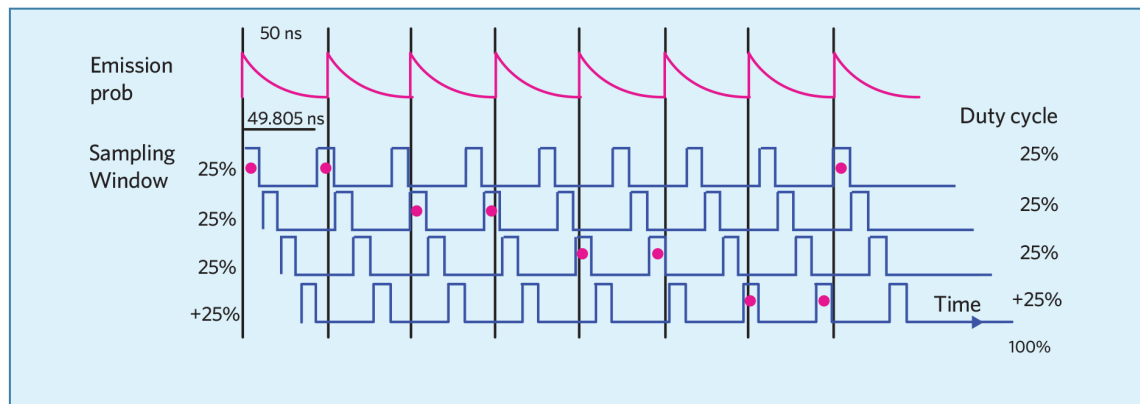


Figure 1: Graphical illustration of the Digital Frequency Domain principle for a light source modulated at 20 MHz. The cross-correlation frequency is 1/8 the sampling frequency (in practice it is 1/256). The fluorescence photons (red dots) are recorded into any of the four sampling windows according 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

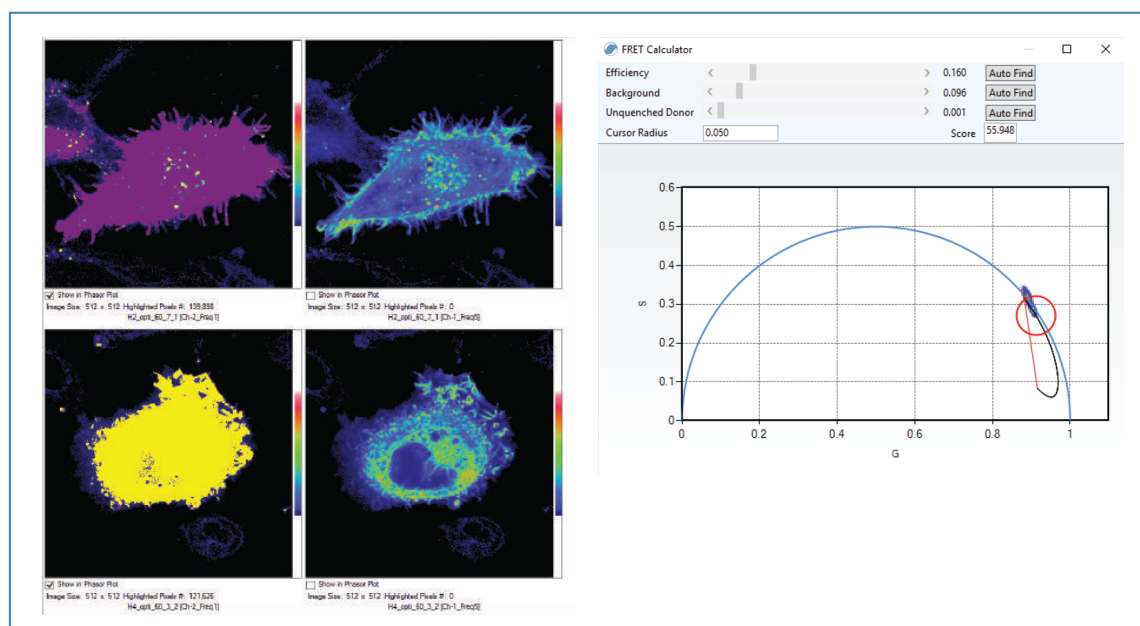


Figure 2: Cells were expressed with EGFP only (left top) and EGFP-mCherry pair (left bottom). The purple area (top left) represents the pixel selected by the purple circle (cursor) on the phasor plot (right). The yellow area (bottom left) represents the pixel selected by the yellow circle (cursor) on the phasor plot (right). In order to calculate the FRET efficiency, the decay times of the 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 the ISS Q2 system coupled to an Evident microscope; excitation is 488 nm and emission is collected through 500 nm - 550 nm filter. (Courtesy of Dr. Hsiao Jye-Chian, Institute of Chemistry, Academia Sinica, Taiwan).

