

SlideBook™ 5.0 FLIM Module

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Introduction

Fluorescence lifetime imaging (abbreviated as either FLI or FLIM) provides an entirely different dimension with which to characterize a sample's fluorescence apart from emission intensity when excited at a particular wavelength.

Long the domain of spectroscopy and biophysics labs, SlideBook's FLI module strives to make fluorescence lifetime an accessible tool for "mainstream" molecular biology research involving live cell imaging. In measuring a different physical property of a fluorophore, fluorescence lifetime imaging (FLI) has advantages in measuring protein proximity, polymerization, relative concentration of different molecules, separation of different labels with spectral overlap, ion concentration, and removal of autofluorescence.

SlideBook's FLIM module has been designed to work seamlessly with all types of acquisition supported by SlideBook: 2D, 2D timelapse, 3D, 4D, multi-channel, and multi-location.

Much like its fluorescence spectra, a fluorophore's lifetime serves as a molecular "fingerprint." As a molecule's lifetime is largely unrelated to its spectral properties, two molecules that may be nearly indistinguishable through conventional fluorescence measurement, such as FITC and GFP, can be easily separated using lifetime.

One of the most interesting properties of fluorescence lifetime images is that the lifetime measurement is concentration-independent (as long as there is sufficient concentration to detect a signal). Concentration issues frequently plague Förster resonance energy transfer (FRET) measurement using conventional microscopy, as precise bleedthrough correction measurements must be made to fluorescence channels that may have widely varying acceptor concentrations. Minor error in the correction measurement can have a large impact on reported FRET.

Using the fluorescence lifetime to measure FRET is convenient, because only the donor needs to be imaged. The presence of acceptor shortens the lifetime of the donor, and the FRET efficiency can be easily calculated directly from the change in lifetime:

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

where τ_{DA} is the donor lifetime in the presence of the acceptor and τ_D is the donor lifetime when no acceptor is present.

Lifetime acquisition

A frequency domain lifetime system measures the phase shift and demodulation of a fluorescent sample's emission, by modulating both the light source and the emitted signal at high frequencies (typically around 50-100 MHz).

A single lifetime plane (either in z or time) is constructed from taking N exposures at equally spaced phase shifts between the excitation modulation and the camera's intensifier modulation. For instance, if $N = 4$, four exposures are taken at 0, 90, 180, and 270 degrees, respectively.

The multiple phase-shifted exposures allow SlideBook to estimate the inherent phase shift and demodulation of the sample using what is called *homodyne detection* theory.

Notation and units

The following will be explained in more detail and used throughout the documentation:

τ Lifetime (ns)

τ_{phase} Lifetime (ns) as determined by a measurement of phase shift

τ_{mod} Lifetime (ns) as determined by a measurement of demodulation

$\phi(\tau)$ Phase measurement of a fluorescent signal with lifetime τ

$M(\tau)$ Modulation measurement of a fluorescent signal with lifetime τ

x x-axis component of polar plot: $x = M(\tau) \cos \phi(\tau)$

y y-axis component of polar plot: $y = M(\tau) \sin \phi(\tau)$

Lifetime data format

Rather than store the original phase-shifted images, SlideBook computes the best-fit phase shift and demodulation on a pixel-by-pixel basis and then transforms these to x and y locations on a polar plot described in Redford and Clegg (2005):

$$x = M(\tau) \cos \phi(\tau)$$

$$y = M(\tau) \sin \phi(\tau)$$

For each FLI exposure, SlideBook creates three channels:

- lifetime x component, designated as (**X**) following channel name
- lifetime y component, designated as (**Y**) following channel name
- mean intensity, designated as (**Int**) following channel name

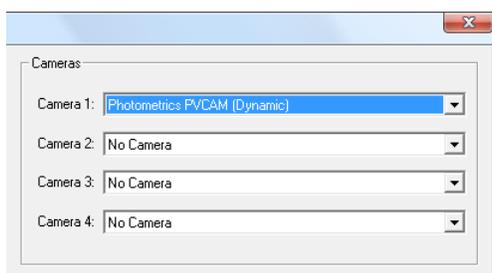
SlideBook stores a lifetime acquisition in three consecutive channels. The first two channels are the x and y locations of a pixel's value after the polar coordinate transform of the fitted phase and modulation lifetimes. The third channel is the mean intensity of the series of phase-shift exposures.

