PRACTICAL MANUAL FOR FLUORESCENCE MICROSCOPY TECHNIQUES

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chapter

Frequency-domain Fluorescence Lifetime Imaging Microscopy (FD-FLIM)

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1. Principle and Theory

The excited-state lifetime is defined as the mean time a molecule spends in the excited-state. The excited-state lifetime of a fluorescent probe provides a robust and sensitive measure of the probe's environment. It can change in response to environmental changes such as micro-polarity and pH. It can also change when a suitable molecule in nearby by a process called fluorescence resonance energy transfer (FRET). In the latter case the excited-state lifetime of the fluorophore decreases in a characteristic fashion with distance between the two molecules. The excited-state lifetime, unlike intensity, is a kinetic quantity and as such largely independent of factors such as concentration or optical path length. When the lifetime is resolved spatially and presented as an image we refer to this as a fluorescence lifetime image. The technology used to collect and interpret a fluorescence lifetime image is called fluorescence lifetime imaging microscopy (FLIM).

The principle behind measuring excited-state lifetimes is to excite the molecule of interest and measure the response of that molecule to that excitation. In the time-domain the excitation is pulsed and the response is a convolution of that pulse with the excited-state decay of the molecule-usually for short pulses the emission appears as an exponentially-decaying signal, see Figure 1.

The frequency-domain technique is less intuitive than the time-domain analogue because we are often used to thinking of decay processes in time. But in fact our circadian rhythms operate in the frequency-domain. We are used to waking and sleeping with a given period or frequency which is controlled by the periodicity of night and day. We can also excite a collection of molecules with light that is continuous but intensity modulated with a given frequency. If the molecules emit photons immediately after excitation, then the emission will appear with the same frequency as the excitation and the shape of the emitted waveform will be identical to the shape of the excitation waveform. This is the situation of zero-lifetime. However, if there is a delay between excitation and emission, due to a finite excited-state lifetime, then the emitted waveform will be shifted in phase. We call this a phase shift or a phase lag. A human analogy is jet lag. The light and day cycle is shifted in phase due to air travel from different time zones and this is out of phase with our internal circadian clock.

In the frequency-domain two parameters are obtained from the detected waveforms that related to the lifetime or lifetime distribution. Not surprisingly, the phase shift, is related to the lifetime of the excited state. As implied from the above discussion, the smaller the phase difference between excitation and emission, the shorter the lifetime of the excited state. Another property of a waveform is the modulation. A time-delay between excitation and emission causes a loss of modulation or demodulation of the fluorescence signal. That is the longer the excited-state lifetime the greater the demodulation.

Figure 2 contains a schematic that illustrates and defines modulation and phase-shift.

For a single exponential decaying system characterised by a lifetime, τ, the intensity remaining, *I(t)*, after time, *t* is given by the expression.

$$I(t) = exp(-t/\tau) \qquad (1)$$

The corresponding phase (ϕ) in FD-FLIM is given by the expression,

$$\varphi = Arctan\ (\omega\tau) \qquad (2)$$

And the modulation is given by the expression,

$$M^2 = 1/(1+(\omega\tau)^2) \qquad (3)$$

In equations (2) and (3) ω is the modulation frequency. The lifetime determined from the phase (equation 2) is often referred to as the "phase lifetime" and the corresponding lifetime determined from the modulation (equation 3) is called the "modulation lifetime". For single exponential processes the phase lifetime is equal to the modulation lifetimes. For non-exponential decay processes (those involving sums of

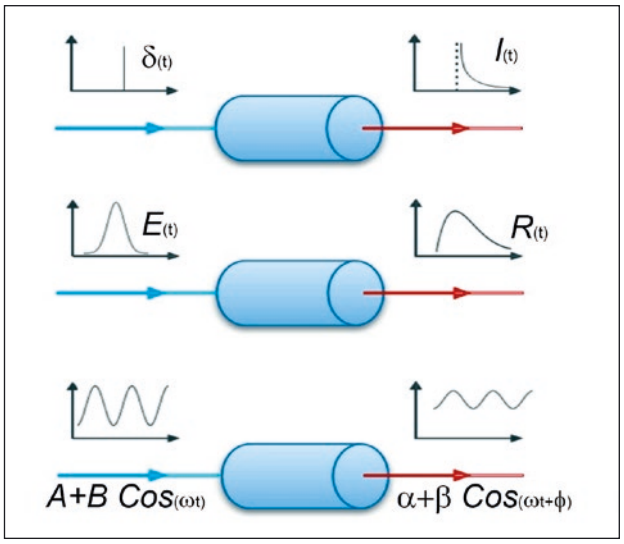

Figure 1 *Schematic representation of the principle behind time-resolved fluorescence measurement techniques. Top: Delta excitation pulse (blue line) excites a fluorescent sample (cylinder) and this sample emits fluorescence with exponential time decay (red line). Middle: If the excitation pulse (blue line) is broad, the response to the excitation appears as broadened emission decay (red-line). Bottom: Sinusoidal-modulated excitation (blue line) and resulting sinusoidal emission (red line). Note the change in shape of the fluorescence due to the finite excited state decay of the fluorophore.*

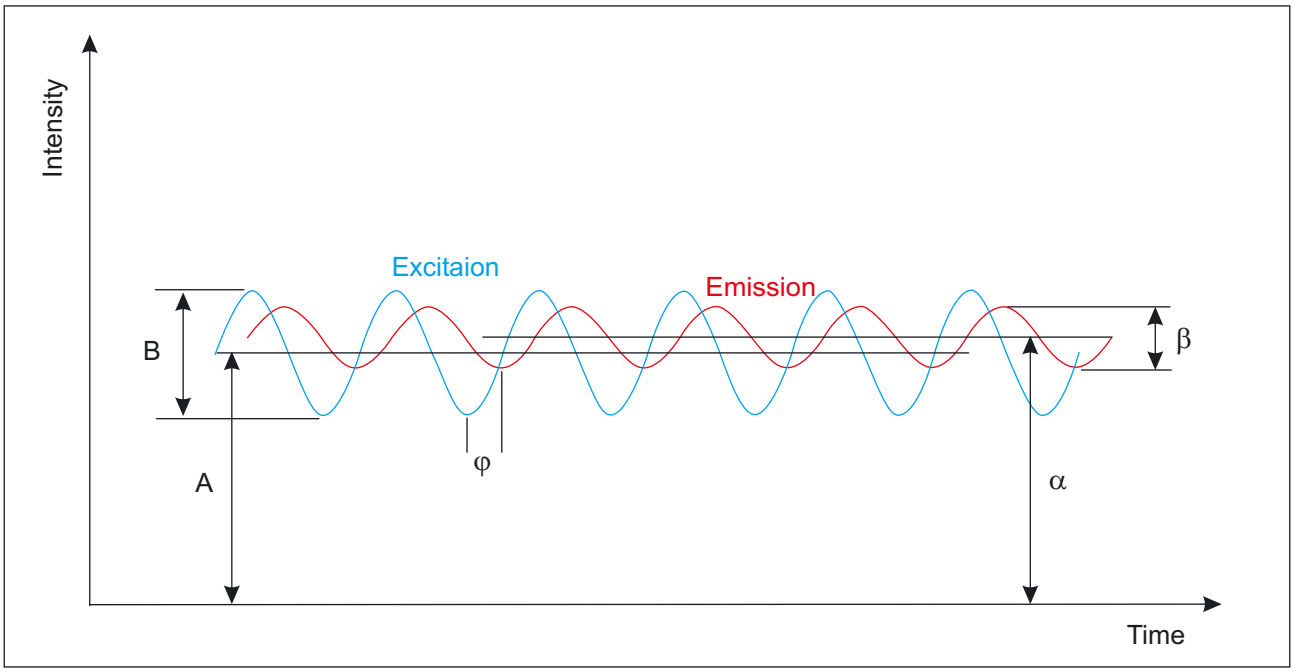

Figure 2 *Schematic representation of excitation and emission waveforms in FD-FLIM. Blue line represents the excitation waveform with average signal intensity A and waveform amplitude B. The red-line represents the waveform of the emission. Due to the finite lifetime of the excited-state, the emission waveform is shifted in phase (φ) and de-modulated, that is the amplitude of the emission waveform (β) divided by the average signal (α) is reduced compared to the modulation of the excitation (B/A).*

exponential functions) the phase lifetime and modulation lifetimes are not equal. Expressions for more complex decaying systems (non-exponential time decays or sums of exponential decays) are given elsewhere. Although determination of these more complex models is possible using multi-frequency methods, in practise measurements of FLIM on biological samples are performed at a single modulation frequency. For questions of biological importance one is usually more interested in a change in the emission decay of a sample through FRET or changes in microenvironment. Importantly, *changes* in the excited-state lifetime of the fluorophore are inferred through a change in the phase and modulation of the emission. Later we will see a representation of this phase and modulation that is particularly convenient and useful for interpretation of FLIM experiments.

How is the Fluorescence Waveform Detected?

Before we go into the "nuts and bolts" of the instrumentation, it is important to consider how the sinusoidal fluorescent waveform is detected. As can be gleaned from equations 2 and 3, to measure lifetimes on the order of nanoseconds requires modulation frequencies of the order of reciprocal lifetimes, i.e. 10-100 MHz. The excitation must be modulated at high frequency and we require the phase and modulation of the emitted high-frequency signal. The determination of the emitted fluorescence signal waveform can be achieved using heterodyne or homodyne detection. In heterodyne detection a high-frequency signal is transformed into a low frequency signal. In homodyne detection the high frequency signal is transformed into a static phase-dependent signal. In both techniques the fluorescence signal is multiplied with a reference waveform derived from a common modulation source.

In the heterodyne technique the gain of the detector is modulated at a slightly different frequency to the frequency of the excitation source. The result of mixing the emission at one frequency with the gain at a slightly different frequency is a new waveform with low frequency and identical phase and modulation to the original (high-frequency) emitted waveform. Time-sampling of this low frequency waveform and subsequent Fourier analysis recovers the phase and modulation information.

In the homodyne method the gain of the detector is modulated at exactly the same frequency as the excitation. This gives a filtered signal that depends only on the phase difference between the emission and the reference waveform. This signal may be sampled by shifting the phase between the detector and the excitation. Repeating this process generates a waveform at each pixel of the image which contains the phase and modulation information.

2. Instrumentation

A schematic of a typical wide-field FD-FLIM is shown in Figure 3. This system is built around a research grade microscope with the light source directed

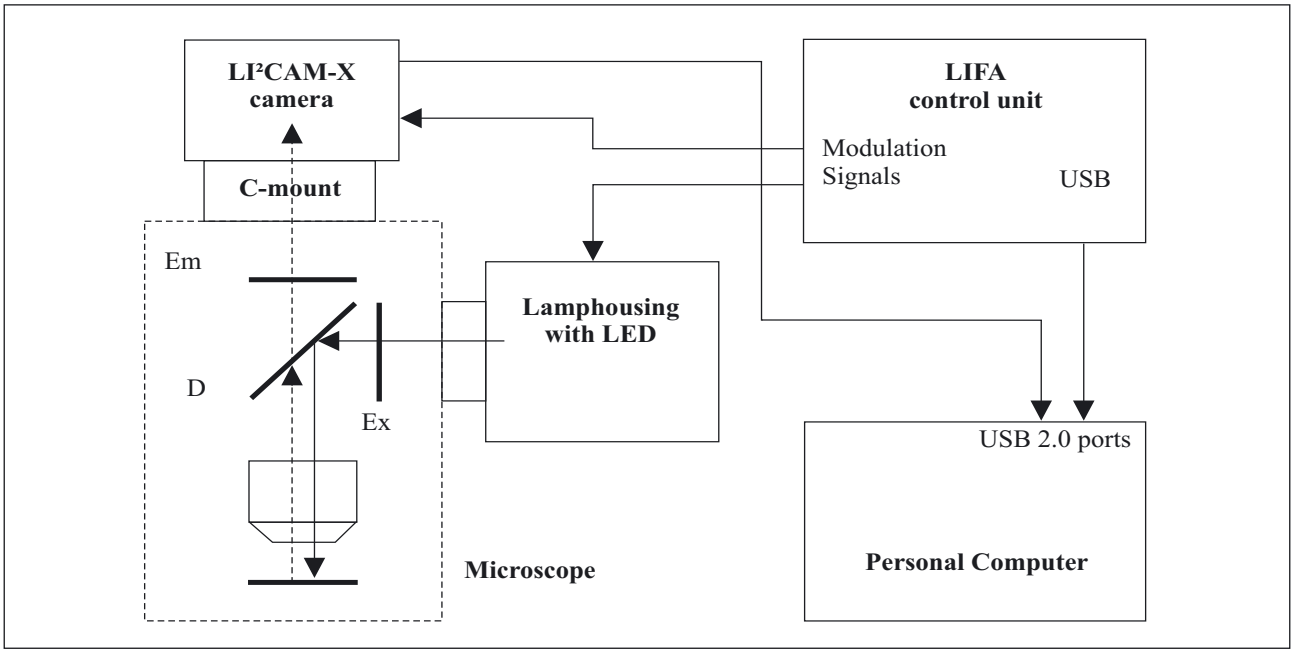

Figure 3 *Schematic representation of the LIFA wide-field FD-FLIM. Components are discussed in the main text. (Diagram from the Lambert Instruments LIFA manual).*

through the back of the microscope and the detector mounted onto an emission side-port (microscope not shown). The difference between a conventional microscope and an FD-FLIM microscope lies in the detector. The heart of this system is the micro-channel plate image intensifier which serves as the mixing device in homo-dyne or heterodyne detection. The gain of the intensifier is modulated at high-frequency under control of the signal generator and this waveform is essentially mixed in the detector with the emission signal waveform that emerges from the microscope. The signal generator sends an identical frequency signal to the light source which provides the modulated excitation waveform. The CCD camera is a detector that provides a digital 2D representation of the image that impinges on the MCP phosphor. The computer contains software that controls the frequency of modulation and shifts the phase between the MCP and light source, reads the images from the CCD camera, and computes lifetime images.

Light Sources

In FD-FLIM any repetitive waveform that excites the molecule of interest is required. For typical lifetimes of 1-10 ns one requires 10-100 MHz frequencies (see equation (2)). Continuous lasers can be used in combination with acousto-optic or electo-optic modulators to provide the periodic, modulated excitation waveform. Pulsed laser systems such as Ti-Sa lasers, have also been used and provide the added advantage of two-photon excitation. Direct electrical modulation of light-emitting diodes and laser diodes has been demonstrated. For example, in the Lambert Instruments LIFA system modulated LEDs or modulated laser diodes are used as the excitation source.

Detectors

The detection of the emitted fluorescence signal waveform can be carried out in a number of ways depending on the configuration of the microscope (scanning or wide-field) or whether the detection is homo-dyne or heterodyne. When scanning is used (either stage scanning with fixed laser or laser scanning with fixed stage) the emission is focussed onto a single detector, usually a photomultiplier tube, an avalanche photodiode or a micro-channel plate detector and the signal is timed with the position of the scanning stage or laser to extract an image. In wide-field FD-FLIM instruments the whole field is illuminated and the image focussed onto an area detector such as a micro-channel plate image intensifier and a charge-coupled device camera.

Microscope

Most FLIM systems are built on a research grade fluorescence microscope. The objective lens is an essential optical element that provides the magnification needed to see objects on the (sub) micron scale. The delivery of the excitation light and the handling of the fluorescence emission differ depending on the type of microscope and the desired imaging modality but most systems employ a dichroic mirror to reflect emitted light to the detector and excitation

and emission filters to select excitation and emission wavelengths.
In confocal systems, hardware is needed to deliver and raster scan a laser beam to the sample and a pin-hole between the emission and the detector is utilised to reject out of focus light. In wide-field systems, no extra hardware is needed aside from the excitation source, signal generator and image intensifier and charge-coupled device camera.