Metabolic state of kidney physiology by monitoring NADH and FAD

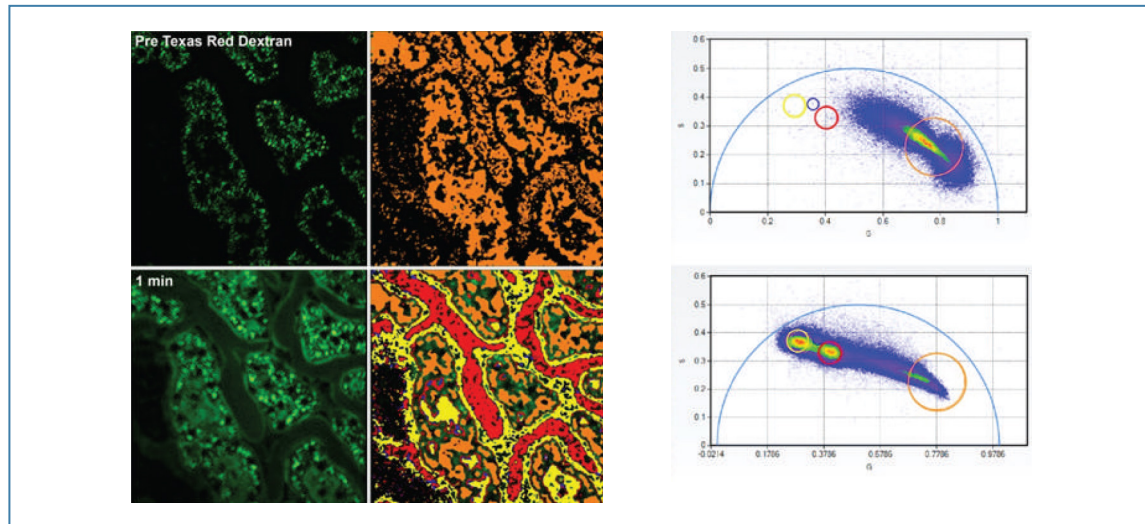


Figure 3: Fluorescence images (left) and FLIM images (middle) for a region of the living rat kidney prior to (top) and 1 minute after injection (bottom) of a small, freely-filtered Texas Red dextran. FLIM image colors derived from regions of interest on phasor plots (right). Top FLIM images and phasor reflect tissue autofluorescence, with lifetimes along the FAD-NADH axis (orange). Bottom FLIM images and phasor plot 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 cell spheroids FastFLIM measures decay times of fluorescence ranging from the sub-nanosecond to the second.