Hardware configuration in SlideBook

FLI hardware can be configured in the **Edit > Hardware Configuration** and **Edit > Hardware Properties** dialog boxes.

Camera

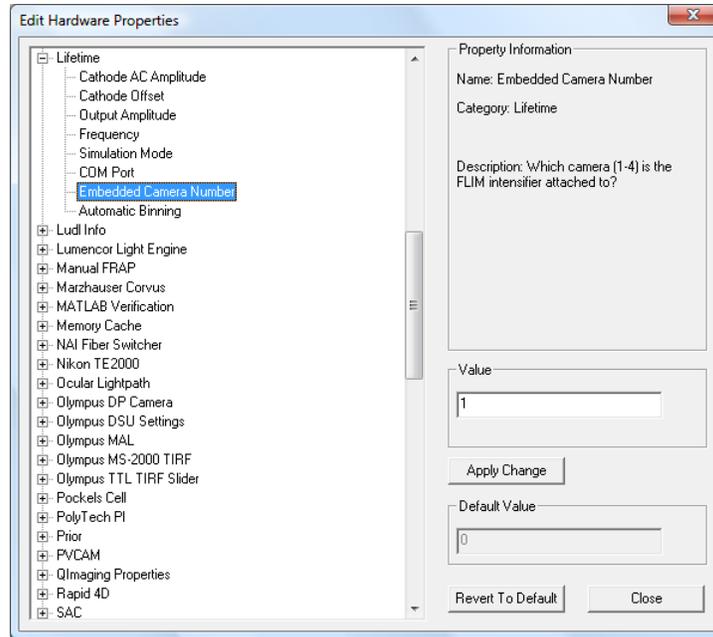
SlideBook controls the intensifier and camera combination as a logical "FLIM camera" that will have different properties than the camera itself. In **Edit > Hardware Configuration**, select the camera driver that corresponds to the camera connected to the intensifier. Typically, this will be **Photometrics PVCAM (Dynamic)** if the camera connected to the intensifier is a CoolSNAP™ EZ.



If you have multiple cameras in the system, you must take note of which camera (1, 2, 3, or 4) corresponds to the one connected to the FLIM intensifier.

Embedded Camera Number

Under **Edit > Hardware Properties**, open the **Lifetime** group and set the **Embedded Camera Number** to which camera in **Edit > Hardware Configuration** is attached to the FLIM intensifier.



NOTE: If you set Embedded Camera Number to 0 or -1, you are effectively turning off the FLIM component of the system, and SlideBook will not try to initialize the intensifier.

Automatic Binning

Set this to **Yes** to have SlideBook automatically bin 2x2 the FLIM camera. Since the intensifier only has a resolution of about 510 x 510, for many cameras (such as the CoolSNAP EZ) this is a good option as it will allow the attached camera to run faster with no real loss of resolution.

Intensifier

Most of the remaining **Lifetime** properties have to do with configuring the intensifier.

COM Port

Set this to the virtual COM port that corresponds to the intensifiers USB connection to the computer.

Cathode AC Amplitude

This number sets the modulation depth of the intensifier. It has the following values: 0 = none, 1 = low, 2 = med, 3 = high. This should usually be set to 3.

Cathode Offset

This number is in the range of 1 to 1200 and sets the offset of the modulation. Usually this is set around 593. If this number is too high, then you will get nice bright images, but low modulation. If this number is too low, you will get little or no signal. This number should be set for a specific intensifier in the factory. Only change it if you have changed intensifiers, computers, etc. To find the optimal setting: change this number by 10, go through the Pockels' cell optimization procedure below, raise this number until you get the brightest image while maintaining a good modulation level.

Output Amplitude

This is a number between 0 and 10 which determines the peak voltage of the output signal to the Pockels' cell. The default is 7. Occasionally this number is reduced to 6 if the Pockels' cell is overheating.

Frequency

This is a number in MHz between 10 and 100. Default is 50. You can change this number if you are mainly working with long or short wavelengths. 50MHz is good for general use between 1 and 8ns. 80MHz is good for lifetimes at or under 1ns. 20MHz would be good for lifetimes at 10ns or greater. Once this number is changed, the whole calibration procedure must be redone.