Software
The output of a FD-FLIM experiment is a stack of images that represents a sinusoidal function at every pixel. There are a number of steps required before the raw data stacks can be converted into a lifetime image. These steps include;

1. Background correction. This can be performed in a number of ways. A small region outside the sample is interactively selected and the average intensity value from that region in each phase image is subtracted. Alternatively, an image is collected with the excitation source blocked and this image is subtracted from each phase-dependent image. In-cell background correction is more challenging but can be done in some circumstances as a post processing step (see details later).
2. Correction for photobleaching. All fluorophores photobleach to some extent and if not taken into account FD-FLIM values can be distorted. The traditional photobleaching correction is to record phase images in one sequence then re-record the phase images in reverse sequence. Averaging the two sequences of images corrects for linear photobleaching. A more recent innovation utilised permuting the recording order so that the phase steps are not sequentially increasing but rather pseudo-random in recording order. This second method is advantageous because it obviates the requirement of recording two series of phase stacks.
3. Correction for instrumental phase shift and demodulation. The instrument has an intrinsic phase bias and a demodulation. In the time-domain this is called the instrument response function and represents the finite width of the laser pulse and the timing jitter in the detector and the electronics. In the frequency domain, the light source, electronics and detector all contributed to a finite demodulation and phase of the instrument. This is readily corrected by recording a phase stack of images with a reference of known lifetime (fluorescein, rhodamine 6G are good examples). Because the reference stacks are from solutions with no microscopic detail spatial averaging is usually performed on these solution images before the phase and modulation images are extracted.
4. Calculation of phase and modulation images of sample and reference. Once the image stacks representing corrected images are stored in memory, the phase and modulation images are required because they contain information about

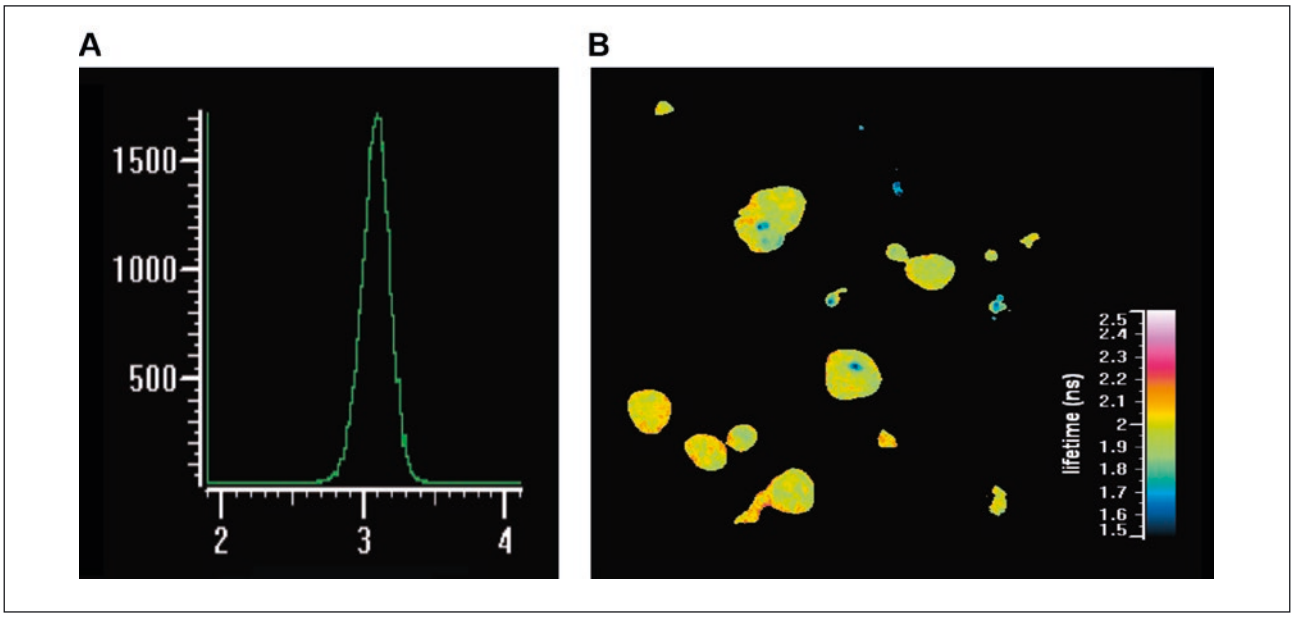

Figure 4 A *Representative lifetime histogram. Plot of the number of pixels versus fluorescence lifetime (in nanoseconds). The large number of pixels in an FD-FLIM image leads to large sample sizes and consequently well-defined lifetime distributions. Even small lifetime shifts of the order of 100ps or less can be readily discerned.* ***B*** *Representative lifetime image. Note the regions in blue that denote very short lifetimes (1.6 ns) compared with the yellow-orange regions (2-2.1 ns).*

the excited-state decay processes at hand. The phase-stacks can be processed efficiently using Fourier Transform methods, namely discrete sine and cosine transformations, which in turn can be manipulated to deliver the required phase and modulations at every pixel location in an image. Direct fitting to a sinusoidal function is also a possibility, which yields the required phase and modulation.

Once the phase and modulation are known then phase lifetime and modulation lifetime images are created (see equations 2 and 3). The lifetime images can be color-coded to aid visualisation of regions with different lifetime. An alternative representation is in terms of histograms. The lifetime is binned into different values on the horizontal axis and the number of pixels in each bin is plotted on the vertical axis. An example of a lifetime histogram is displayed in Figure 4A and an example of a color-coded FLIM image is shown in Figure 4B.

A very useful and convenient visualisation of data is achieved with a plot called the polar plot (or phasor or AB-plot). The phase and modulation is transformed into point on a 2D plot. For a given phase, ϕ, and modulation, M, the coordinates of the point on the polar plot are;

$$x\text{-axis}=B=M\cos\phi \quad (4)$$

$$y\text{-axis}=A=M\sin\phi \quad (5)$$

For a single species the time-decay of the fluorescence emission is represented by a single point on the polar plot at location ($M\cos\phi$,$M\sin\phi$). If the emission decay is single exponential, the phasor will be located somewhere on a semi-circle circumscribed by the points (0,0), (1/2,1/2) and (1,0) and the position on that semi-circle reveals the actual lifetime value. For more complex heterogenous decays the phasor will be located inside the semi-circle. For excited-state reactions involving sensitised acceptor emission or solvent relaxation, the phasor will be located outside the semi-circle.

The polar plot can also reveal data from different experiments (different samples, or same sample different conditions) or data as a function of image location or time or any other hidden variable. The resulting spread of data is often referred to as a polar plot trajectory. The use of the polar plot has many advantages.

(a) Irrespective of the complexity of the fluorescence decay, any fluorophore can be represented as a single point in the polar plot.

(b) Mixtures between different species are represented by the vector sum of the phasors of the

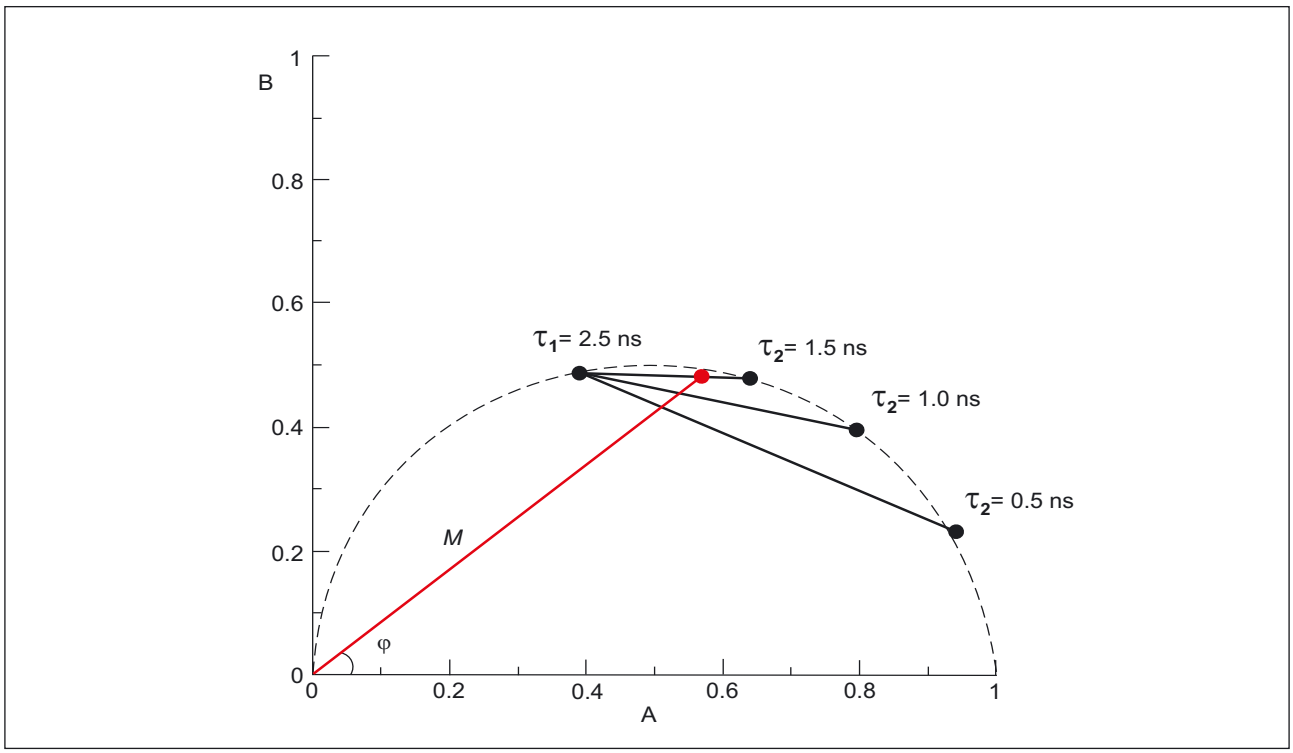

Figure 5 *Polar plot or AB-plot. The red dot represents one fluorescent species with a given fluorescence decay profile. The length of the red-line is the modulation of the emission and angle subtended by the red-line is the phase. Selected single-exponential lifetimes are denoted by the black dots on the dashed semi-circle. Binary mixtures of different lifetime species are denoted by the chords linking the dots. A,B,M and* φ *are defined in the text.*

individual species. All possible mixing combinations fall on a line connecting the individual species. For the mixture of three species the mixture falls inside a triangle. For N-species this will be a polygon with N-vertexes.

(c) FRET experiments can also be simulated taking into account background fluorescence and contributions from non-FRET states.

(d) Data from only one modulation frequency is required.

(e) Analysis of a potentially complex multi-exponential decay problem is reduced to simple rules of vector algebra and trigonometry.

3. Method

Sample: General Considerations

The most important sample in FD-FLIM is the reference solution! The reference must have a defined, single exponential lifetime that is spatially-invariant. If these conditions are not met then the lifetime measurements of the sample will be in error. We have found that a dilute (1-5 μM) solution of rhodamine 6G in distilled water provides an excellent and robust reference solution with a lifetime of 4.1 ns. A few drops (100-200 μL) of this solution applied to a coverslip. Other standards in use include fluorescein (4 ns), rhodamine B (lifetime 1.7 ns), erythrosin (0.080 ns). Scattered light (0 ns) in principle can also be used but care is needed to avoid spurious multiple reflections can cause artefacts.

The sample for FD-FLIM should be prepared in the same way as for standard fluorescence microscopy. That is the cells should be live or fixed, as appropriate, and the molecules or cellular structures of interest need to be specifically tagged with a fluorescent probe. FD-FLIM is compatible with standard dyes (the Alexa dyes, fluorescein, rhodamine) and genetically-encoded probes (GFP, CFP, YFP, and other flavours). One has to be mindful that experimental conditions such as pH, temperature, fixation and mounting can all affect lifetimes. This needs to be taken into account in the experimental design and also when comparing results from different datasets or different laboratories.

For FRET studies one normally compares the lifetime of the donor with the lifetime of the donor in the presence of an energy transfer acceptor. In these cases more samples need to be prepared. One sample with donor-only, one with donor and acceptor, one with acceptor only and an unlabelled set of cells.

Sample: Specific Examples

GFP-fusion construct transfection into cells

A procedure we routinely use for transfecting epidermal growth factor receptor-GFP into HEK293 cells is given below.

1. Sub-confluent HEK293 cells (2.5 X 10^5 cells) were seeded onto sterilized coverslips housed in 6-well plates in 5 mls DMEM + 10% foetal calf serum and cultured at 37°C in 5% CO2. After 6 hours, the media volume was brought up to 5 mls with DMEM + 10% fetal calf serum.
2. Immediately prior to transfecting the cells, the media volume was reduced by aspiration to 1 ml.
3. EGFR-eGFP cDNA was complexed to the non-liposomal transfection reagent FuGENE 6, at a ratio of 1:6, in serum-free DMEM, incubated for 30 mins at room temperature, then aliquoted dropwise onto the cells.
4. After 6 hours, the media volume was brought up to 5 mls with DMEM + 10% fetal calf serum.
5. The cells are left for at least 24 hours to allow transfection.

Immuno-staining protocol

The protocol for antibody-staining and imaging is shown below.

1. Seed A431 cells on sterile round cover slips, washed with PBS.
2. Grow to ~80% confluence.
3. 1x wash with warm (37°C) PBS – aspirate fluids.
4. Fix cells – add 4% PFA to each well to cover the cells (0.5-1.0ml). Incubate 25min @ RT.
5. Wash 2x PBS
6. Incubate with mAb528 – 50μg/ml in FACS buffer (PBS/5% FCS). 18μl of 11.7mg/ml in 4.2ml FACS buffer, allowing 0.7ml/well for total of 6 wells. Incubate 15-30 min RT.
7. Wash 1-2 x PBS
8. Incubate with anti-mouse-IgG FITC-secondary mAb (sheep anti-mouse IgG (Fcgamma chain) FITC conjugate from Jackson Immunoresearch #515-095-071) –50μl of 1.5mg/ml in 3.8ml FACS buffer, allowing ~0.7ml/well for total of 5 wells (final concentration of ~20μg/ml). Incubate 15-30 min @ RT in the dark.
9. Wash 1-2 x PBS
10. Mount cells on slide (no glycerol between the coverslip)

Protocols for FRET studies

For FRET studies one needs ideally four samples;

1. Donor-only sample
2. Acceptor-only sample
3. Donor-Acceptor sample
4. Cell background-sample with no transfection or labelled molecules introduced.

Samples 1 and 2 are needed to compare lifetime of donor with lifetime of the donor in the presence of acceptor (see below).

Samples 2 and 3 can be used to determine FRET through sensitized emission (see below).
For donor-detected FRET studies sample 2 ensures no spectral bleed-through from acceptor into the donor channel.
Sample 4 is to correct the data for background fluorescence signal.

4. Image Acquisition

The reader is directed to the Appendix provided by Lambert Instruments on the operation of the LIFA instrument and obtaining a lifetime image.

5. Data Analysis

The lifetime image takes a bit of getting used to. It is a map of kinetic processes not the intensity or concentration of species as in normal fluorescence microscopy. As a consequence lifetime images can sometimes appear to have less contrast than a fluorescence intensity image. Careful analysis and display of lifetime images can provide improved interpretation.

Histogram Analysis of Regions of Interest
Spectroscopists are used to measuring absorption or emission spectra and measuring shifts in spectra. Lifetimes can be displayed in a similar fashion using histograms- a plot of the no. of pixels versus lifetime. Differences in lifetime between different regions of interest of the same image can be revealed by plotting the lifetime histograms of these regions of interest. Using the ROI tools one can select successive regions, which will be numbered 1,2,3 etc. Then going to the statistics tab tick the boxes corresponding to the lifetime histogram and the ROI number. A color-coded histogram will appear in the window. The statistics function also provides information on the mean, standard deviation and the number of pixels in the ROI. The histogram analysis can also be applied to different experiments. For example in FRET one compares the lifetime of cells containing a donor with cells containing a donor and acceptor. A shift in the donor histogram to lower lifetime values in the presence of acceptor indicates FRET from the donor to the acceptor.

Polar Plot Analysis
Another way of visualising a FLIM experiment is to use the polar plot. This can be accessed using the polar plot tab in the LIFA software or alternatively one can use Enrico Gratton's Globals for Images software. As mentioned before the polar plot represents the phase and modulation values of an image on a two-dimensional graph. For images the polar plots usually appear as a cloud of points instead of a single point. A selection tool is used to point to specific regions of the polar plot and pixels with these phase and modulation characteristics are highlighted onto the intensity image.

Interpretation of Results
Tests of statistical significance
For cell biophysical studies, where biological variability is the rule, statistical tests are an important way of testing whether two sets of observations are significant or insignificant. The simplest implementation is to analyse 20-50 cells (number of observations, N_1) from one treatment and 20-50 cells (number of observations, N_2) from another treatment and compute the corresponding mean phase lifetimes (x_1 and x_2) and variances (σ_1 and σ_2) in the phase lifetime from each treatment dataset.
The t-value, which provides a measure of whether the mean values from each dataset are significantly different, is given by the expression,

$$t = (x_1 . x_2)/((\sigma_1^2/N_1) +)((\sigma_2^2/N_2))^{0.5} \qquad (6)$$

The number of degrees of freedom is given by N_1+N_2-2. Using the number of degrees of freedom and the t-value, a t-table can be examined to determine the significance level of the t-value. For example if 10 cells per treatment condition is measured, the number of degrees of freedom is 18. Inspection of a t-table reveals that for t-values greater than 2.1 the means of the two datasets are significantly different at the 95% significance level.

Background Mixing
For cells containing a high level of fluorescence label background fluorescence is usually ignored in FD-FLIM. However as meaningful, biologically-relevant studies demand protein expression at physiological levels background fluorescence can become an inevitable component of the detected emission. There are generally two types of background. Off-cell background arises from camera offset, room lights, immersion oil, buffers and cover-slips. This type of background can be examined by selection of regions that do not contain cells and subtracted or taken into account in analysis. Cellular autofluorescence is the other source of background and arrises from native (not extrinsically-labelled) molecules contained within the cell eg flavins, collagen etc. This type of fluorescence must be measured in unstained cells before it can be subtracted.
The effect of background mixing into the (desired) sample is given by the simple equations,

$$A\ total = \alpha A\ sample + (1-\alpha)\ A\ background \quad (7)$$

$$B\ total = \alpha B\ sample + (1-\alpha)\ B\ background \quad (8)$$

Where A and B are the sine and cosine components of the phasor (defined above), and α is the fractional fluorescence contribution of the sample emission to the total emission. Equations (7) and (8) can be applied to cell populations, single cells, or at the pixel level. Significant background mixing can be visualised in an FD-FLIM image from the polar plot as an elongated cloud of points that begins at (B,A) *sample* and stretches out to (B,A) *background*.