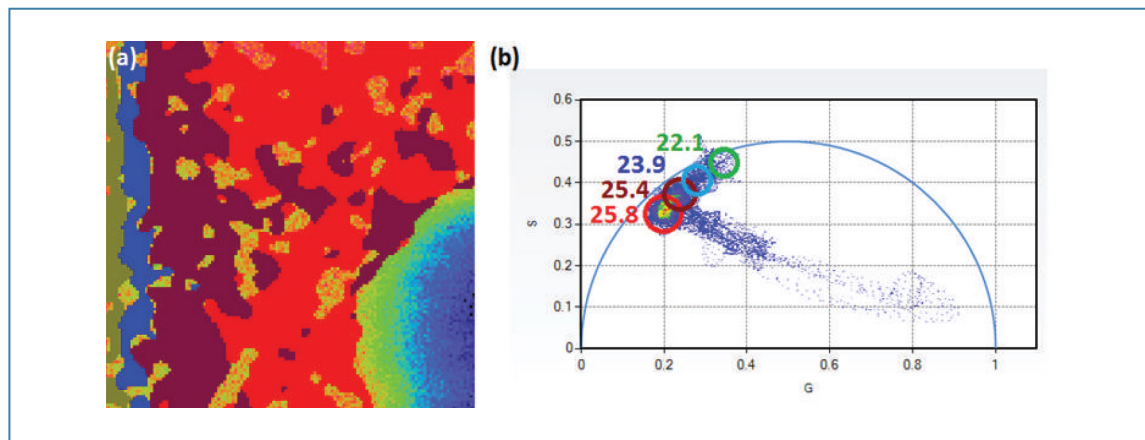


Figure 4: Lifetime map of oxygen consumption in breast cancer cell spheroids (MDA-MB-231 cell line) embedded in a fibrin gel. Oxyphor G4 dye, introduced in the media lines, is used to monitor the local oxygen concentration by measuring the decay times, which are sensitive to the local concentration of oxygen. Measurements were acquired on an Evident (Olympus) FV1200 equipped with a 60X water objective; excitation was at 635 nm using a modulated laser diode (by ISS) and emission was collected through a 80 nm-bandpass filter centered at 809 nm by the NIR detectors (ISS). (Left) lifetime image, 159 x 159 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 biexponential 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

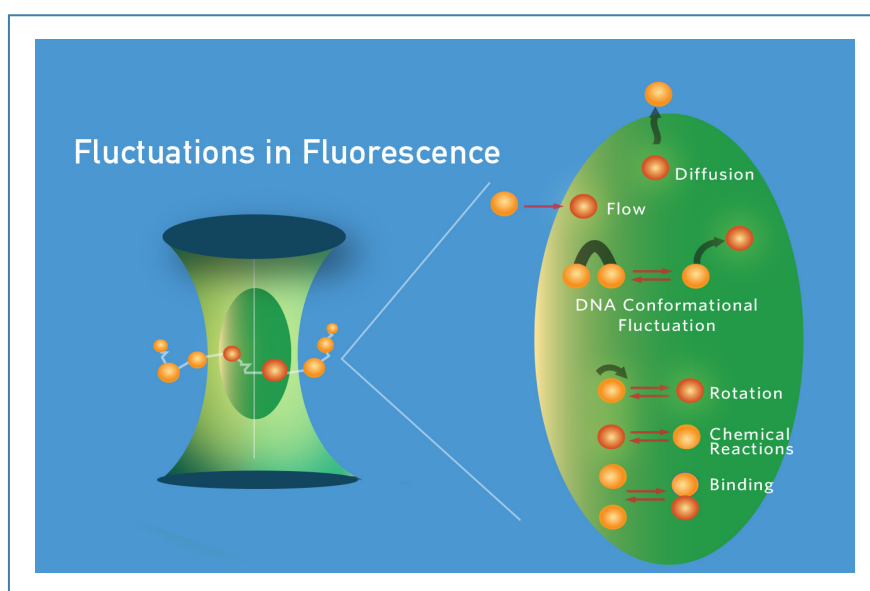
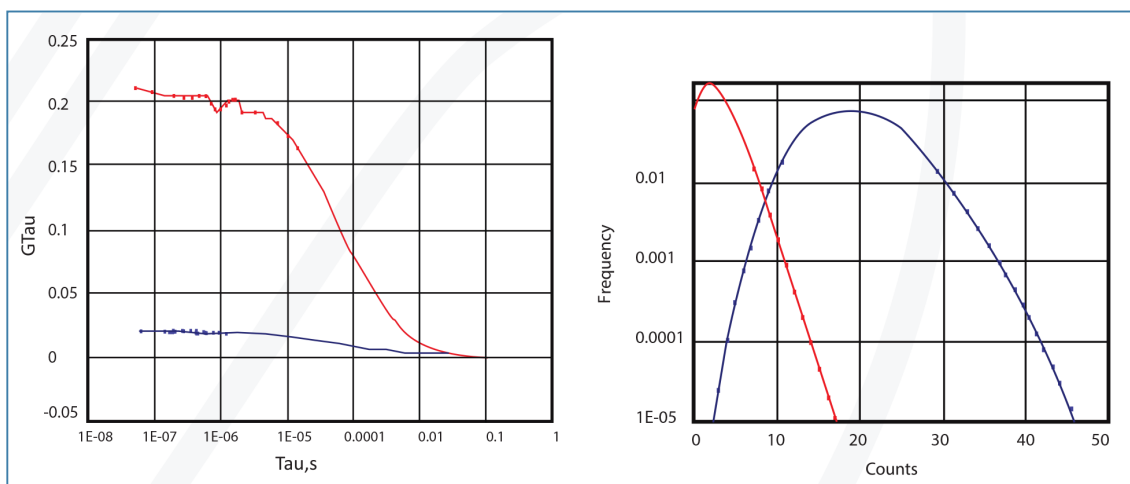