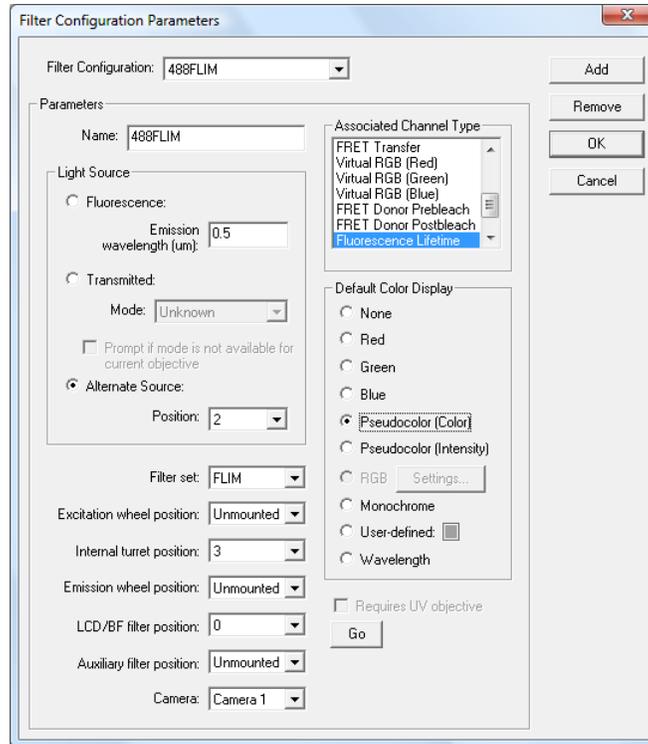
Simulation Mode

If you want to demonstrate the functionality of the system without having the hardware attached, you can set the **Simulation Mode** property to **Yes**. You will also need to set the **Embedded Camera Number** property to 1 (or whichever other test camera you want to assign it to). When you go to collect a FLIM image, you will get a field that has four distinct vertical bands. If you calibrate the left band to 1 ns, then the remaining bands will fit to 2, 3, and 4 ns respectively.

Defining Filters

Define a filter for each laser line that will be used for lifetime imaging. The Associated Channel Type should be set to Fluorescence Lifetime and the Default Color Display to Pseudocolor (color). The turret, shutter, and alternative source channel need to be set correctly just as with any other filter configuration.

NOTE: It is important that you set the **Camera** field to the specific camera that is attached to the FLIM system.



Control and Calibration of FLIM

The next sections will walk through hardware control and calibration of the system. Control of the system is available in the **Focus Controls**, and complete calibration consists of the following steps:

- Dark value correction
- Flat field correction
- Lifetime calibration

Focus Controls

Most of the FLIM-related control and feedback is available in the **FLI** tab in the Focus Controls. There are a few other components of the Focus Controls outside this tab that you will also need to use, however.

The FLIM camera works in three different modes:

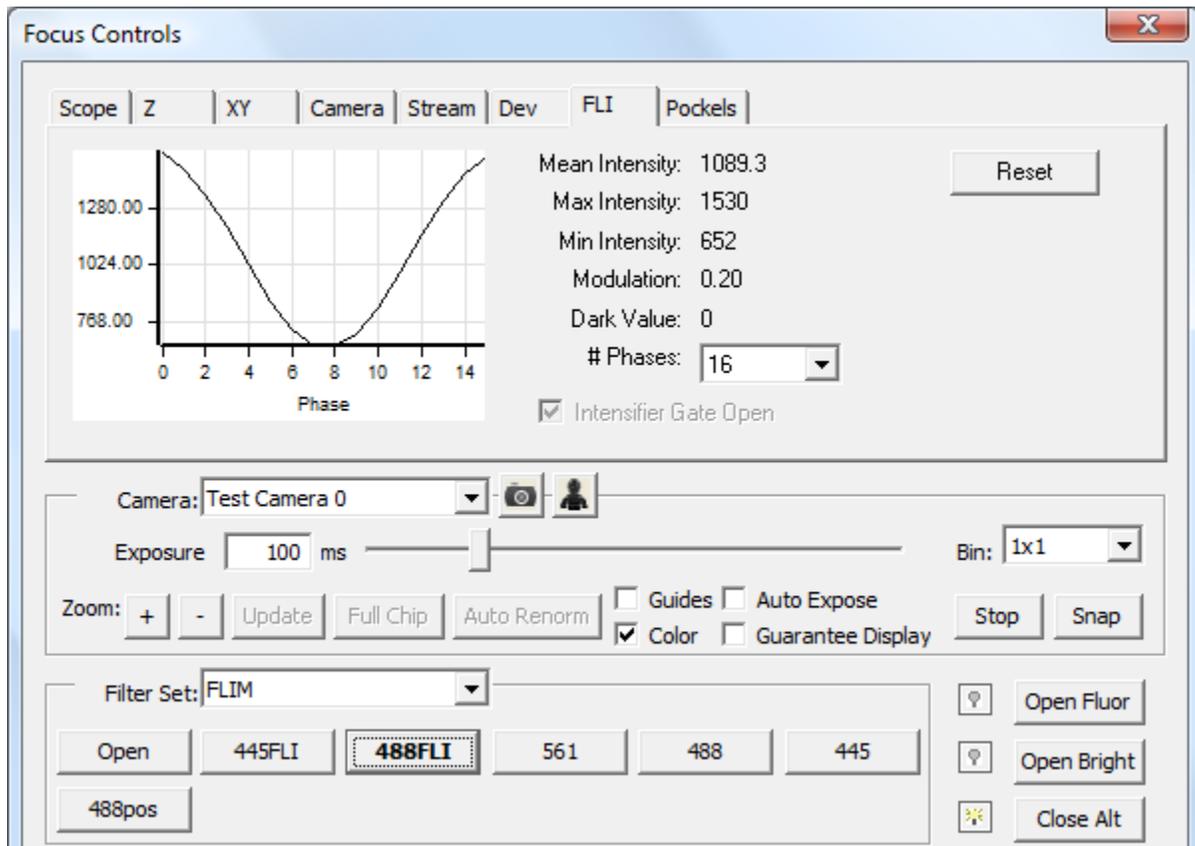
- Modulation and intensification off – The intensifier's state acts as an electronic shutter for the attached camera. If the intensifier is off, no light is reaching the camera.
- Intensification on, modulation off – With the intensification on, but high-speed modulation off, the FLIM camera acts like any other intensified camera, with the intensification dictated by the Intensification slider on the Camera tab of the Focus Controls. This is a useful mode to find your sample and ensure that the intensification is not set too high.
- Intensification and modulation on – This is the mode where a sequence of phase-shifted images are acquired and assembled into a lifetime image.

To turn on the intensification, click on the **Intensifier Gate Open** checkbox in the **FLI** tab.

To turn on the modulation, click on the **Color** checkbox in the main part of the Focus Controls. Note that if you select **Color** (modulation) then SlideBook will automatically lock **the Intensifier Gate Open** state to on.

WARNING: The intensifier is a sensitive device and can be damaged by too much light or intensification. There is a built-in shutoff circuit but please take care to turn the **Intensifier Gate Open** or **Color** checkboxes on with the **Intensification** set to a minimum level. Slowly raise the **Intensification** value until the mean intensity of the sample is approximately 2/3 of the dynamic range of the CCD, and no more. If you do accidentally overload the intensifier, a red light will appear on the control box and you will need to reset the intensifier (and restart SlideBook).

When modulation is on (i.e., **Color** is checked), the **FLI** tab will display a graph of the mean intensity of the phase-shifted exposures, as well as relevant statistics. **Mean Intensity** reports the mean intensity across all phase exposures. **Max Intensity** is the maximum of mean intensities of the phase exposures and **Min Intensity** is the minimum of the mean intensities of the phase exposures. **Modulation** represents the depth of modulation, and a good target value is around 0.2. **Dark Value** will be described later.



Dark value correction

SlideBook handles dark correction slightly differently for FLIM than for other types of capture.

When you acquire an image using **Image > Capture** and select **Dark Field** for a FLIM channel, SlideBook will first take an exposure with no illumination and compute the minimum value of the result. SlideBook then stores this value as the **Dark Value** (which will be subsequently visible in the **FLI** tab of the **Focus Controls**). All future FLIM acquisitions will subtract this value from each phase shift exposure. While the camera offset of the camera typically attached to the intensifier (a CoolSNAP EZ) has a relatively low offset (a value around 90 out of 4095) it can still impact proper modulation measurements particularly with low signal levels.