FRET or no-FRET?
Arguably FD-FLIM is of greatest use in FRET applications for detecting interactions (or conformational changes) between labelled biological macromolecules. In FRET excitation of the donor molecule results in non-radiative transfer of energy to the acceptor molecule. If the acceptor is fluorescent it can emit a fluorescence photon. The requirements for FRET are restrictive. The spectral properties of the fluorophores, the orientation between the fluorophores and the distance are important determinants on the efficiency of the FRET process. These aspects are discussed in detail elsewhere.
Detection and measurement of FRET by FD-FLIM is relatively straightforward but depends on the experimental design. The measurement method is a consequence of the photo-physics of the FRET process itself.

FRET induced donor lifetime quenching in FD-FLIM
FRET adds a non-radiative decay channel to the excited state of the donor. As a consequence FRET decreases the lifetime of the donor molecule in the presence of the acceptor. To detect FRET one measures the lifetime of the donor in the absence of the acceptor (τ_d) and then measures the donor lifetime in the presence of the acceptor (τ_{da}). The FRET Efficiency, E, can be computed with the relation,

$$E = 1 - (\tau_{da}/\tau_d) \quad (9)$$

The donor lifetime can be determined from a sample containing the donor-only (with no acceptor). Alternatively, the donor-only sample can be prepared from the donor-acceptor sample photo-chemically by photobleaching the acceptor (see acceptor photobleaching chapter). It is very important that in the donor lifetime method the donor is uniquely excited and the emission represents the emission from the donor only. In FD-FLIM lifetime is often the phase lifetime or modulation lifetime. The FRET can also be calculated using the polar plot and is visualised as a movement of the donor phasor in a clockwise direction along the universal-circle.
Methods exist for using the polar plot to analyse FRET in the presence of background emission or in the situation of variable amounts of FRET and non-FRET states. The reader is referred to the publications for more detailed accounts.

Sensitized Emission
FRET results in a delayed emission from the acceptor fluorophore because the initially excited donor transfer energy is transferred (albeit invisible, non-radiatively) to the acceptor. This delay gives an additional phase shift to the acceptor emission (over that associated with the normal excitation and emission from the acceptor). This extra phase can cause an effect known as lifetime inversion, that is the lifetime calculated from the modulation becomes less than the lifetime calculated from the phase. This effect also causes the phasor of the acceptor to move in a counter-clockwise movement outside the semi-circle of the polar plot.

Artefacts and Trouble shooting
Photobleaching
Photobleaching can dramatically distort lifetime measurements and in some cases cause an inversion of modulation and phase lifetimes even for simple fluorophores. Reducing the excitation intensity and measurement times can reduce photobleaching. When photobleaching is unavoidable, pseudo-random phase recording can help reduce the effects of photobleaching on lifetime measurements. Consideration of background is needed if photobleaching deteriorates signal to background levels.

Roomlight
Roomlight adds a DC signal to the data. This systematically causes a demodulation of the signal and will distort the lifetime computed from the modulation (i.e. the modulation lifetime will increase). The phase lifetime will not be effected for pure DC signal background. This can be visualised in the polar plot as a line that connects the origin (0,0) to the fluorescence signal. This can be eliminated by turning off the light, covering the sample, or ensuring a background correction image is recorded and subtracted from the phase stacks.

Sample Movement
An FD-FLIM image is a single image derived from several individual images obtained at different times (or different phase steps). An implicit assumption is that there is no movement during image acquisition or perhaps more precisely that the concentration distribution of fluorophores in the image is time invari-

ant during the FLIM acquisition. This is often a good assumption (where fluxes in the cell ensure pseudo-steady-state) or cells are fixed. However, in some cases “comets” can appear in the lifetime images and correspondingly, streaks in the polar plot. These are due to motion of a small number of particles in the image. Whole cell motion will give the effect of shadowing whereby there is a distinctive gradient of high to low lifetime. Motions of a large number of particles will broaden lifetime histograms and cause a blooming of polar plots. In some selected cases this is useful for determining translational diffusion coefficients[23]. Stabilising the sample and decreasing exposure times is the best way to reduce these effects.

Instrument Drift
Drift can sometimes occur due to lack of temperature stabilisation on LEDs or AOMS or electronics. If left unchecked, drift can give erroneous impressions of time-dependent biological phenomena or give erratic results. The simplest way of diagnosing and correcting drift is to measure a lifetime standard or any stable sample periodically. Small lifetime fluctuations (<0.1 ns) are probably due to random fluctuations.
However, any monotonic change in the lifetime of the standard is evidence for drift.
A good way to avoid drift is to carry out drift tests during instrument warm up until stability is confirmed. We have found drift to be a rare problem with our set-up with stability of better than 50 ps over a period of hours. Another way of safe-guarding against drift is by permuting sample collection order so that the same sample or reference is collected at several different times.

Fixation, Antifade
We have found that fixation can alter the lifetime of a YFP-tagged cell surface receptor and more anecdotal evidence suggests it can effect lifetimes of GFP-tagged proteins. The exact reason for this phenomenon is not currently known but it is important to understand that the lifetime of a fluorophore in living cells is not necessarily the same as in fixed cells. Antifade has also been anecdotally attributed to lifetime changes. Because the composition and quantity of antifade may vary from batch to batch or sample to sample it is not recommended to use this with FLIM experiments.

Temperature
Most cell studies are carried out a 4 degrees centigrade, 37 degrees centigrade or ambient temperature (often undefined). The excited-state lifetimes of nearly all organic fluorophores depend on temperature with a decrease in lifetime with increasing temperature. Where possible it is preferable to control the temperature or at least note the ambient temperature at the time of the measurements.

Polarisation Effects
For molecules excited with polarised light, the time-dependent detected emission depends on the excited-state lifetime, the rotational motion of the fluorophore and the emission collection geometry. This can be useful for measuring rotational dynamics of fluorophores. However this effect can also perturb lifetime measurements. Use of a polariser in the excitation (or a laser which is polarised) and an analyser in the emission path oriented at the magic angle (54.7 degrees) is the traditional way to exclude polarisation artefacts in time-resolved spectroscopy. This approach is rarely employed in FLIM probably because of the reduction in attendant signal. Instead, lasers are sent through polarisation scrambling fibres to produce excitation light that is not linearly-polarised. Unpolarised light sources from lamps or LEDS also reduces but does not guarantee complete removal of the effects of polarisation on FLIM measurements.

Noise
Noise is not really an artefact but a reality of the measurement process. Clearly a trade-off exists between reducing photo-bleaching and reducing effects of movement, which requires use of low excitation and fast acquisition, and collection of enough emission photons to ensure nicely resolved FLIM images. The signal to noise ratio can be increased by using averaging or increasing the exposure time. Increasing the averaging or exposure time by a factor of N will increase the signal to noise ratio by a factor of $\sqrt{N}$. An alternative approach, for advanced users, is to use de-noising routines as a post-acquisition step in cleaning up FLIM images. A very detailed and excellent account of such an approach has been published by Professor Clegg’s laboratory.

Optical Elements in the Excitation or Emission Path
Optical elements such as ND filters can add to the optical path length and consequently cause a phase delay in excitation or emission. Consequently care should be taken in ensuring that when extra optical elements are introduced into a sample measurement they are preserved in the measurement of the reference as well.

4. Technique Overview

Applications
A selection of applications is collected in Table 1. The list of FLIM applications is growing rapidly. FLIM is popular in biophysics and cell biology as a means

to measure interactions between biological macromolecules in the cellular environment. Not only is it useful for detecting the presence of these interactions but also is highly quantitative allowing detection of stoichiometry of these interactions as well. FD-FLIM has the distinct advantage of rapid acquisition (up to video rate) making it favourable for detecting dynamics on cellular timescales. FLIM can provide a robust readout of fluorescent biosensors because it is independent of signal intensity and biosensor concentration. FLIM has also been proposed as an alternative tool to biopsies in the clinical setting because autofluorescent lifetimes have been shown to be a function of metabolic state or pathological state of cells and tissues.

Limitations

FD-FLIM requires specialised instrumentation but commercial options are available.

References and Further Reading

Table 1 *Selected FD-FLIM applications and artefact corrections*

Application	Labels	Comment	References
Reviews on FLIM			Berezin et al[1] Wouters et al[2],[3],[4]
Ligand-ligand interactions	Fluorescein/ rhodamine	FRET	Gadella et al[5]
Receptor phosphorylation	GFP, Cy3-antibody	FRET, Global analysis	Verveer et al[6]
Sub-unit assembly	Cy3 and Cy5 direct	FRET	Bastiaens et al[7]
Rotational dynamics	GFP	- FLIM+polarisation	Clayton et al[8]
Spectral FLIM		Prism-based spectrograph	Hanley et al[9]
FLIM with ICS	AlexaFluor488/546 GFP/Alexa555 GFP/mRFP		Clayton et al[10] Clayton et al[24] Kozer et al[25]
Biosensor Application(s) Pre-clinical applications Graphical representation/ analysis	 Unstained tissue Unstained tissue	Phase-suppression, pH gradient11, Ca concentration TD-FLIM,Cancer TD-FLIM,Cardio	Eichorst et al[12] Hansen et al[11] Lakowicz et al[13] McGinty et al[14] Marcu[15] Clayton et al[16] Redford et al[17] Digman et al[18]
Photo-bleach correction	YFP		Van Munster et al[19]
De-noising routines			Spring et al[20]
Fixation effects Lifetime calibration Movement	YFP Rhodamine 6G Beads		Ganguly[21] Hanley et al[22] Lajevardipour et al[23]

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Appendix: Lifetime Acquisition and Analysis from Lambert Manual

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4 System Setup guide

This tour will guide you through the process of recording lifetime images with LI-FLIM. The purpose of this chapter is to get you up and running, and to lead you through the most common tasks that you will do with LI-FLIM. It does not try to optimise your experiment in any way. If you want to start doing real lifetime measurements, then you should read chapter about "Analysis", to get a more detailed description of the effects of different hardware settings and parameters on the measurement speed and accuracy. Likewise, if you come across some menu options or toolbar buttons in LI-FLIM, that are not explained in this guide, please refer to "Reference guide" chapter for a complete reference of all options, features and functionality of LI-FLIM.

Last but not least is the LIFA system and camera, and possibly other hardware correctly connected to the computer (via USB and possibly other cables) and the widefield fluorescence microscope (camera, lamp housing with adjusted LED) and that all drivers are installed and are working correctly. Such that the system is set-up in such a way that by pressing 'Reference' and 'Sample' subsequently, LI-FLIM is capable of taking correct lifetime measurements.

Warning!
The fiber-optically coupled image intensifier to the CCD camera is a relatively expensive device. Therefore it is important to take all the warnings mentioned in the Hardware manual into account. Although the software detects potential damaging levels, it remains important to be aware of possible harm to this device.

4.1 Preparations

Before one can obtain a lifetime measurement one should take care of the following:

Filter cube
Select the proper filter cube with the excitation filter that matches the wavelength of the LED excitation and the emission filter that matches the wavelength of the emitted fluorescence light of the specimen. It is important that the LED emission is completely blocked by the dichroic and the emission filters.
Be aware that for fluorescence resonance energy transfer (FRET) experiments, a band pass emission filter cube is required for the emission of the donor fluorophore only. The emission of the acceptor fluorophore should be eliminated from this signal completely in order ONLY to measure the lifetime of the donor fluorophore and to calculate the difference in donor lifetime before and after FRET. Some examples are given below. For more information on the FLIM FRET theory, see the "Reference guide" chapter and the referenced literature.

Objective
In principle every objective is fine for doing FLIM. However, the higher numerical aperture (NA), the more light is coming to the sample, the less intensification is required and the better lifetime value will be obtained.

Light path of microscope
All ND filters should be eliminated from the light path and the field diaphragm and aperture should be opened as much as possible. The more light is coming to the sample, the less intensification is required and the better lifetime value will be obtained.
During measurements, 100% of the light has to be directed to the camera port. When starting up and between experiments, all the light should be directed to the eyepieces of the microscope.

Reference solution
For the frequency domain method a reference solution is required. The ideal reference is a material or solution with a uniformly distributed concentration of fluorescent molecules and a known single lifetime component that can be used with the same filter cube as the sample. It is not necessary that the reference has the same lifetime as the sample. Advisable is to have comparable brightness for reference and sample. Some possible references are listed below:

HPTS
Sigma Aldrich: "Fluka 56360". HPTS (8-hydroxypyrene-1,3,6-trisulfonate) has an excitation maximum at 460 nm and an emission maximum at 510 nm and thus can be used for e.g. GFP transfected cells (use 1 µM) as well as for CFP transfected cells by using a more concentrated solution. HPTS has a single lifetime component of 5,3 ns and is pH insensitive.

Fluorescein
Sigma Aldrich: "Fluka 46955". Fluorescein has an excitation maximum at 490 nm and an emission maximum at 514 nm and can thus can be used for e.g. GFP transfected cells (use 1 µM) as well as for CFP transfected cells by using a more concentrated solution (use 10 µM fluorescein). This fluorescein solution has a single lifetime component of 4.00 ns at pH above 10. A disadvantage is that the solution is prone to bleaching.

Rhodamine 6G
Sigma Aldrich: "Fluka 83697". Rhodamine 6G has an excitation maximum at 528 nm and an emission maximum at 547 nm and thus can be used for GFP transfected cells (use 50 µM in saline??). Rhodamine 6G saline solution has a single lifetime component of 4.11 ns.

FluorRef slides
The advantage of FluorRef slides (http://www.microscopyeducation.com/fluorrefslides.html) is that each slide has a stable lifetime value and is not prone to bleaching. These slides can be used for evaluation of the LIFA system after some doubts of correctness in lifetime calculations. Note that the slides are very bright; extra ND (neutral density) filters should be used, not to damage the photocathode (see hardware manual for explanation).

Object glass with cavity
The reference solution can the best be used with an object glass with cavity. E.g. from Fisher Scientific: Object glass with cavity; Menzel; Glass thickness 1.2 – 1.5 mm. Diam. Cavity 15 - 18 mm with depth of 0.6 – 0.8 mm.

Erythrosin B:
The lifetime is 86 ps. this gives better (more accurate) results because its lifetime is closer to zero (i.e. the measured phase and modulation are close to the phase and modulation of the excitation light). one need 1 mg dissolved in 1 ml of pure water, fresh (one day it can not be saved). This gives a deep pink solution. One should focus clearly within the drop, preferably far away from the glass. For example, for a YFP emission filter. The excitation can be anything up to 532 nm.

A schematic drawing of the setup with a standard LIFA system, and an upright wide field fluorescence microscope is given in figure 4.1.

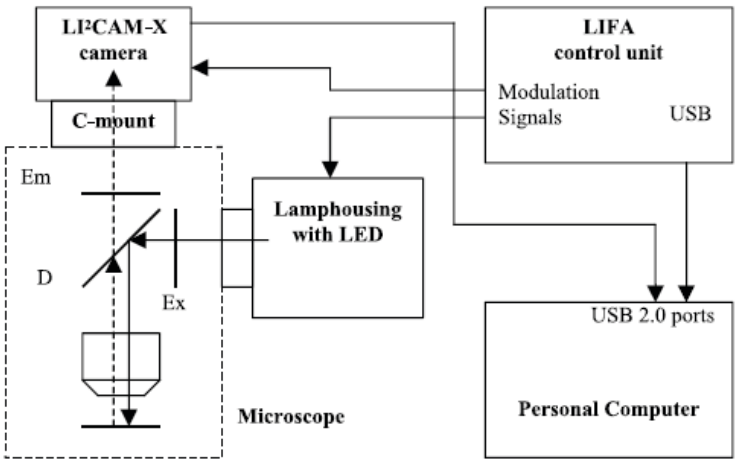


Figure 4.1: *Schematic overview of the LIFA setup in **Modulation** mode with wide field microscope. The filter cube is represented as an excitation (Ex) and emission (Em) filter, and a dichroic mirror (D).*

The excitation light from the LED first passes an excitation filter (Ex). Then it is reflected into the objective lens by the dichroic mirror (D). The sample is excited by the incoming photons, and emits light of a longer wavelength: we have fluorescence. This emission signal passes through the dichroic mirror and is filtered by an emission filter (Em). It then reaches the photocathode of the TRiCAM, to be intensified by the built-in image intensifier tube. You should select the filtercube that matches the wavelength of the LED (excitation), and the wavelength of the emitted fluorescence light of the specimen and the reference material figure 4.2. It is important that the LED emission is completely blocked by the dichroic and the emission filters.