Figure 5: As a particle enters into the observation volume, a burst of photons is emitted and collected. The temporal correlation provides the FCS curve (left); the distribution of the amplitudes provides the PCH (right). Measurements were acquired on two solutions of Rhodamine 110 at 3 nM (red curves) and 24 nM (blue curves) using an Evident (Olympus) IX73 microscope equipped with a 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 an 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 photo-damage 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 a fixed distance from the object's surface; as the position of the laser spot and the distance from the surface are a 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.

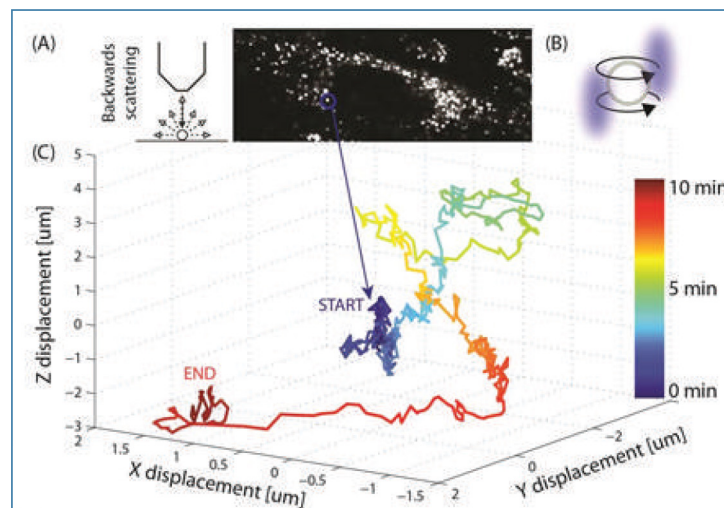


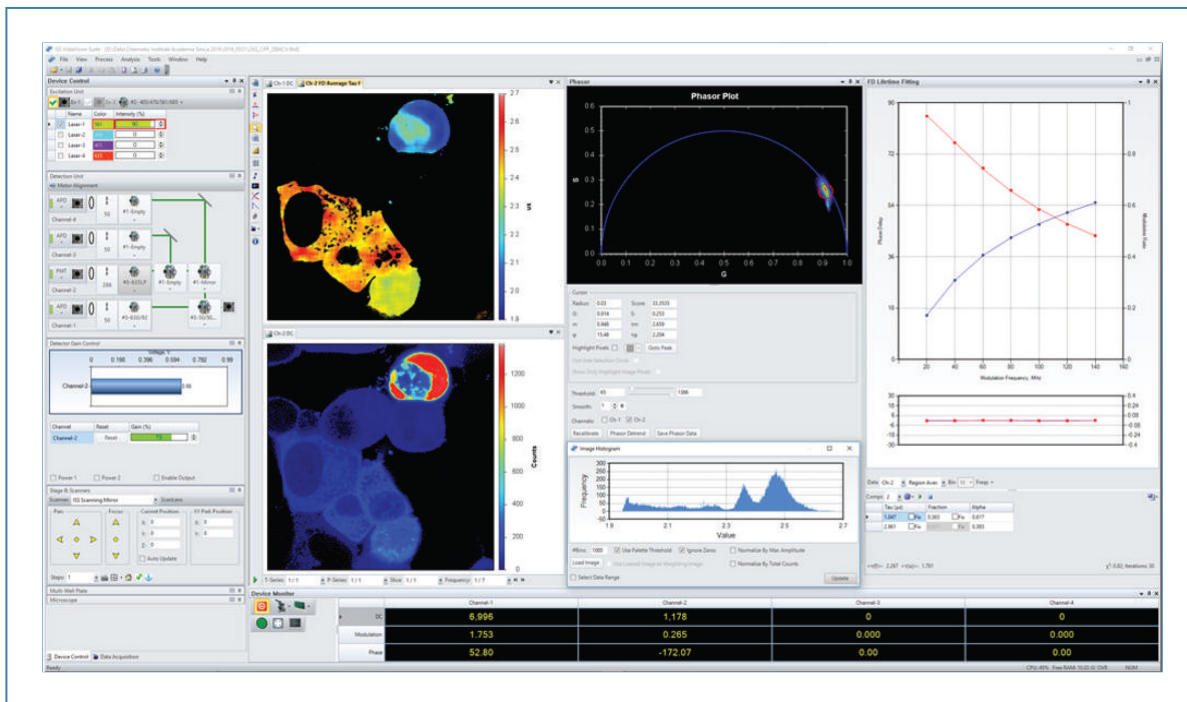
Figure 6: Given their dense core and size in the 200-400 nm range, which is similar to the wavelength of light, insulin granules are highly reflective due to Mie scattering of visible light. Such a robust, imperishable signal is used here to track granule position with high accuracy. Here a single granule 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).