Phase flat field correction

It is common for there to be some variation in phase across the intensifier cathode. This is referred to as *phase rollover*. To correct for this, SlideBook can take an image of a sample with uniform, finite lifetime (such as fluorescein) and create a phase flat field, which can be stored and applied to images just like intensity-based flat fields.

Ideally, phase flat fielding should be performed even before calibrating the system with an image of a sample with known lifetime.

To create a phase flat field, place a homogenous sample on the microscope. You can monitor the unmodulated fluorescence signal by opening the **Focus/Capture Window** and **Focus Controls** and selecting the appropriate lifetime filter configuration. Click on the **FLI** tab and check **Intensifier Gate Open**.

Make sure the signal is well below saturation by clicking on the **Camera** tab and looking at the histogram. You should adjust exposure time and lifetime intensifier intensification so that the brightest pixel is about 2/3 the dynamic range of the camera.

Then, run a test exposure in **Image > Capture** and make sure to check the **Dark Field** option.

After that, collect a full-chip FLIM image at 1x1 binning by going to **Image > Capture**. Select only one FLIM channel for capture. Select the **Dark Field** checkbox but leave the **Flat Field** option unchecked.

Once captured, select the image in the slide view in which it appears and select **Image > Lifetime (FLI) > Compute Phase Flat Field**.

If you want to verify that it was created, and looks reasonable, select **Edit > Setup Guides > Display Flat Fields**. You should see an entry that corresponds to the FLIM filter configuration. A proper phase flat field should present a smooth image. If the FLIM camera's chip size is larger than the intensifier screen, you may see the signal as a clipped circle within a larger rectangle of background. Depending on the level of phase rollover present in the system, it may look like either smoothly varying concentric rings or a flat disc.

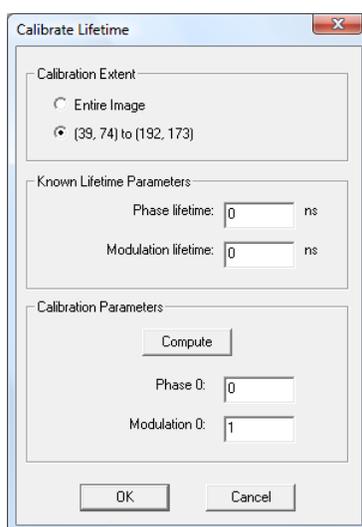
Lifetime calibration

Now, in order to calibrate the system for lifetime computation, you need image a homogenous sample with either a known single lifetime or known τ_{phase} and τ_{mod} components. Typically, this is a thin layer of fluorescein (with a known lifetime around 4.0).

If the system has been previously calibrated, you must first clear the existing calibration from SlideBook. Do this by selecting **Image > Lifetime (FLI) > Clear Calibration**.

Once you capture the image and open a view of it, select a homogenous region of the sample with the marquee tool, and then choose **Image > Lifetime (FLI) > Calbrate....**

The following dialog box will appear:



If the known sample has a single lifetime, enter the lifetime for the sample in both the **Phase lifetime** and **Modulation lifetime** edit fields. Otherwise, enter the known τ_{phase} and τ_{mod} components in the corresponding edit fields.

Click on the **Compute** button.

SlideBook will determine the calibration parameters.

When you click **OK**, SlideBook will use these new calibration parameters for all subsequent image captures. Old images that you load, however, will still have the calibration parameters in effect when you collected the image.