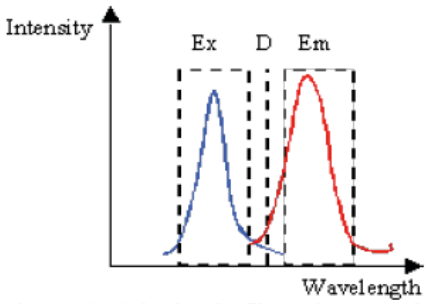


Figure 4.2: *Selecting the filter cube to match the excitation (blue line) and emission wavelengths (red line).*

In the case of FRET detection by FLIM, the LED and filtercube should match the excitation and emission wavelengths of the Donor fluorophore. The emission filter should be a bandpass filter that blocks the emission of the Acceptor fluorophore. See for example the following two FRET pairs:

CFP-YFP FRET pair:

- LED of around 440 nm,
- Filter cube with EX: 436/20 nm, DM: 455 nm, BA: 480/30 nm,
- CFP (Donor): Excitation peak = 439 nm, Emission peak = 476 nm,
- YFP (Acceptor): Excitation peak = 514 nm, Emission peak = 527 nm.

GFP-mCherry FRET pair:

- LED of around 480 nm,
- Filter cube with EX: 480/30 nm, DM: 505 nm, BA: 535/40 nm,
- GFP (Donor): Excitation peak = 484 nm, Emission peak = 507 nm,
- mCherry (Acceptor): Excitation peak = 587 nm, Emission peak = 610 nm.

The fluorescence lifetime is determined on a per-pixel basis, by modulating the LED light source and the gain of the camera at different phase shifts with respect to each other. This process is automated by LI-FLIM to easily acquire all data needed to calculate the spatial distribution of the lifetime(s) of a specimen. Recording a lifetime image usually takes only a few seconds. For a more detailed description of the theory behind frequency-domain FLIM, please refer to chapter “Theory”, and the referenced literature.

4.2 Startup

Before you power up the LIFA system, make sure that the microscope is set up to direct all the light to the eyepiece. This ensures that no light can reach the intensified camera by accident. Switch on the LIFA system, and then launch LI-FLIM by clicking on the LI-FLIM 1.2.19 icon on the desktop, or the LI-FLIM 1.2.19 entry in the Windows Start menu, under the Lambert Instruments section.

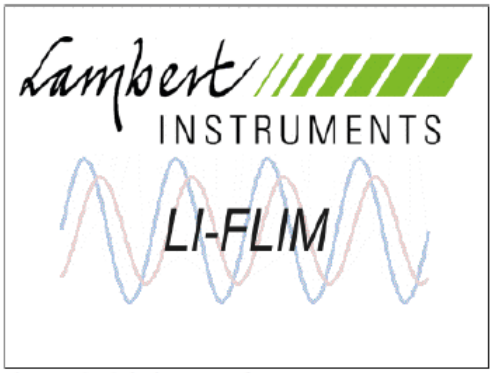


Figure 4.3: *Splash screen of LI-FLIM.*

When LI-FLIM is starting up, it will show a splash screen (figure 4.3). During this time, the software is trying to detect all connected hardware. If the LIFA control unit and the TRiCAM camera are correctly connected, and powered, then the software should detect the presence of these two devices. You can also use LI-FLIM without any hardware connected, but then you will not be able to do lifetime measurements, but only analysis of data that was recorded earlier.

4.3 Layout of the main window

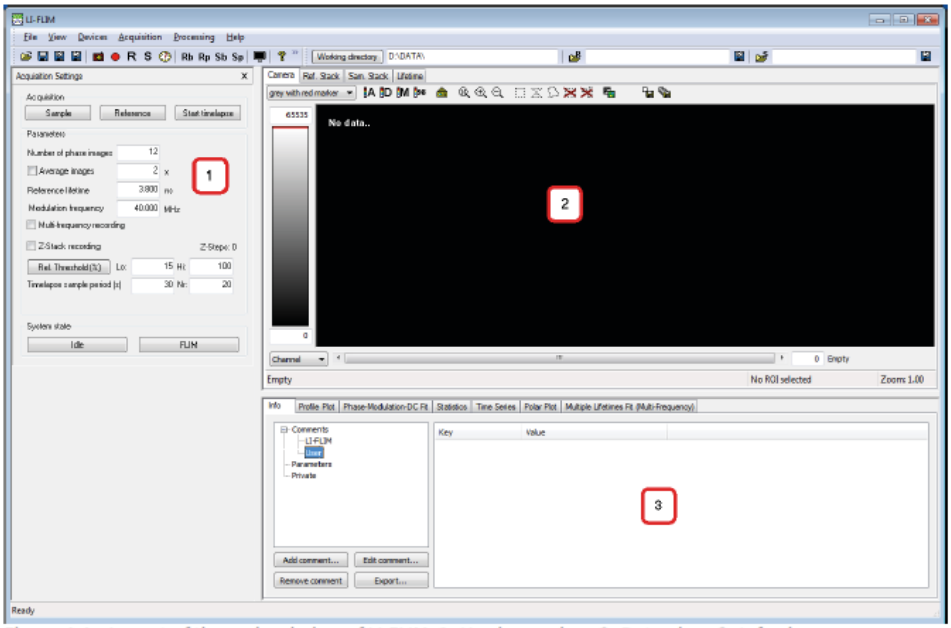


Figure 4.4: *Layout of the main window of LI-FLIM. 1. Hardware view, 2. Data view, 3. Info view.*

LI-FLIM's main window consists of three parts (figure 4.4). At the left, there is the *Hardware view*section. It contains the Acquisition Settings window, and the hardware control windows. In the displayed image there are no control windows present, standard there is one for the LIFA and for the TRiCAM. If your system has other hardware that is controlled by LI-FLIM, the corresponding control windows should show up in this part of the main window. You may need to use the scrollbar at the right side of the Hardware view section to see all device control windows. If not all devices show up in this section, then you should close LI-FLIM and check if all systems are connected correctly and are powered. If the missing devices still do not show up after restarting LI-FLIM, please contact Lambert Instruments for support. At the top right we have the *Data view* section. The recorded images and the calculated lifetime data will show up in this part of the main window. You can select which data you want to see by clicking on one of the tabs at the top of this section. We will see how this works when we record a Reference. At the bottom right part of the main window, we have the *Info view*section. By selecting one of the tabs at the top of this section, you can see various types of information about the recorded data. For example: you can see the statistics of all defined regions of interest on the lifetime data. Or you can inspect the sine that was fitted through the recorded phase-images in a single pixel. To summarize: at the left side, the Hardware view, you control the hardware and the acquisition of images. At the top right side, the Data view, you can see the recorded images, and the calculated lifetime data. At the lower right, the Info view, you can see various types of information about the recorded and calculated data. Note: this layout is set as default when LI-FLIM is started for the first time. You can change the layout to suit your needs by using the *View –> Options* menu. Please refer to “Reference guide” chapter for more detailed information on changing the layout. Recently a 'basic' and 'expert' view have been added to LI-FLIM since version 1.2.10. As a standard LI-FLIM is used in basic view, where only a limited number of settings can be used in the Acquisition Settings, the Lifa Settings and the TRiCAM settings. For less experienced users, the LI-FLIM software will be easier to use.

4.4 Recording a Reference

Before LI-FLIM can determine the lifetime of a specimen, it must know the “system phase” and “system modulation” of the entire setup. The fluorescence lifetime for a single pixel is calculated by recording the phase shift and decrease in modulation depth of the emitted fluorescence light coming from the sample (see chapter “Theory”). By recording a Reference, the software knows the system's phase and modulation for a lifetime of t=0ns. Every deviation in phase and modulation of the sample's emitted fluorescence corresponds to a certain lifetime. In this guide, we will first record a Reference, and then a Sample. Often, you will want to do this the other way around. The specimen of which you want to determine the lifetime may emit very little fluorescence. In that case you would want to optimise the measurement of the Sample, and afterwards record the Reference. Most of the time it is easier to change the amount of fluorescence coming from the reference material (by changing the concentration for example), than from the sample of which the lifetime is to be determined. If you start with a very bright Reference material, you may find that you have to use inconveniently long exposure times or very high intensifier gain settings for recording the sample. It is generally best to keep the intensifier gain setting the same between a reference and a sample recording for getting the most accurate lifetime measurements.

We start by placing the reference material (in our case a piece of fluorescent plastic) under the microscope. We make sure that all light is directed towards the eyepiece of the microscope, and then switch on the LED by clicking FLIM (figure 4.5) The Green box “Gated Closed” should become yellow “Gating active” to signify that the camera is active and the LED is indeed “on”.

Figure 4.5: *Switching on the LED by pressing "FLIM" in the Hardware view section.*

Now try to bring the reference into focus by looking through the ocular of the microscope. This is often not an easy thing to do, because for example a fluorescein solution or fluorescent plastic does not have any structures on which you can focus. You can focus by finding the position that results in the brightest image. You could also try to set the field diaphragm half-open, and then try to focus on the projected diaphragm shadow.

We are ready to start the Live video mode, by pressing “FLIM” an image appears in the Camera tab of the Data view section. The intensity values from the camera images are mapped to displayed image colors using the colormap shown at the left side of the Camera window. In our case, intensity values below 9.3 are set to black, values above 114.5 are set to white, and values in between will get their corresponding level of grey (figure 4.6). Because the emission light is directed to the eyepiece of the microscope, and not to the camera, we should see noise only. If you see a completely black or completely white image, you should adjust the color limits. To change these limits, you can either enter new values by hand, or click one of the four toolbar buttons (figure 4.6)

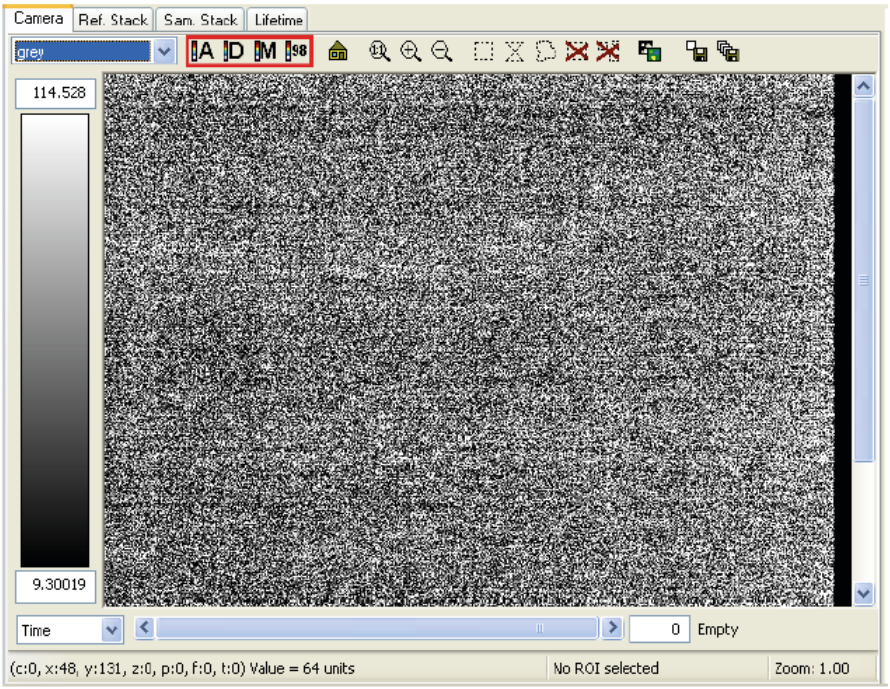


Figure 4.6: *Toolbar buttons to change the colormap limits. A - Scale to Absolute minumum and maximum. D - Scale to minimum and maximum data value. M - Scale to average plus and minus one standard deviation of the entire image data. 98 - Scale to fit 98% of the image data. Only camera noise is visible and displayed with the Grey Colormap*

To see the noise, you would click the "98" toolbar button, to fit the colormap limits to the minimum and maximum values in the camera image, ignoring outliers (dead or hot pixels). Now we can direct the emitted fluorescence light to the camera port of the microscope. Although the gain of the intensifier is low (usually 400V, 450V after starting LI-FLIM), you should already start seeing the image become brighter. You may need to click the "98" toolbar button again to see a dim image appear instead of pure white. As a side note: if your microscope has an 80/20 camera port, and not a preferred 100/0 port, then the sample or reference is partly illuminated by ambient or background light from the environment (e.g. sunlight) that enters the oculars. If this is the case, make sure that the oculars are covered with black cloth to avoid any unexpected background light influencing the measurements. At this point, it is best to select the "grey with red marker" colormap (figure 4.7). This map has a clear indication for overexposed areas of the camera. We are going to increase the MCP voltage to increase the gain of the image intensifier until we have an image on screen that makes use of the full dynamic range of the camera. To do this easily, we want to see overexposed parts of the image clearly.

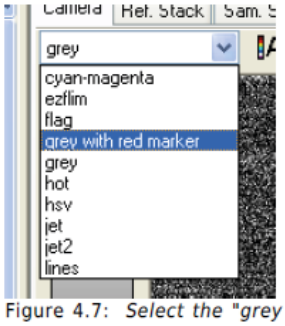


Figure 4.7: *Select the "grey with red marker" colorbar from the drop-down list in the toolbar.*

Now click the "A" button in the toolbar to set the colormap limits to Absolute (0 - 65535). Before we can take a reference we first need to make sure that we setup the right MCP value and therefore we need to find the phase setting that results in the brightest image on screen. The emission and detector signal will go in and out of phase with respect to each other while stepping through the 0 – 360 degrees, resulting in a sinusodial variation in intensity. Keep the phase setting at the position that results in the brightest image, to make use of the full dynamic range of the camera start increasing the MCP voltage until you have an image that is as bright as possible, without overexposing any part of it. If needed, you can zoom the image in and out quickly by using the scrollwheel of the mouse while the pointer is over the image. You may have to click once inside the image area to make it "active" before the scrollwheel works.

We are now set to record the reference phase stack. Click the "Idle" button in the Acquisition Settings window, and fill in the correct lifetime of the reference material (figure 4.8). In our example, this is 3.8ns for a fluorescent plastic. Then enter the number of phase steps taken during recording, in our example, we use 12 phase steps. This is normally a good compromise between measurement speed (less phase steps is faster) and accuracy (taking more phase steps results in more accurate measurements). If everything is set, click the Reference button (figure 4.8). The system will now be set to record a background image first while the lightsource is off, and then the 12 phase images using the "FLIM" settings we stored earlier. After the recording is finished, the system will return to Idle state automatically. During a measurement, a progress indicator is displayed along with a "Cancel" button. If you want to cancel the measurement for some reason, you can click this button. The system may need some time to react. After cancelling a measurement, the system will return to the Idle state.

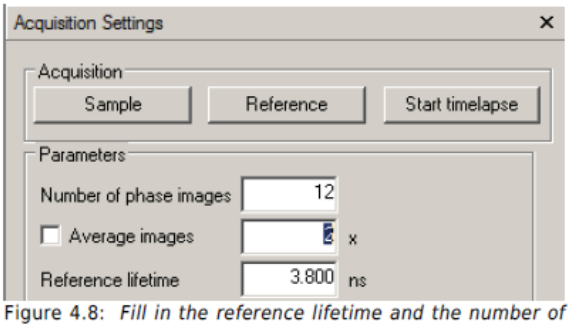


Figure 4.8: *Fill in the reference lifetime and the number of phases. Then click the "Reference" button to start recording the reference.*

The "Ref. Stack" data tab has been filled with new data it contains the recorded phase images. Let's look at the "Ref. Stack" by clicking on the corresponding tab. We can walk through the phases by selecting the

"Phase" dimension and moving the scrollbar at the bottom of the Data view section. We can have a look at the intensities of a single pixel and the sine that is fitted through those intensities, by selecting the "Phase-Modulation-DC fit" tab in the Info view section. You can move the cursor by clicking somewhere in the phase stack image with the left mouse button (figure 4.9).