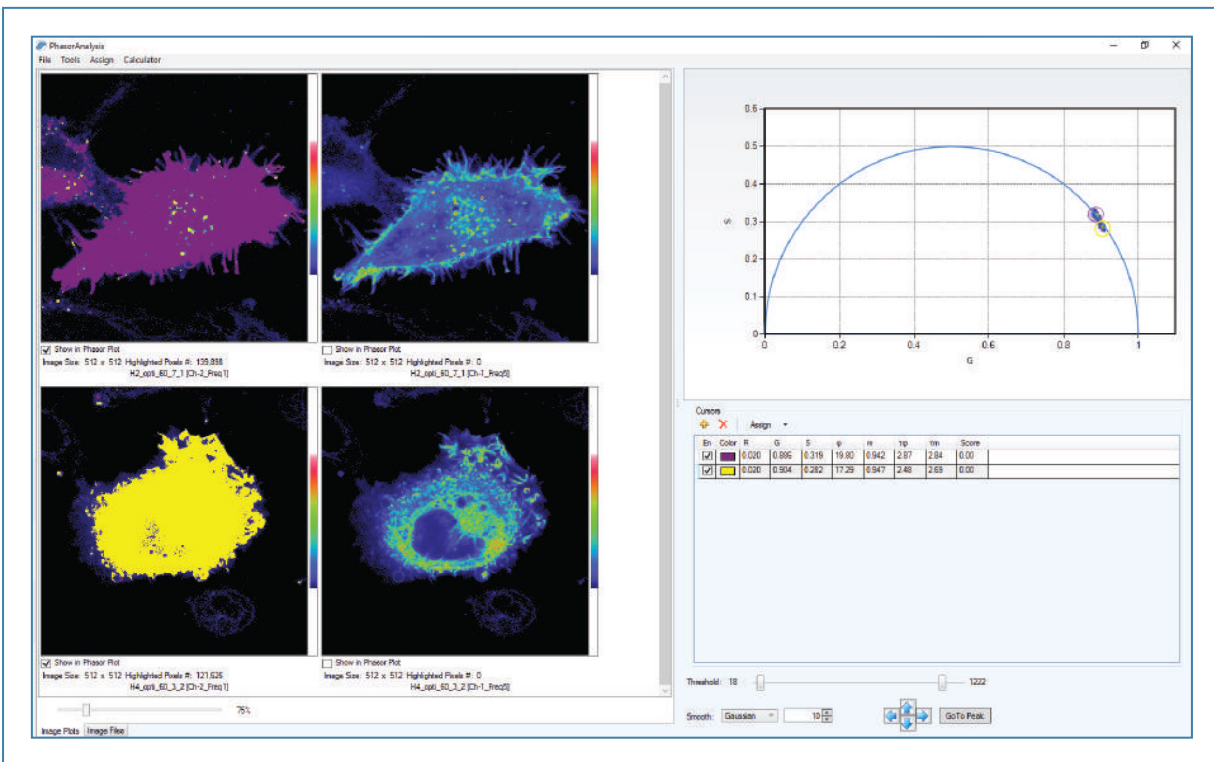
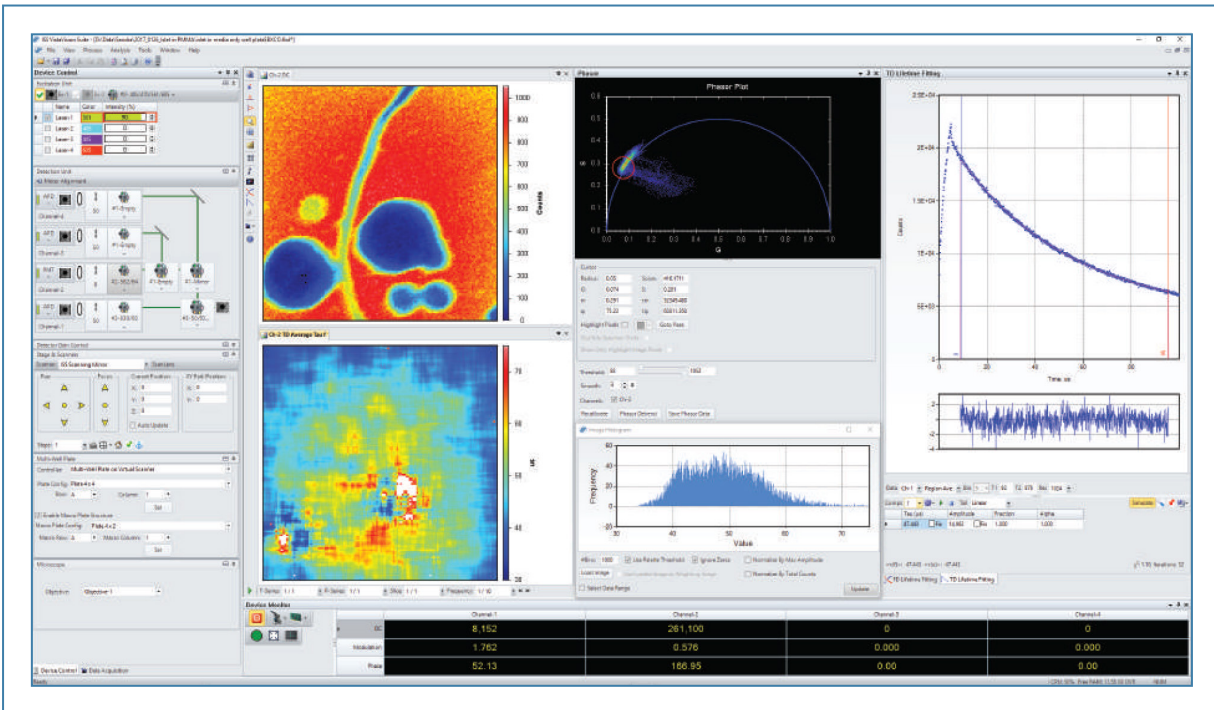
VistaVision Software