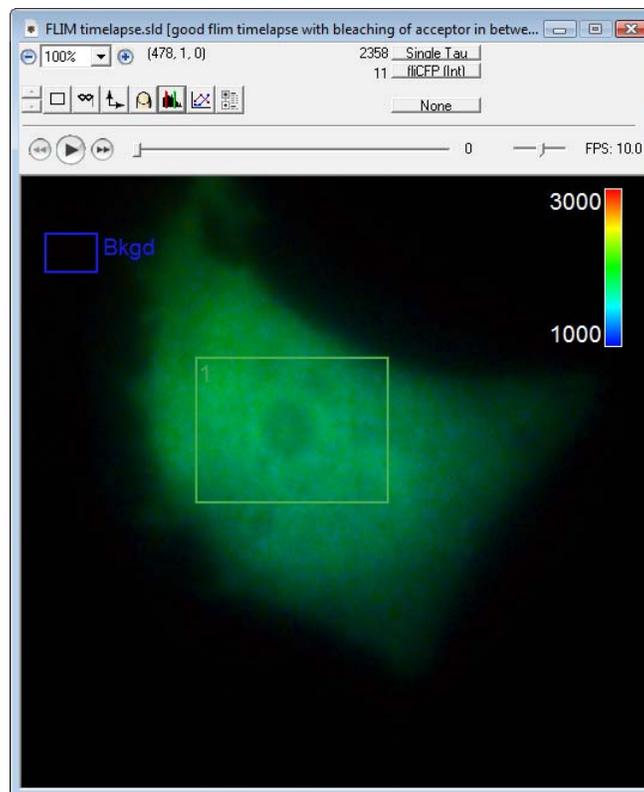
Acquiring and viewing a lifetime image

Acquiring a lifetime image, once the system has been calibrated, is easy. Simply select the lifetime channel that corresponds to the excitation wavelength that you want when you are setting up a new experiment in **Image > Capture**. You can collect several lifetime channels or you can mix lifetime and non-lifetime channels.

Creating a single- τ channel

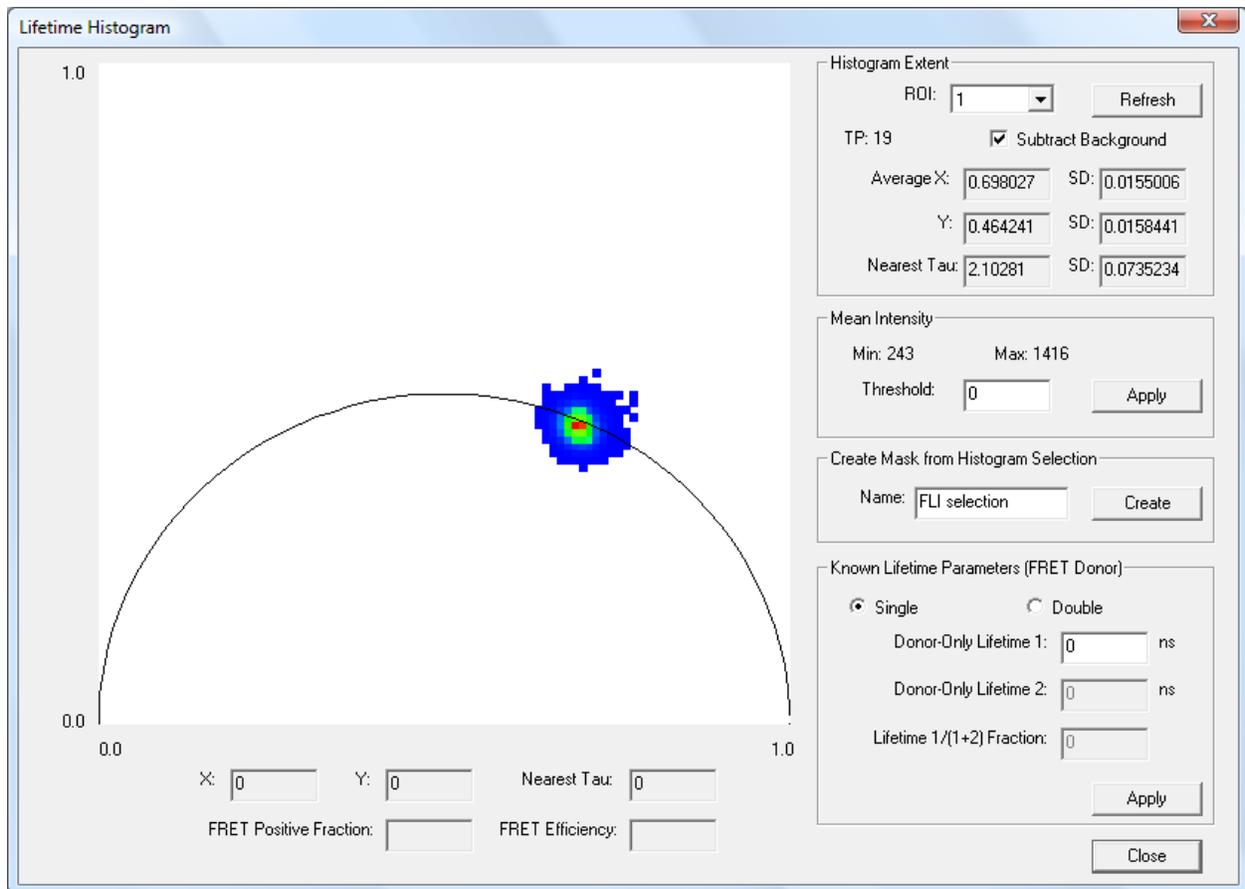
Acquiring a FLIM image produces three component channels, (X), (Y), and (Int). While the x and y component values are related to lifetime, and can be useful to generate simple lifetime-based contrast, a proper scaled display requires that you create a fourth channel that maps to lifetime directly. You can do this by selecting **Image > Lifetime (FLI) > Create Single Tau FLI Channel**.