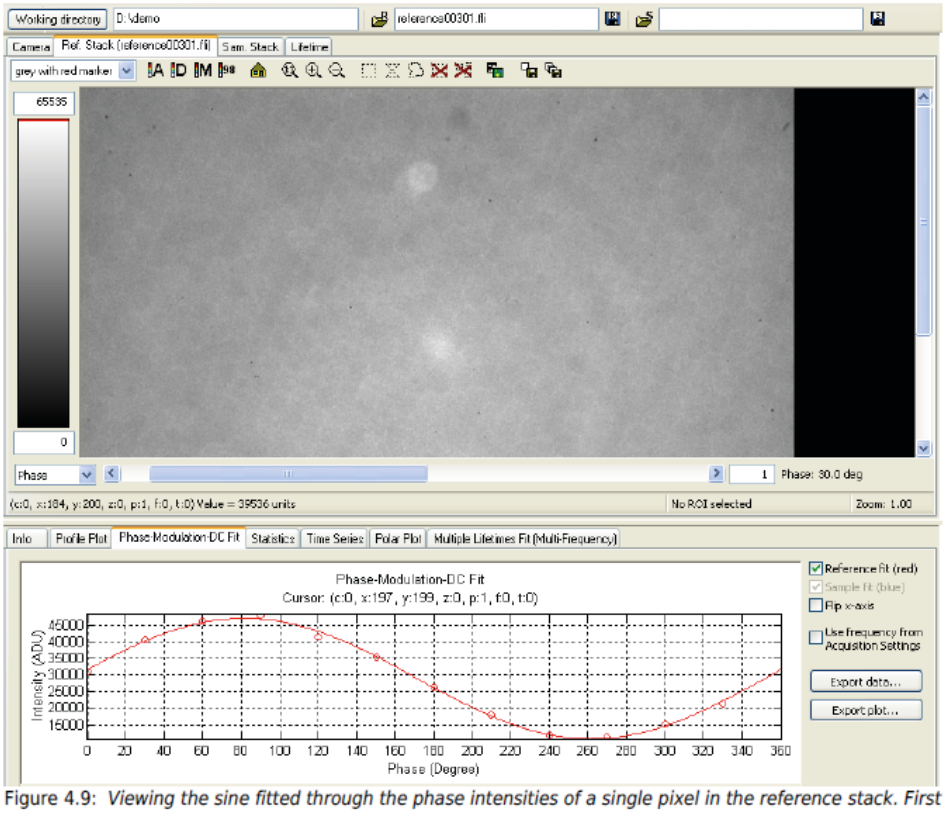


Figure 4.9: *Viewing the sine fitted through the phase intensities of a single pixel in the reference stack. First select the Ref. Stack tab , then walk through the phase images by selecting the Phase channel (Dropdown below the colormap) and using the horizontal scrollbar to see the images that we recorded. In the Info View section, you can see the sine fit through the phase intensities by selecting the Phase-Modulation-DC Fit tab.*

The reference phase stack has automatically been given a new (file)name, in our case it is reference00301.fli. By default, the auto-saving option is off (see Chapter "Reference Guide", the LI-FLIM Options window for more information on using auto-saving), so we will save the recorded data by hand. First, select a suitable folder in which we are going to save all our data by clicking the "Working directory" button. You can select an existing folder, and also create new folders in the window that pops up. Next, click the "Save Reference" button to save the Reference phase image data we have just recorded.

We are now done recording the Reference. It is time to place a specimen of which we want to determine the lifetime under the microscope.

4.5 Recording a Sample and calculating the lifetime

The specimen that we are going to measure in this example has different fluorescent properties than the reference material. Most notably, there is a lot less emission light coming from the sample. We can overcome this by increasing the exposure time of the camera, and changing the Neutral Density filters if

present. These two parameters are also the only two things you can safely adjust between recording a reference and a sample. The MCP voltage should ideally not be changed, in some case it is allowed to change but ideally not more than about 5 to 15 Volt, the amount of allowed voltages can be found in the Testsheet, there is a special lookup table. All other parameters should be kept the same, otherwise the reference will be invalid, and as a result the calculated lifetimes too. Note that changing the setup of the microscope, by selecting another filter cube, changing the diaphragm or selecting another objective lens, can also invalidate the reference. Let's start by placing the specimen under the microscope and focussing it using the eyepiece. We set the LED to 200 mA to get a bit more light. When the specimen is in focus, we turn off the LED and switch the microscope to output 100% of the light to the camera port.

We will now take three steps in one to get a brighter image. We will increase the exposure time of the camera to catch more photons per image than we do now with 100 ms exposure time. We will also increase intensify of the excitation light by removing an ND filter. And, while in the process of adjusting these two parameters we will switch to FLIM mode, and find the brightest phase, and finetune the exposure time further, in the same way as we did when recording the reference. By increasing the exposure time, you catch more “primary photons” and reduce the relative photon noise, at a cost of longer acquisition times. By increasing the MCP voltage, you also intensify the photon noise, but you can do faster acquisitions on an image that uses the full dynamic range of the camera, decreasing the relative contribution of (digitisation) noise from the camera itself.

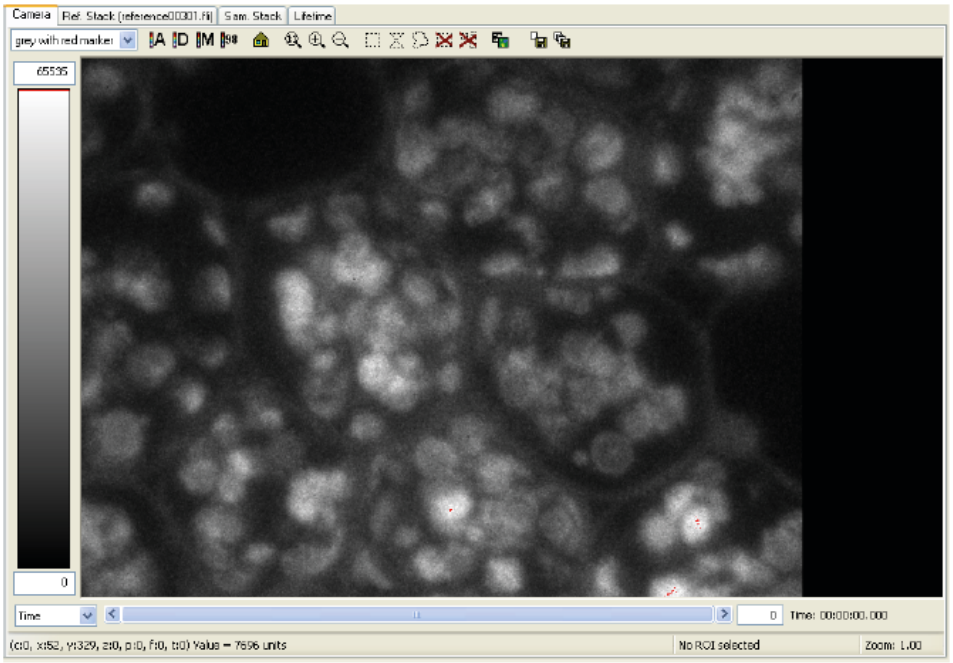


Figure 4.10: *The system is in the FLIM state (modulation signals are on), and the MCP voltage and exposure time have been adjusted to make use of the full dynamic range of the camera at the brightest phase.*

Now we can record a Sample by clicking the “Sample” button in the Acquisition Settings window. A new background image is recorded, and stored in the (invisible) “Sam. Back” tab. Then the phase images are recorded, and stored in the “Sam. Stack” tab. Finally, the phase, modulation and DC data (the invisible “Sam. PMD” tab) and the lifetime image are calculated (“Lifetime” tab). The system is switched to Idle state automatically (figure 4.11).

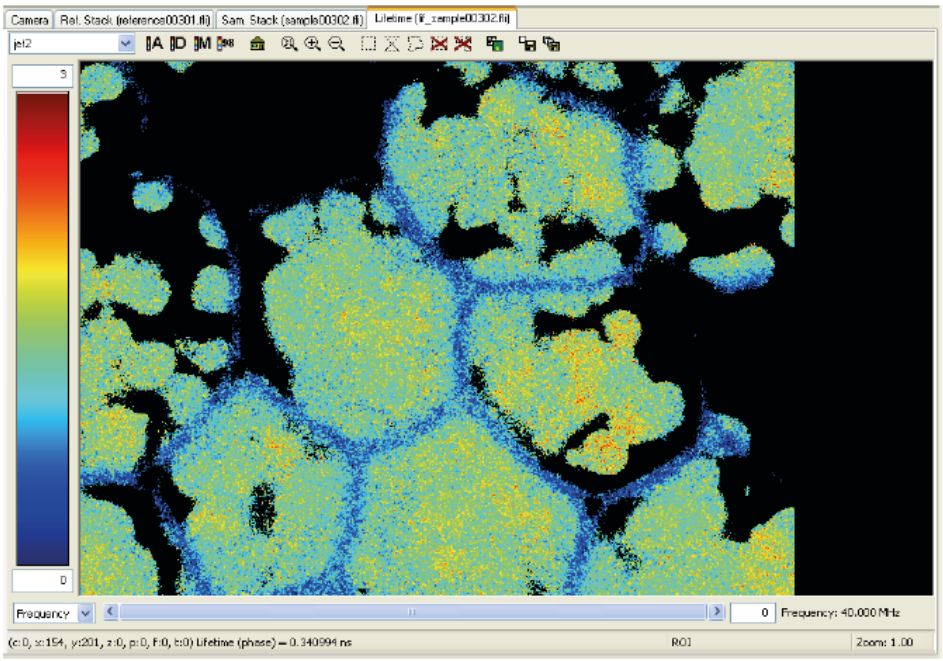


Figure 4.11: *The resulting lifetime image. We selected the "jet2" colormap, and set the lower limit to 0 ns (blue) and the upper limit to 3 ns (red). Because the "Threshold" was set to 15%, some parts of the lifetime image contain no data (black).*

The resulting lifetime image shows the per-pixel lifetime calculated from the phase shift of the Sample's fluorescent emission. The lifetime data has three channels: Lifetime from phase, Lifetime from modulation and DC intensity. The DC intensity channel is the average of the 12 phase images in the Sample stack. Because the "Threshold" value in the Acquisition Settings window of the Hardware View part was set to 15%, some parts of the image will have no lifetime data. At those pixels, the DC intensity was below the value of 15% of the maximum DC intensity in the image. If you want to change this value, you will have to select *Processing –> Recalculate Sample* in the main menu to let this change have any effect on the lifetime image. At this point we want to make sure that we save all data needed for analysis afterwards (see Chapter "Analysis"). An experiment is completely described with the Reference phase stack and the Sample phase stack. All other data (Phase-Modulation-DC and lifetime image) can be recalculated exactly from these two datasets. We already saved the Reference phase stack (as reference00301.fli), so we now have to save the Sample phase stack. Click the "Save Sample" button on the toolbar. In our case, it is saved as sample00302.fli. The FLI file format in which the files are saved by default is described in detail in Appendix B. Saving all these stacks can become cumbersome. LI-FLIM has an autosave option, that will automatically save every new Reference and Sample stack. See "Reference Guide" for more information on this subject. It is good to note that if you had first recorded the Sample, and afterwards the Reference, the lifetime image will not have been calculated automatically: that only happens after a new Sample is recorded, and not after recording a Reference. To calculate the lifetime image after recording a new Reference, you would click *Processing →Recalculate Sample* in the main menu. See figures figure 4.12, figure 4.13 and figure 4.14, on how to do some simple statistical analysis on the data, and how to combine the intensity image with the lifetime image. The Reference and Sample that we recorded will be used in Chapter "Analysis" as basis for a more detailed description on how to analyse the results. We have now recorded our first lifetime image (time for some coffee?). The next two sections show two acquisition modes that we have not mentioned yet: Time lapse recordings and Multi-frequency recordings.

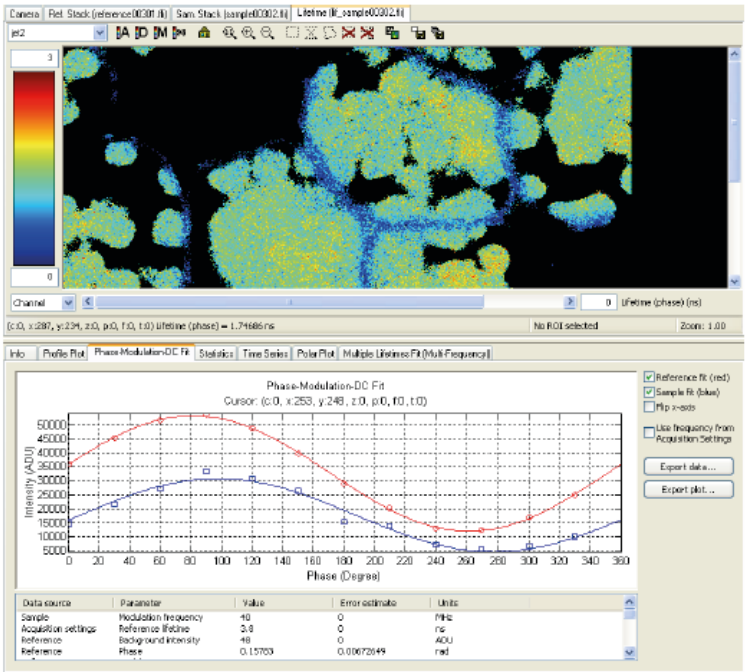


Figure 4.12: *Showing the two sine fits through the phase intensities of a single pixel: the red line is the reference and the blue line is the sample at the same (X, Y) location. The blue line (sample) is shifted in phase and has a different modulation depth than the red line reference).*

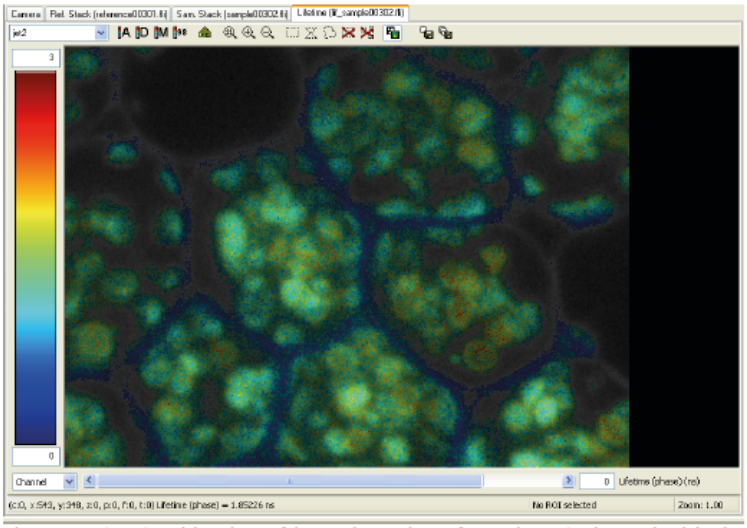


Figure 4.13: *Combination of intensity values from the DC channel with the colormapped lifetime values of the lifetime channel. To do this, first go to the DC Intensity channel using the horizontal scrollbar, then adjust the colormap limits to get a nice image, then go back to the lifetime channel and select a colormap. Finally click the "Combine color" toolbar button.*

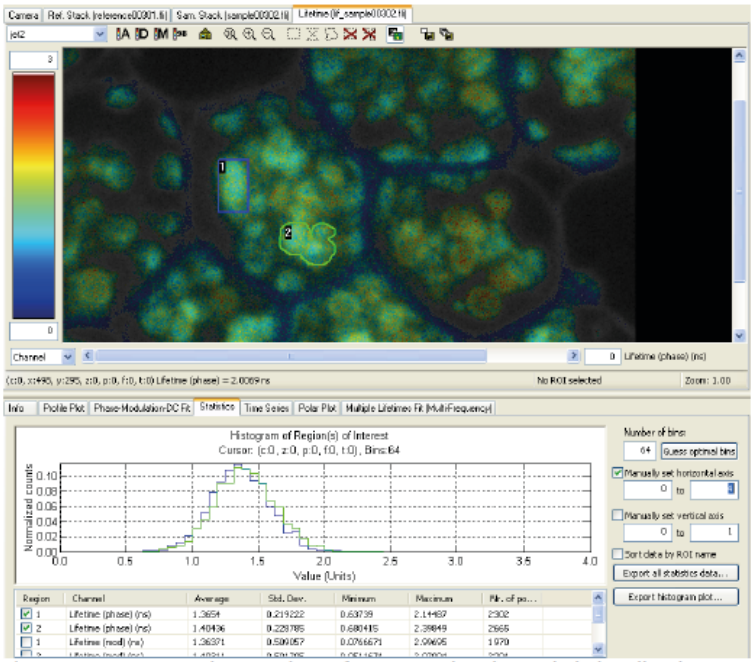


Figure 4.14: *You can draw Regions of Interest using the encircled toolbar buttons, and see statistics in the Statistics tab in the Info View section. You can move regions of interest around by holding the SHIFT key, and click + drag the ROI to a new location.*

4.6 Timelapse recordings

You can record samples at regular intervals to see changes in lifetime over time. To do this, we need a valid Reference, and a single Sample phase stack recording. In fact, only a sample background is enough: the system will record only phase-image stacks during a timelapse recording and uses the current background image for background subtraction. The easiest way to begin with a timelapse experiment, is to first record a normal Sample, to see if everything (intensifier gain, number of phase images you want to use, etc.) is set up OK, and then start the timelapse measurement. In this way, you will automatically have a valid sample-background image, and you have an idea about how long it takes to record a single sample stack.