VistaVision (Windows 11, 64-bit), is a user friendly software that interfaces to the software running on the Evident (Olympus) microscope system for a smooth and easy operation.

The VistaVision Imaging and FLIM Module is for image acquisition, processing, and image display. The acquisition parameters entered in the Evident (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).

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 the global analysis with either autocorrelation functions or photon counting histograms. Plots are exported to popular formats (png, jpeg, gif, tiff, bitmap).





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 Evident (Olympus) confocal microscope, the upgrade package adds the following measurement 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
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Time-Domain FLIM	Acquired in digital time-correlated single photon counting (TCSPC)
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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.
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Cross-Correlation (FCCS)	The FCCS function provides the temporal correlation of the fluctuations related to events occurring simultaneously on two or three channels.
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Photon Counting Histogram (PCH)	The PCH function plots the distribution of the photon counts at the specified time interval. It provides the number of molecules in the observation volume and their brightness.
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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.
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Fluorescence Lifetime Correlation Spectroscopy (RICS)	The knowledge of the fluorescence lifetime allows for the correlation of detector artifacts, scattering, and the recovery of dual species diffusion coefficients when the diffusion coefficients are similar.
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Raster Image Correlation Spectroscopy (RICS)	It retrieves the diffusion coefficients at each pixel of a confocal image.
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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.
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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.
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Edge Tracking	Studies dynamics on interface surfaces like cell membrane.
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Nanoimaging	Provides image for a micrometer size extrusion with nanometer diameter like microtubules.
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Components of the upgrade package

Three components are required for the upgrade package and their selection is easy; the data acquisition unit, the detector unit, and the lasers. All of our units are controlled through the USB port of the computer (ISS VistaVision software runs on Windows 11 OS). ISS laser launchers can accommodate 3-, 4-, and 6-lasers made by ISS, B&H, Coherent, and other laser manufacturers.