If you use the pseudocolor display mode for a view, and set the hue channel to the generated *Single Tau* channel, and the intensity channel to the (Int) component channel, and set the renormalization range of the *Single Tau* channel to a reasonable range of ns (x 1000), such as a **Low** of 1000 and a **High** of 3000, then the display will map color to nearest single τ with a range determined by the renormalization range. In the case below, we see a pseudocolored cell displayed in a range where blue is 1 ns (1000 ps) and red is 3 ns (3000 ps):



The lifetime histogram

The lifetime histogram displays the lifetime for a selected region using the polar plot representation:



In order to bring up the lifetime histogram, create at least one region in the image. If you believe there is a significant background component, you should also create a background region. Then, select **Image > Lifetime (FLI) > Lifetime Histogram**.

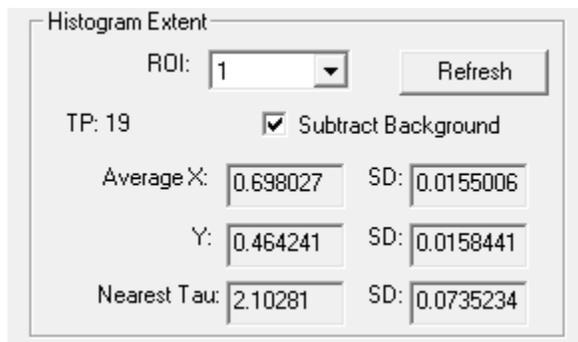
The polar plot itself gives a reading of the number of pixels in the selected region that have specific x and y values. The redder the display, the higher the number of pixels that have that corresponding x, y value.

Lifetimes with a single exponential fall on the semicircle. The lower right position at (1.0, 0.0) represents zero lifetime, and the lower left position at (0.0, 0.0) represents infinite lifetime.

If you see a single, circular, relatively compact cluster, as in above, you are looking at a region of spatially homogenous lifetimes with good signal to noise. If you see a multi-modal cluster or multiple clusters, you are looking at a region with a spatially inhomogeneous mixture of lifetimes. If the cluster is spread out, or potentially filling much of the plot, you probably have insufficient signal to make a good determination: one of the nice properties of the polar plot is that the compactness of the cluster correlates with the degree of error.

Histogram Extent and background correction

The upper right corner of the dialog allows you to select which region to plot as well as whether it is performing background correction. It also gives useful statistics about the lifetime within the selected region.



The screenshot shows a dialog box titled "Histogram Extent". It contains the following elements:

- ROI: 1 (dropdown menu)
- Refresh button
- TP: 19
- Subtract Background
- Average X: 0.698027, SD: 0.0155006
- Y: 0.464241, SD: 0.0158441
- Nearest Tau: 2.10281, SD: 0.0735234

In order to change to view a different ROI, simply select a different region using the **ROI** dropdown.

If you move a region on the corresponding view, or change which timepoint that view is displaying, then you will need to click the **Refresh** button to update the display.

If you click on **Subtract Background**, the plot will compute the lifetime of the background region and shift the plot accordingly. Note that background here is strictly defined to be regions that have fluorescent signal but are not the label of interest. So, for instance, if you are imaging a GFP-RFP FRET construct that is expressed in either only a fraction of cells or a spatially-resolved compartment within each cell, against a background autofluorescence, you would want to select a background region that contained only the autofluorescence.