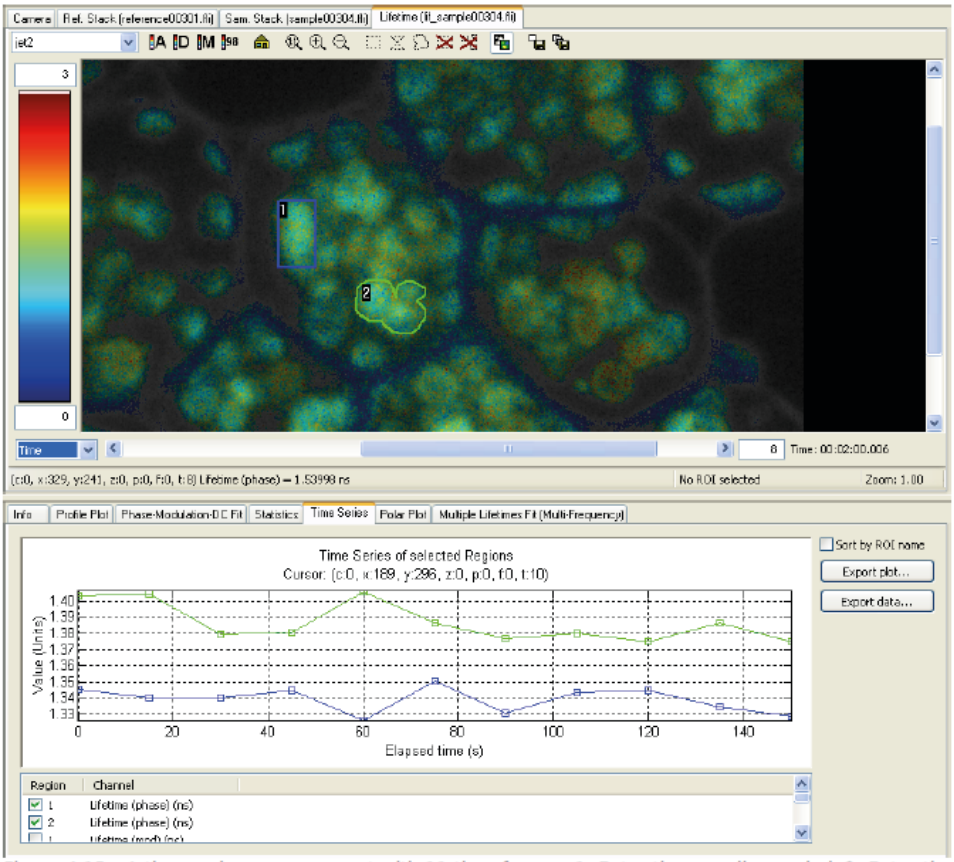


Figure 4.15: *A time series measurement with 11 time-frames. 1. Enter the sampling period. 2. Enter the number of samples to record. 3. Enable, or disable, the Idle state between recordings (only in expert view). 4. Select the Time Series tab and select the regions of interest you want to follow. 5. Start the timelapse recording 6. Scroll back and forth through the data in the time dimension after the timelapse recording has completed.*

To start a timeseries experiment, enter the sample-period, the number of samples you want to record, and decide if you want the system to return to "Idle" state between samples (figure 4.15). If you want to measure as fast as possible, then you can set the period to 0 seconds, and disable the "return to idle state" option. You should enable the "Always return to Idle" option if you are planning to measure for longer periods (say more than 10 minutes). Modulating the image intensifier for long periods of time has a negative effect on its lifespan. You should also create some ROIs on interesting locations, and select the data you want to follow during the timelapse by selecting the checkboxes in the "Time Series" tab. You cannot do this once the timelapse recording has started. Click "Start Timelapse" to begin the measurement. A progress indicator window pops up which shows you the number of timeframes that were already recorded, and an estimated time left to finish the entire recording. Click "Cancel" to prematurely stop the recording. Note that in this case, the sample is *not* automatically saved if you have enabled the autosave option of LI-FLIM. With the "Time Series" tab in the Info view section, you can plot the average values of the selected ROIs over time. This plot is continuously updated while recording a time series experiment. You can also scroll back and forth in the time dimension in the Data View tabs. All data collected (and calculated) for the "Sam. Stack", "Sam. PMD" and "Lifetime" tabs are available. However, LI-FLIM may feel a bit sluggish if you scroll through big time-series files. All data is stored in temporary files, and the time frame that you are viewing is loaded into the computer's RAM memory for fast access. If you scroll through the time dimension, new data must be loaded from these temporary files, which takes some small amount of time. Similarly, if you have a timelapse

experiment with a lot of timeframes, it will be difficult to move around ROIs (by holding the SHIFT key and dragging them with the mouse). Every time the ROI is displaced, all timeframes must be revisited to recalculate the ROI's statistics. The best way to move ROIs in the case of a large number of timeframes is to first select the "Info" tab, then move the ROI(s), and then select the "Time Series" tab again.

4.7 Multi-frequency recordings

Instead of recording a Reference and a Sample at a single modulation frequency, you can also choose to record at multiple frequencies. This has the advantage that, in theory, you will be able to determine multiple lifetime components per pixel and their relative fractions. However, this method is very sensitive to noise, so in practice we will not be looking at multiple lifetimes in single pixels, but instead we will take an entire ROI at once and find the lifetime components that are present in, for example, a single cell. There is one difficulty in recording a multi-frequency phase stack: the modulation depth of the LED varies greatly with the frequency, so you may find that at lower frequencies your Reference or Sample stack is overexposed at some parts of the image, while at higher frequencies you are not using the camera's dynamic range fully. With this version of LI-FLIM it is difficult to prevent this from happening. You will have to experiment with the exposure time setting of the camera or the MCP voltage setting of the LIFA to find reasonable values for the entire frequency range. In this example we will record phase stacks in the 10 – 80 **MHz** range, and we will use the settings that were found to be OK in the 40 **MHz** single frequency case. We will ignore a little amount of overexposure in the lower frequency phase stacks. Click the "Setup" button next to the "Multi frequency recording" checkbox in the "Acquisition Settings" window. The window shown in figure (figure 4.16) will pop up. We enter 10 **MHz** for the lowest frequency, 80 **MHz** for the highest frequency, and 7 for the number of frequencies. Next, click the "Generate logarithmically distributed list" button to update the frequency list at the bottom of the window. This exact list of frequencies will be used during the Sample and Reference recording. You can edit the list by hand if needed. Make sure that the "Number of exponentials to fit" (= number of lifetimes) is set to 1. This will speed up the per-pixel lifetime calculation, and we can always re-calculate the lifetime image using another number of exponentials. The other settings can be left at their default values. They are discussed in more detail in section 5.8.

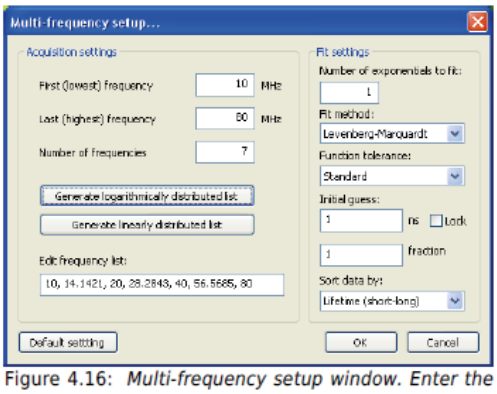


Figure 4.16: *Multi-frequency setup window. Enter the lowest and highest frequencies to be used, and the number of frequencies. Then click the "Generate logarithmically distributed list"-button to update the frequency list at the bottom. Also make sure the "Number of exponentials to fit" is set to 1 for now.*

After clicking "OK", we enable the "Multi frequency recording" checkbox in the Acquisition Settings window. As we have our Sample still under the microscope, we click the "Sample" button. The system will start with taking a background image at every frequency, and continue with recording an entire phase stack of twelve images at every frequency in the list. When the sample is recorded, we save it to disk, resulting in a ~45 **MB** file, which contains 7 background images and 7 x 12 = 84 phase images, all loss-less compressed into a single file. Next we place the Reference material under the microscope and find the optimum settings for 40

MHz. Then, we make sure the "Multi frequency recording" checkbox is still enabled and click the "Reference" button to record a multi-frequency Reference. In this case, the lifetime image is not automatically recalculated after recording the Reference (that is done only after recording a new Sample), so we will do that by hand: select *Processing ->Recalculate Sample* from the main menu. Note: The frequency will not automatically be reset to the single frequency value it had before.

If we look at the Statistics tab we see that the lifetime image now has four channels: A *Lifetime 1*, *Fraction 1*, *Chi-square* and *DC intensity* channel. If you had entered more than one exponential in the multi-frequency setup window (figure 4.16), then you would find more channels here: every extra exponential adds a lifetime and fraction channel. In this single-lifetime-fit case, the lifetime image looks fine. But when fitting multiple lifetimes per pixel, the data will usually look very noisy, having pixels with lifetime components of billions of nanoseconds, with a very tiny fraction for example. In that case, the Statistics tab does not help very much in the analysis of the results. Instead we will select the "Multiple Lifetimes Fit" tab in the Info View section for analysis (figure 4.17).

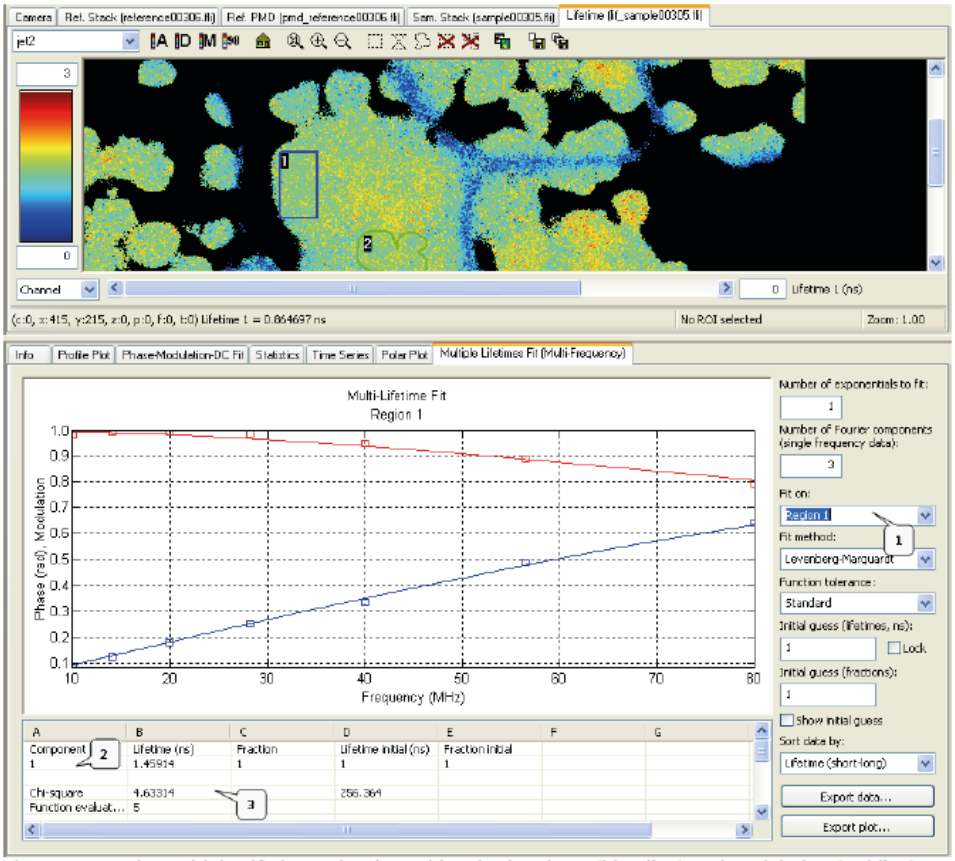


Figure 4.17: *The Multiple Lifetimes Fit tab. In this tab, the Phase (blue line) and Modulation (red line) are plotted for a single pixel, or region(s) of interest. In this case, Region 1 is selected (1). The table (2) lists the Lifetime and Fraction for every lifetime component found by trying to find the best fit for the selected number of exponentials through the phase and modulation data. Chi-square is a measure of the goodness-of-fit (3).*

In the Multiple Lifetimes Fit tab (figure 4.17), we can extract multiple lifetime components from a single pixel, a region of interest, all regions combined, or the entire image. In our case, we want to look at region 1. If you know the number of lifetime components present beforehand, you can enter them in the "Number of

exponentials to fit" edit box at the top left of the Multiple Lifetimes Fit tab. In this example, we do not know how many lifetime components should be present. In that case, we start with fitting for a single component, and note the value of Chi-square. Then we start increasing the number of components and see if Chi-square gets smaller by a significant amount (say 2x smaller). If Chi-square hardly gets any smaller, or increases, then we know that our new "model" is wrong.

Table 3-1. *Chi-square values for different number of exponentials to fit. This shows that the single-exponential model is the best one for our sample.*

Number of exponentials to fit	Chi-square
1	4.6
2	5.5
3	6.8 (figure 4.18)

Table 3-1 shows the results in our case. The single-exponential model is the best one for our sample (at least for the data in region 1). When fitting more exponentials, we get an increasing Chi-square. What you will generally see in the list of lifetime components when you are fitting too many exponentials, is that several components have almost exactly the same lifetime, or wildly different lifetimes (say thousands of nanoseconds) with tiny fractions (0.00001 for example, see also figure 4.18). This is also a sign that the model is a bad one, and the number of exponentials to fit should be reduced.

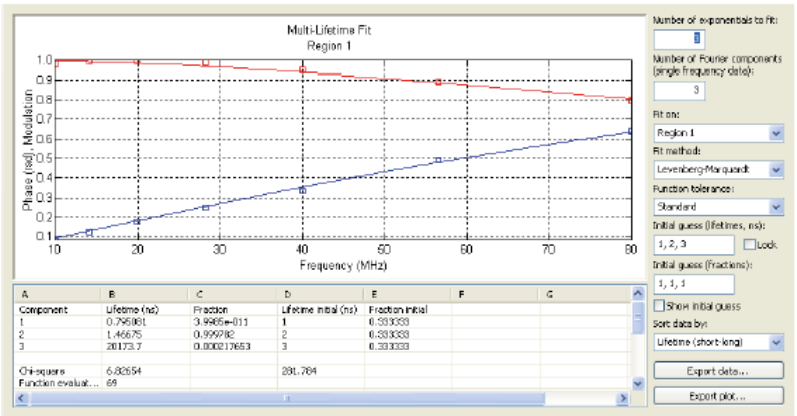


Figure 4.18: *Three component fit. Two lifetime components (1 and 3) have very small fractions: a sign that there are less than three lifetime components present in the sample.*

We now have seen the three basic measurement types: a single Reference and Sample, a Timelapse sample, and a Multi-frequency Reference and Sample. The next chapter will go into more detail about how to optimise your measurements. Chapter "Analysis" shows you how to analyse the samples you recorded in more depth: the buttons, edit fields, check boxes etc. that we did not discuss in this chapter will be explained there.

4.8 Summary

1. **Recording the Reference**
    - I. **Start LI-FLIM software**
    - II. **Place reference material with known mono-exponential lifetime under the microscope**
    - III. **Switch the light beam to the eye port of the microscope**
    - IV. **Set the LED current to 100 mA or laser to 10 mW**
    - V. **Switch the LED/laser on by clicking the LED DC/MOD. DC checkbox**

VI. **Focus using the eyepiece**
VII. **Click “Idle” to switch off the LED/laser**
VIII. **Switch the light beam to the camera port of the microscope**
IX. **Set the MCP voltage to minimum (400 V) and the Exposure time to 100 ms**
X. **Click “FLIM” to switch on the LED/laser and the image intensifier in modulation mode**
XI. **Draw a region of interest in the middle of the camera image**
XII. **Enable the Statistics tab and find the average ADU value**
XIII. **Determine the brightest phase (= highest average ADU) by sliding the phase control from 0 to 359 (if you do not see a difference between phases incrementally increase the MCP voltage by 10 V)**
XIV. **To make use of the whole dynamic range of the camera, increase the MCP voltage until the maximum ADU in the statistics reaches 55.000-65.000 (anything above 65.000 means overexposure and thus possible damage to the camera). You might also want to adjust the Exposure time**
XV. **Click “Reference” to record a reference phase image stack**
XVI. **Click “Save Reference” to store the stack on disk (obsolete if Autosave is enabled)**
XVII. **Remove the reference material**
XVIII. **Switch the light beam to the eye port of the microscope**

2. **Recording the Sample**
   I. **Unless noted here do not change any parameter of the system between reference and sample!**
   II. **Place specimen under the microscope**
   III. **Switch the LED/laser on by clicking the LED DC/MOD. DC checkbox**
   IV. **Focus using the eyepiece**
   V. **Click “Idle” to switch off the LED/laser**
   VI. **Select “Edit comment” on the Info tab and give a description of your sample (very useful afterwards)**
   VII. **Set the MCP voltage to minimum (400 V) to prevent overexposure**
   VIII. **Switch the light beam to the camera port of the microscope**
   IX. **Click “FLIM” and determine the brightest phase and optimal MCP and Exposure time settings. Best results are obtained with approximately the same MCP voltage (± 15 V) as used for the reference. Exposure time may be increased**
   X. **Refocus the microscope using the camera image if necessary**
   XI. **Click the “Sample” button to record the sample phase stack and get a lifetime image**
   XII. **Don’t forget to save your data!**

Note that you can safely change the

- Exposure time of the camera
- ND filters

between recording a reference and a sample. Every other parameter should be kept at the same value. You should also *not* change the filter cube, objective lens of the microscope or diaphragm between reference and sample. Changing one of these things may result in inaccurate or invalid lifetimes. If you exit LI-FLIM, all acquisition settings and hardware parameters will be stored. The next time LI-FLIM is started, these values will be restored, except for the MCP voltage (to prevent accidental overexposures).

6 Analysis

This chapter will explain how to analyse the samples you record. Either directly after recording them, or afterwards after loading previously stored samples. You can also run LI-FLIM on a computer with no hardware attached to analyse stored data.

We will load the files that were stored while performing the steps in Chapter "Optimising lifetime measurements". The results are completely uninteresting from a biological point of view. Instead, we will focus on how to do the analysis and not on the results we get.

The currently selected Info tab generally shows information about the currently selected data tab: if you have selected the Statistics tab and the Lifetime tab, then you are watching the statistical data of the Lifetime image.

Most Info Tab windows will update themselves "live". For instance, the Statistics tab can show live histograms and statistical data while the camera is running. This means that the computer may become a bit sluggish if you have drawn many regions of interest, and are displaying the statistics and histograms during live video mode. Similarly, if you select the Time Series tab, and you have a Sample with many timeframes, it will take some time before the tab is displayed: all time frames stored in a temporary file on the hard disk are visited, to update the statistics of all ROIs before the data can be displayed.

Note that the "Reference Guide" refers to this chapter for a detailed description of the various Info View tabs.

6.1 Preparation

Before we begin describing the different Info View tabs, make sure that you have a Reference and a Sample file ready. Either by recording a new Reference and Sample, or by loading them from disk using the File menu or the Working Directory toolbar. When loading a Reference or Sample directly after starting LI-FLIM, or when loading a Reference or Sample phase stack that has a different number of pixels, you may see the window in figure 6.1 being displayed. LI-FLIM was not able to calculate a lifetime image using the Reference and Sample data that are currently loaded. Once both Reference and Sample tabs contain compatible phase stacks, then a Lifetime image can be calculated automatically.

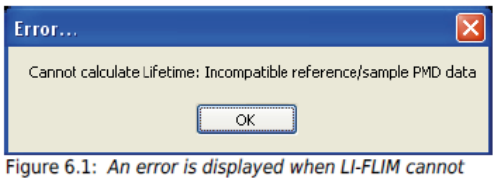


Figure 6.1: *An error is displayed when LI-FLIM cannot recalculate the lifetime image after loading a new Reference or Sample stack. You can turn off automatic recalculation of the lifetime image in the LI-FLIM Options to prevent this error message from being displayed.*

Make sure that you now have a valid lifetime image. Check if the entered Reference lifetime is OK. If not, enter a new value and select *Processing -> Recalculate reference* and *Processing ->Recalculate sample* if needed.

6.2 The Info tab

The “Info” tab (figure 6.2) shows data in text format that is stored with the image data currently selected.

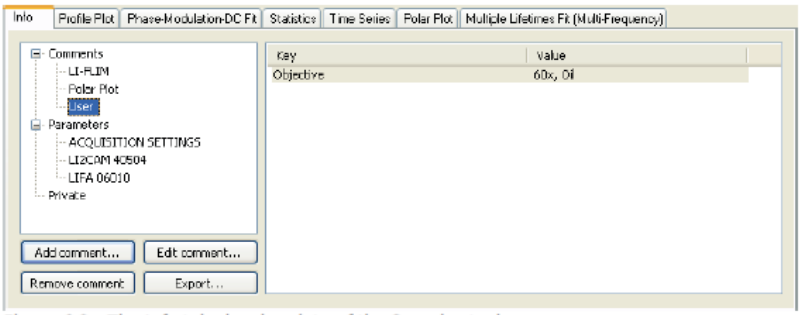


Figure 6.2: *The Info tab showing data of the Sample stack.*

You can add, edit and remove comments. They take the form “Key = value”. In this example, a user comment “Objective = 60x, Oil” has been added. Every Data view tab has its associated Info. When you enter a new comment for the Sample stack, it will not show up in the Reference stack. When the data (i.e. the Sample stack) is saved, this info, including the user comments, are stored with it.

LI-FLIM remembers the last entered user comments for every Data View tab. When LI-FLIM is restarted, those comments will already be present, even if no data is loaded/recorded yet.

For every new Sample, the Parameters section (figure 6.3) is updated with the parameters used for recording it. Here you can see which settings were used for a specific sample.

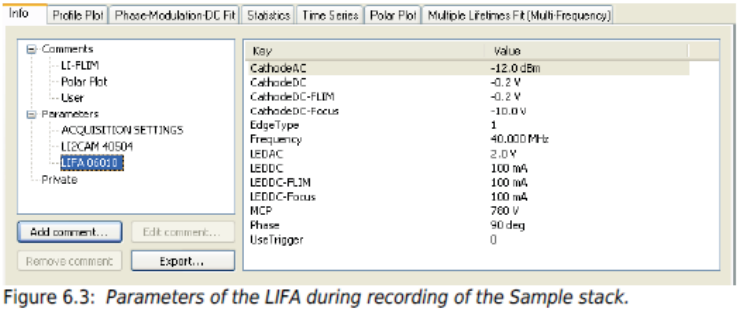


Figure 6.3: *Parameters of the LIFA during recording of the Sample stack.*

With the “Export” button you can store all Info data as a text file. Note however that this text file cannot be used to set all connected hardware back to the state that it was in at the time the image stack or lifetime image was recorded/calculated.

6.3 The Profile Plot tab

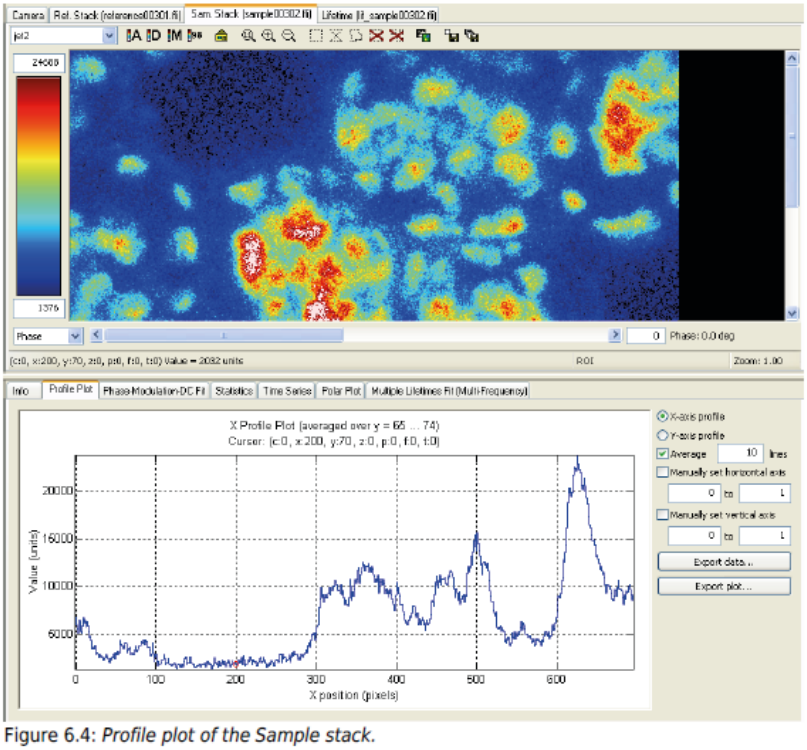


Figure 6.4: *Profile plot of the Sample stack.*

The profile plot shows the pixel values of the selected Data tab (figure 6.4) along the X or Y direction at the current cursor location. You can change the cursor location by left-clicking in the image in the Data View section.

You can select an X or Y axis profile (see right hand side of figure 6.4), and the number of lines to average. In this example, we have set averaging to 10, to smoothen the plot. The ranges of the horizontal and vertical axes of the plot can be set to fixed values, to prevent auto-scaling.

The data of the plot can be exported (“Export data” button) as a Comma Separated Value file, or an Excel sheet. The plot itself can be exported as a bitmap image (“Export plot” button).

A small red circle in the plot shows the actual cursor location along the selected axis (in this example it is displayed at x = 200).

6.4 The Phase-Modulation-DC Fit tab

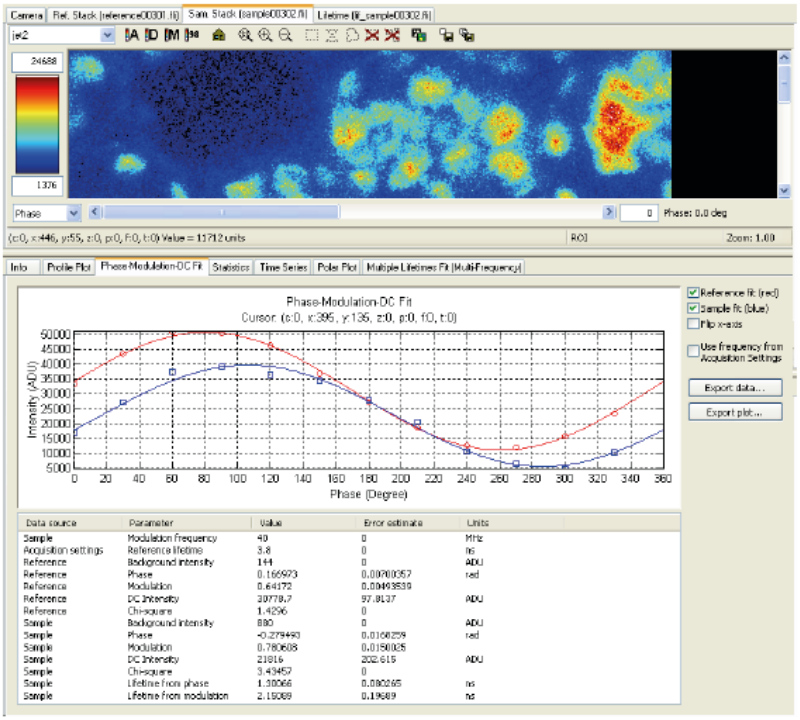


Figure 6.5: *The Phase Modulation DC Fit tab shows the phase intensities of a single pixel in the Sample and Reference phase stack.*

The Phase-Modulation-DC Fit tab (figure 6.5) shows the phase intensities of a single pixel of the Reference and Sample phase image stack. You can change the cursor location by left-clicking inside the image displayed by the currently selected Data tab. It is not important which Data tab is currently selected, because the data from the Reference Stack and Sample Stack tabs is always used. You may want to select the Lifetime tab, and set the cursor to interesting locations in the image, for instance.

The red line is the sine fit through the Reference phase stack intensities. The blue line is the fit through the Sample phase stack data. The hollow squares are the actual measured intensities at the cursor location.

You can export the data (“Export data” button) as a Comma Separated Values text file, or an Excel sheet. The plot itself can be exported (“Export plot” button) as a bitmap image.

The table below the plot shows the calculated values for Phase, Modulation, DC Intensity and lifetime along with an error estimate. These error estimates, (and the Chi-square), are really just that: estimates. LI-FLIM does not directly fit a lifetime for a pixel: it fits sine waves through the Reference and Sample data, using a Fourier transform, and from those sines, the lifetime is calculated. Because the image intensifier gain and the LED lightsource are not modulated with a pure sine wave, there will be higher order components present. LI-FLIM cannot determine easily if deviations from the fitted first order sine are due to noise, or to higher order components. For the error estimates, all deviations from the fit are assumed to be caused by noise. This usually gives an error estimate that is higher than the actual value, but still gives an indication about the accuracy of the measurement.

In practice, you can use this Info tab to quickly determine if something went wrong during a Sample or

Reference recording. You can see at a glance if there is very noisy data, invalid data (e.g. half of the sine is OK, but for the last part all phase intensities are equal), overexposure (if the actual phase intensities do not follow the sine at its top, but are clamped to the maximum intensity value of 65535), etc.

6.5 The Statistics tab

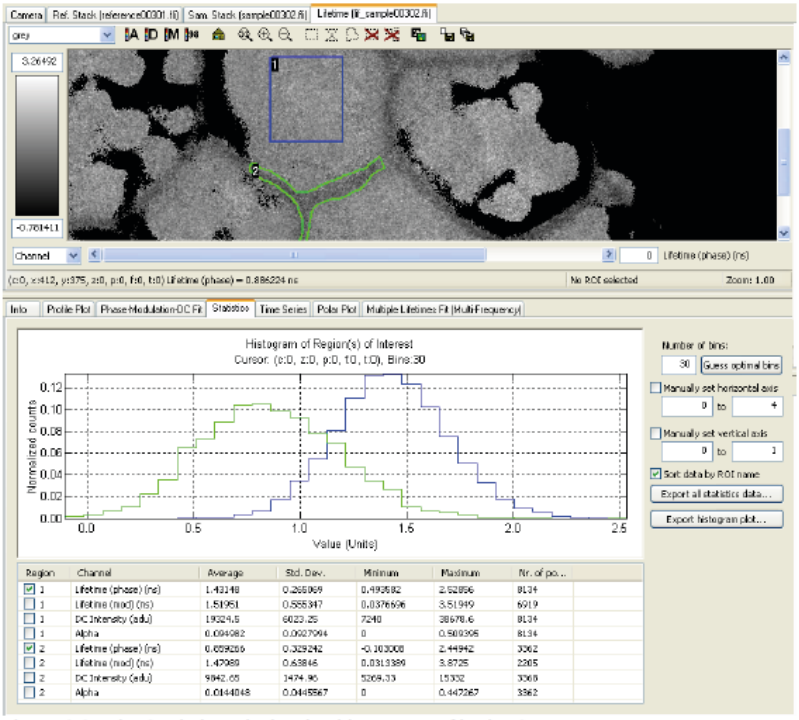


Figure 6.6: *The Statistics tab showing histograms of both ROIs.*

With the Statistics tab (figure 6.6), you can see statistical data of the pixels within the defined regions of interest. The statistics of the currently selected Data tab are shown, at the currently selected cursor location (phase, Z, frequency, time) of every channel present. In (figure 6.6), the Lifetime image is selected, and the four channels Lifetime (phase) (ns), Lifetime (mod) (ns), DC Intensity (adu) and Alpha are listed in the table for every region of interest.

Every line in the table shows the Average, Standard Deviation, minimum value, maximum value and number of valid pixel values of the ROI. Different channels within the same Region of Interest may show a different value for “Nr. of points”. In this example, the “Lifetime (phase)” channel contains a different number of “not a value” pixels, which are ignored for the statistics, than the “Lifetime (mod)” channel.

To display a histogram of the pixel values of an ROI, select the checkboxes in front of the data that you want to see. The displayed lines have the same color as the ROI that they belong to.

When viewing the statistics of intensity images, you can click the “Guess optimal bins” button to change the number of bins for the histogram to such a value that no “gaps” occur. The TRiCAM produces 16 bit intensities, with a 12 bit resolution. This means that some values will never occur, and this could result it the histogram showing gaps, which may be annoying.

When looking at the Phase/Modulation/DC data, the phase may have a value close to +π or -π. If the actual

phase is near one of these values, then because of the noise, some of the pixel values will have "wrapped around" to the other end of the [-π,+π] interval. This will result in unexpected values for the average phase (it may end up close to 0.0!).

When looking at lifetime images, you may want to create a small region of interest, and move it around with SHIFT + left mouse button, to see the histogram changing as the ROI moves. In that case, it could be useful to enable the "Manually set axis" checkboxes, to fix the horizontal and vertical axes of the histogram plot.

If you select the lifetime channel of a multi-exponential lifetime image, you may get unexpected results for the average value of some ROIs: because single pixel data is often too noisy to get accurate lifetime results, there is a chance that one or more pixels in a region have extremely long lifetimes (say 10^{15} ns). Such a pixel immediately pulls the average up to a nonsense number.

For calculating the lifetime from modulation of a ROI, two methods exist in LI-FLIM. Standard LI-FLIM takes the averages of the calculated Lifetimes and displays it in the Statistics table, but when one enable the "Use average Modulation" checkbox, LI-FLIM first calculates the average of the modulation and secondly calculates with that the Lifetime value.

Finally, you can export the statistics data and the plot as a Comma Separated Values text file, or an Excel sheet. The histogram plot itself can be exported as a bitmap image.

6.6 The Time Series tab

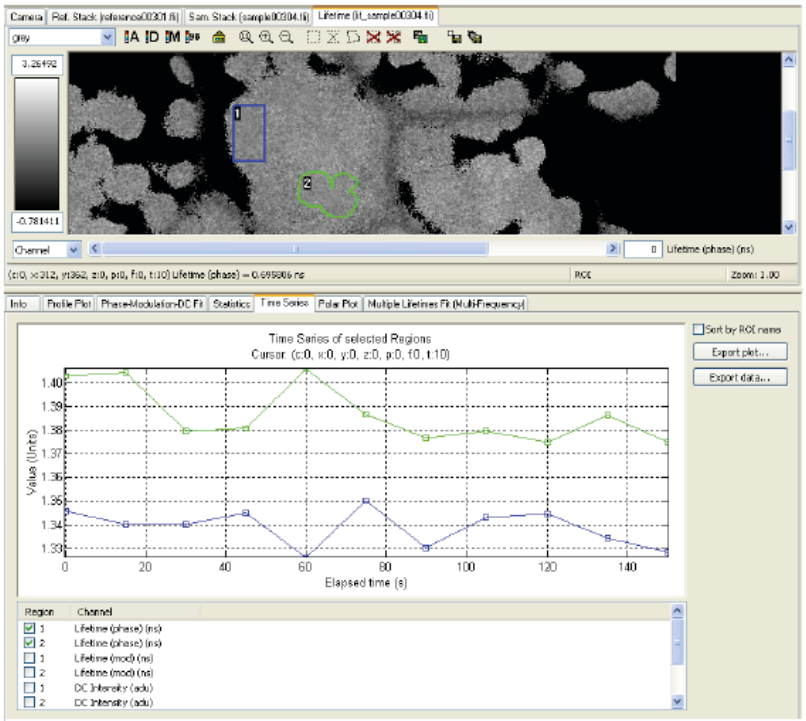


Figure 6.7: *The Time Series tab, showing the average value of the Lifetime from phase of regions 1 and 2 plotted agains the elapsed time in seconds.*

With the Time Series tab you can see changes in the average value of a Region of Interest over time. In figure 6.7, the Lifetime tab is selected, and the average lifetime from phase is plotted for regions 1 and 2.

Note that when you have a large number of timeframes, then the Sample Stack, Sample PMD and Lifetime data will have their timeframes stored in temporary files that are several gigabytes in size. That means that every time you switch to the Time Series tab, or switch between the Sample's phase stack, PMD or Lifetime tab, LI-FLIM has to reprocess all timeframes and recalculate the statistics. This may take several seconds. The same applies when you move ROIs. In that case, it is best to first switch to another Info view tab, and then move the ROI.

You can export the displayed plots as a Comma Separated Values text file, or an Excel sheet. For every selected ROI and every point in time, the average, standard deviation and number of pixels is written to the file. The plot itself can be exported as a bitmap image.

To determine if a change in measured lifetime is due to noise or to some biological effect, you can use the following rule of thumb: the standard deviation of the average value of the ROI, is equal to the standard deviation of the pixel values in the ROI divided by the square root of the number of data points. If you see a change in average lifetime that is larger than 2 of those standard-deviations-of-the-average, you are approximately 95% certain that this change in lifetime is not due to noise, but due to a change in the actual lifetime of the sample. Note that this is only valid if the lifetime inside the region of interest is supposed be homogeneous, and all variations between pixels are caused by noise with a Gaussian distribution.

6.7 The Polar Plot tab

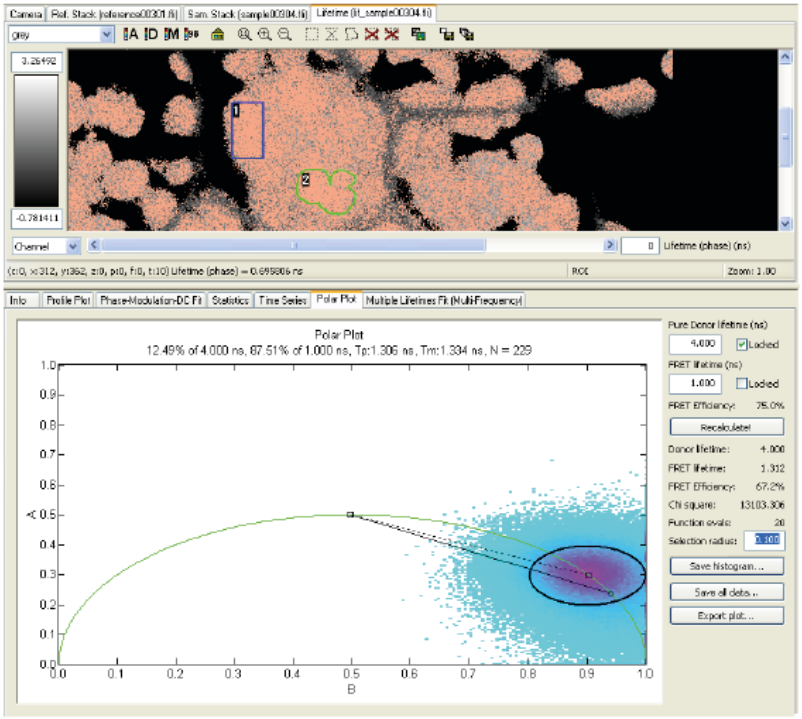


Figure 6.8: *The Polar Plot tab. The left mouse button is being held down in the center of the black circle. All pixels that fall within the circle (depending on their phase and modulation) are highlighted in the Lifetime tab.*

The Polar Plot tab performs a Global Analysis on the entire image. For details on the theory behind this, please refer to section 7.4.

The Polar Plot analysis can be used if the following two statements are true:

1. **The sample contains exactly two different lifetimes.**
2. **The lifetime components appear in different relative concentrations throughout the sample.**

An example would be two cells, one of which has 100% donor molecules, while the other one next to it contains donor and acceptor molecules, and exhibits FRET at a well defined donor-acceptor separation distance. In this case we have two cells in our phase stack images, with a total of two lifetimes (requirement 1): the donor lifetime and the shorter FRET lifetime. The second requirement is also met: inside one cell we have 100% donor molecules, while in the other, the concentration might be 25% non-FRETting donor molecules and 75% FRETting molecules.

The Polar Plot of a sample is represented as a two dimensional histogram. Every pixel in the sample phase stack (or lifetime image) corresponds to a location on the polar plot. You can picture this as a pixel having a relative phase shift and a relative modulation decrease. You can draw a line with an angle equal to the phase shift, and length equal to the modulation decrease to arrive at the correct location in the plot. The plot is divided in 200 x 200 bins, and all pixels of the lifetime image end up in one of the bins depending on their phase and modulation. Bins with little pixels are blue-ish, bins with many pixels are bright purple.

The green half-circle represents locations of 100% pure lifetime components. Mixtures of multiple lifetimes end up somewhere inside the circle. In figure 6.8, a single dot (more like a cloud actually) shows up at a lifetime of 1.3 ns, because the vast majority of the pixels have this lifetime.

LI-FLIM tries to fit a straight line through the histogram. This is shown as the dotted line in figure 6.8. The line starts at the pure Donor lifetime and ends at the pure FRET lifetime. You can lock one or both lifetimes to prevent them from being fitted to another value. All points in between the two fitted lifetimes represent a mixture of both lifetime components. All pixels in the lifetime image are then projected perpendicularly onto this line to find the relative Donor concentration in that pixel. These values are stored for every pixel in the Alpha channel of the lifetime image. In figure 6.8, the Donor lifetime was locked to 4.0 ns, and you can see in the plot that most pixels would have a low value of alpha (nearly 0% donor concentration, near 100% “FRET” concentration).

The solid line is the line between the two entered lifetimes under “Pure Donor Lifetime” and “FRET lifetime” (in this case 4.0 and 1.0 ns). This can be used to play around with the location of the line and see the values for alpha displayed in the title of the plot as you move the mouse cursor around. When you click inside the plot, a circle is displayed, and all pixels that fall within the circle are highlighted in the currently active Data tab (i.e. the lifetime image). This allows you to quickly identify Donor and FRET cells. The selection radius can be adjusted by entering a new value at the right hand side of the plot.

By clicking the “Recalculate!” button, a new fit is made, and the lifetime image, including the Alpha channel is recalculated. Note that the lifetimes that were found can also be viewed in the Info tab, under the “Polar Plot” item in the “Comments” section.

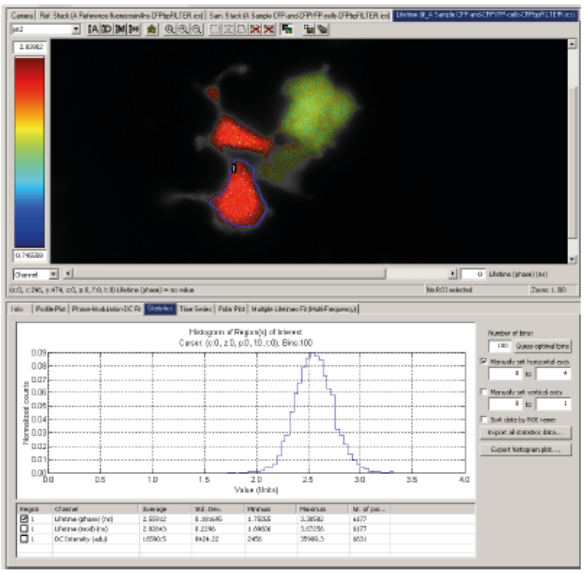
With the three buttons at the lower right side of the Polar Plot tab, you can save the histogram data (the number of pixels that fall inside the bins), a list of all data (as sets of [B, A, X, Y] values where B and A are polar plot coordinates and X, Y are pixel coordinates), and a bitmap image of the polar plot.

6.8 The Polar Plot tab - FRET Efficiency map

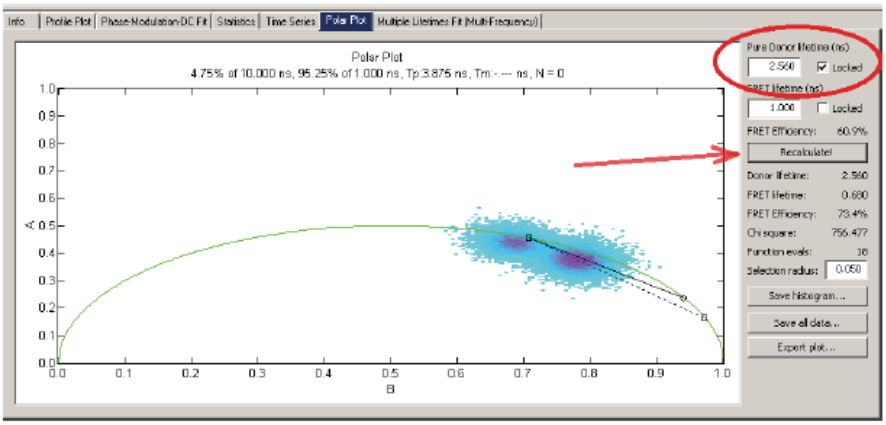
This adds a new channel to the Lifetime image, showing a per-pixel FRET efficiency value calculated from the decrease of lifetime-from-phase with respect to a given pure donor lifetime value.

Short how-to on how to start using this feature:

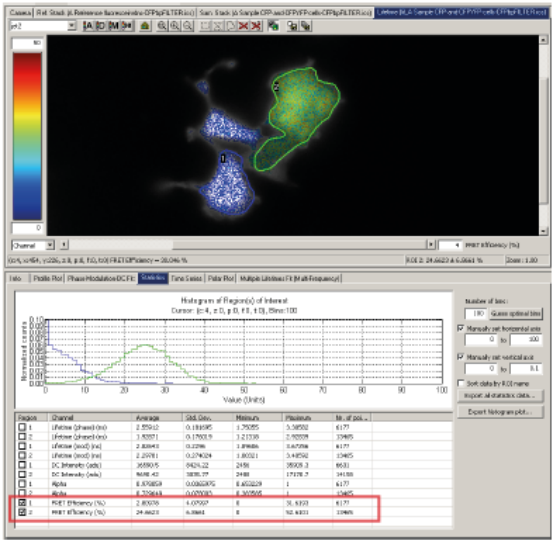
1) Record a lifetime image of a sample that contains pure Donor cells. Determine the lifetime-from-phase of the pure Donor. In this example, it is 2.56 ns:



2) Enter that value in the *Pure Donor lifetime* edit box of the *Polar Plot* tab. Lock this lifetime by clicking the *Locked* checkbox next to it. Then click the *Recalculate!* button.



3) The FRET Efficiency map is now enabled, and will be recalculated/updated automatically every time a new sample is recorded, relating the measured lifetime-from-phase to a per-pixel FRET efficiency based on the value given for the Pure Donor lifetime.



6.9 The Multiple Lifetimes Fit (Multi-Frequency) tab

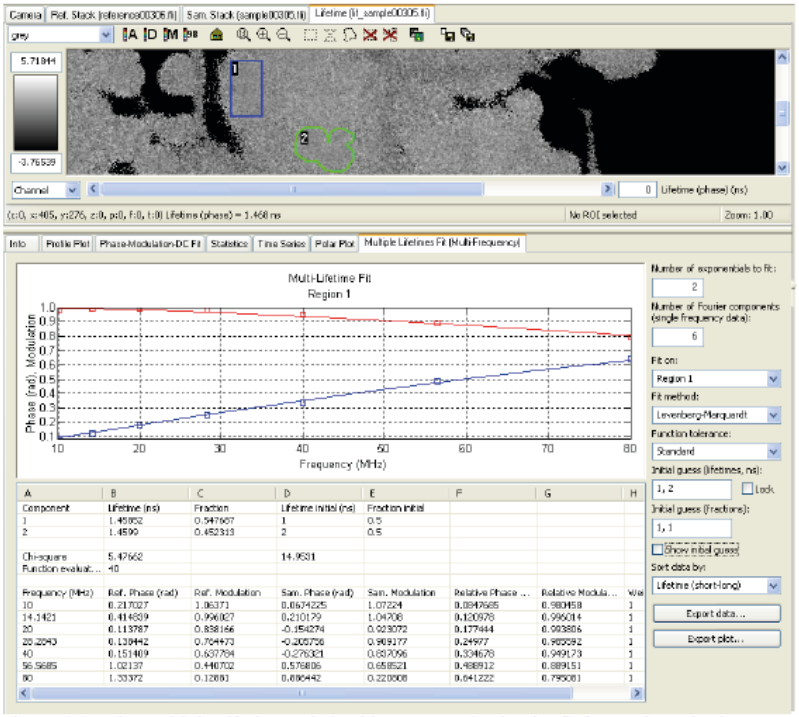


Figure 6.9: *The Multiple Lifetimes Fit (Multi-Frequency) tab. The fit for Region 1 is shown.*

The Multiple Lifetimes Fit (Multi-Frequency) tab shows a plot of the relative modulation (red line) and phase

shift (blue line) of the sample at different frequencies. The solid line is the result of the fit through the actual data points (hollow squares). If the sample has been recorded at multiple frequencies, then those frequencies are shown, while in the case of a single-frequency sample, the value entered in the "Number of Fourier components" box determines the number of higher-order components that are shown. If you recorded a single frequency sample at 40 **MHz**, and entered "5" as the number of components, then the phase and modulation data are plotted for 40, 80, 120, 160 and 200 **MHz**.

The "Number of exponentials to fit" at the upper right side determines the number of lifetimes that the fit procedure should find. It will always find that number of lifetimes, but some of them may be bogus. The table below the plot shows the results. In our case, two lifetime components that are nearly the same were found. This is a clear indication that there is actually only one lifetime present.

You should carefully watch the value of Chi-square: if that does not improve by a factor of 2, after changing the number of exponentials to fit, then the new model is not better than the one you had. In our case, 1 exponential is a better model than 2 exponentials, the Chi-square was larger in the latter case.

Below the found components and the Chi square, a table displays the phase and modulation data used for the fit, and the plot.

At the right hand side of the tab, you can select on what data you want to fit the lifetime(s): a single pixel (can be selected by clicking the left mouse button inside the Data view tab), a region, all regions combined, or the entire image combined. Single pixel data is usually very noisy. When you select a region, the phase image intensities are combined, and a single phase and modulation is calculated from the combined data. This results in much more accurate exponential fits.

The "Fit Method" lets you choose the type of fit. The "Levenberg-Marquardt" method is usually the most stable. You can use the "Simplex Weighted" in the case of single-frequency data, when the higher order components have a low modulation and hence result in noisy phase and modulation data. A weighting factor is applied in that case.

The "Function tolerance" determines at what accuracy level the fitting procedure decides that it is close enough to the best fit. A low tolerance (for fit errors) means that the resulting lifetimes will generally be the best possible fit, but many iterations may be needed to reach that result, taking a relatively long time. A high tolerance produces less accurate results, but is faster. The "Standard" setting is the best compromise in most cases.

The initial guesses for lifetimes and fractions provide a starting point for the fit. You can display the phase and modulation curves that correspond to the initial guess by clicking "Show initial guess". The fractions that you enter are internally normalised to 1. This means that if you enter 1, 2, 2 as fractions for three lifetime components, the actual relative fractions used for a starting point of the fit are 0.2, 0.4, and 0.4. This adds up to 1.0.

As the fit does not necessarily give *the* correct answer (it is a trial-and-error process), choosing your initial guess can make a significant difference to the resulting lifetime components. You should always visually check if the fit (solid curves) is following the data points. If that is not the case, you can change the lifetimes and fractions for the initial guess and see if it improves the fit.

Next, you can choose the order in which the results are presented (lifetimes sorted from long to short or the other way around, etc.). The data can be exported to Comma Separated Value text files, or Excel sheets. The plot itself can be exported as a bitmap image.

As a final note: experience shows that you will need a very good (little noise) reference and sample phase stack before you can get sensible lifetimes out of the data, especially when looking for more than 2 exponentials. If the lifetimes are very close together (say 2.0 and 2.2 ns), it will be difficult to reliably find those two components. However, if you know beforehand which lifetimes are present, you can enter them as initial guess and "lock" them. In that case, only the fractions will be fitted for, which may give more accurate results than fitting both lifetimes and fractions.

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