Each laser beam can be turned ON/OFF and its intensity can be regulated independently through a computer-controlled variable filter. The laser beams are superimposed in the laser launcher, using a combination of dichroics and mirrors and focused on a single-mode fiber that delivers the light to the microscope.

The customer can select any device from the different groups in this chart:

Data Acquisition Units



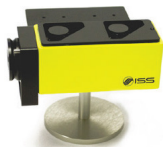
FastFLIM

Frequency-Domain Data Acquisition

SWISS

Digital TCSPC Data Acquisition

Detector Units



Two on- Line Detector

The unit is connected directly to the confocal head of the AX. Filters and dichroic are replaced manually.



Detector Unit

The unit is connected directly to the confocal head via fiber optics. Controlled through the USB port, it includes an automatic dichroic wheel, two filter wheels, and two shutters.

Laser Launcher and Lasers



3-Laser Launcher

Single photon laser wavelengths include: 375, 405, 440, 473, 488, 514, and 640 nm. (for all other wavelengths contact ISS)



4-Laser Launcher

6-Laser Launcher (not shown)

Specifications

Laser Sources

- 1p Excitation
 - ISS laser launcher (models 3, 4, and 6 laser diodes), wavelengths available from 375 nm to 640 nm; adjustable output power, pulse interleave excitation (PIE)
- 2p Excitation
 - Ultrafast femtosecond pulse Ti:Sapphire lasers
 - Ultrafast femtosecond pulse fiber lasers

Data Acquisition Unit FastFLIM (frequency-domain FLIM)

- Input Channels: 4 simultaneous
- Time Resolution: 5 ps
- Count Rate: > 10 million counts per second (CPS) at each channel
- Dead Time: 10 ns
- Computer Interface: USB

Data Acquisition Unit Digital TCSPC (time-domain FLIM)

- Input Channels: 8
- Time Resolution: 1 ps
- Count Rate: up to 167 million counts/s
- Dead Time: 6 ns
- Computer Interface: USB

Detectors

- GaAs PMT (Hamamatsu H16722P)
- Hybrid PMTs (Hamamatsu R10467U models)
- APDs

Software: VistaVision (Windows 11 OS, 64 bit)

Computer & Monitor

- 3 GHz Processor, 32 GB RAM, 200 GB hard drive for 64-bit VistaVision
- 32" 1280 x 1024 resolution monitor
- Windows 11 64 bit professional

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

VistaVision FFS Analysis Features

Statistical Function Utilized for FFS Data Analysis

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

Single Set & Global Fitting Models Available in the FFS Mode

- One or two species using:
- 2D- or 3D-Gaussian PSF
- 3D-Gaussian-Lorentzian PSF
- One-Photon Excitation
- Presence of Flow
- Global Analysis Fitting

Parameters Determined by the FFS Module

- One or two species using:
- Diffusion Coefficient
- Concentration
- Triplet State of Decay Time Constant
- Triplet Function
- Flow Rate
- Size of Excitation Volume

Statistical Function Utilized for FFS Data Analysis: User Defined Equation

Single Set & Global Fitting Models Available in the FFS Mode

- Up to 50 different user defined equations kept in the panel list for user to choose
- Equation could include Sin, Cos, Exponential, etc. Except the integral unclosed form equation
- Global Analysis Fitting

Parameters Determined by the FFS Module: Up to 30 parameters allowed in the equation

VistaVision Image Analysis Features

FLIM Processing

- Lifetime Fitting (Marquardt-Levenberg minimization algorithm)
- Phasor Plots

FLIM Display

- Lifetime Image
- Fractional Contributions Image
- Pre-Exponential Factors Image

Image Export Format: Export to ImageJ

Plots Export Format: GIF, TIFF, JPEG, PNG, BITMAP

Movies Export Format: AVI

For more information please contact ISS at iss@iss.com or visit us at iss.com



1602 Newton Drive
Champaign, IL 61822 USA
Phone: 888-359-8681
iss@iss.com
www.iss.com

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