The plot displays the average and standard deviation for the *x* and *y* values as well as the *nearest tau*, which is the point on the semicircle closest to the weighted center of the region's cluster in the plot.

Calculating FRET using lifetime

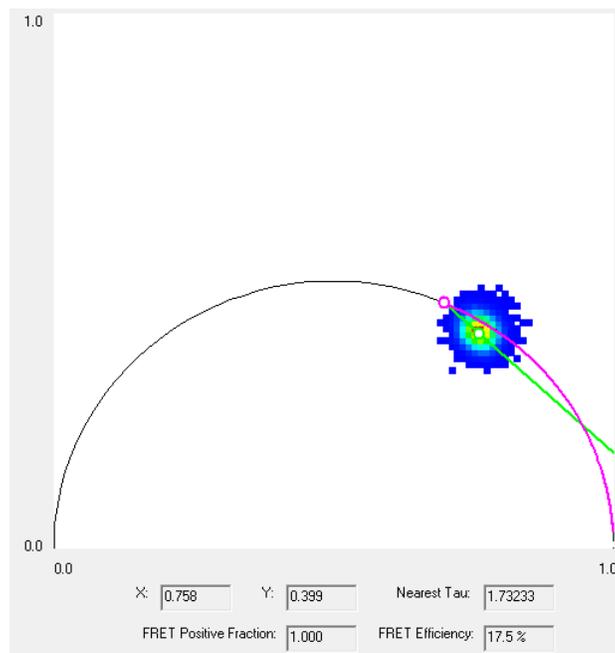
In order to calculate FRET using lifetime data, you must either know the lifetime of the donor in the absence of any acceptor, or measure it in a control experiment. Probably the best way to do the experiment is to take a sample with the same FRETing constructs as your intended experiment and bleach out the acceptor.

A quick visual aid for determining FRET for a region is available in the lifetime histogram.

For instance, in the histogram example above, we are looking at the lifetime of a region of a sample after progressive bleaching of the acceptor for 19 timepoints. If we assume that we have fully bleached the acceptor, the donor-only lifetime is 2.1 ns. Since it lies so close to the semicircle, we will assume it is a single exponential.

If you enter select **Single** in the **Known Lifetime Parameters (FRET Donor)** section of the histogram dialog box, and enter 2.1 as the **Donor-Only Lifetime 1**, then press **Apply**, the histogram will draw a magenta circle at the 2.1 ns position along the semicircle and then re-color the portion of the semicircle that represents the 0% FRET to 100% FRET “trajectory” as magenta as well. Any (potentially) FRETting sample with the same donor will lie either somewhere along this magenta portion or within the chord that goes from the 2.1 ns position to the (1.0, 0.0) endpoint.

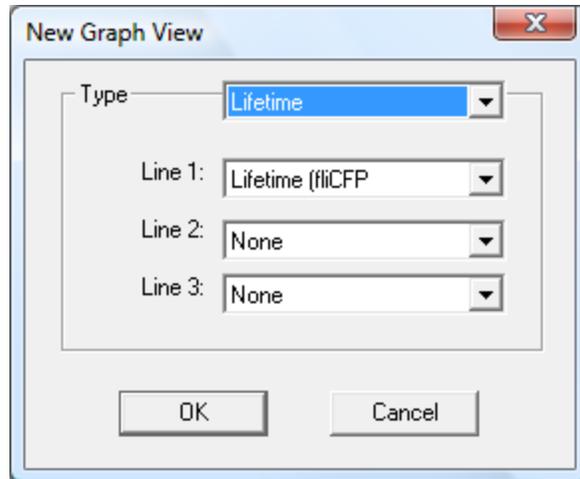
If we double-click on the cluster in an image—in this case it is the first (pre-bleach) timepoint of the same timeseries—then the histogram will draw a straight line from the 2.1 ns position, through where you double-clicked, and on through the semicircle.



Under the plot, the dialog box reports the nearest single-exponential τ of where you clicked as well as the average FRET efficiency (in this case 17.5%) of the region.

Graphing lifetime

Graphing lifetime in timelapse images is straightforward. Create regions (and optionally a background) and select **View > Graph**:



Select **Lifetime** for the graph **Type**, and then choose which lifetime channel(s) you wish to display.

The resulting graph will show lifetime versus timepoint:

