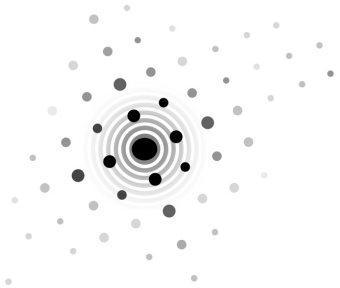
CBIS ADVANCED FLIM MANUAL

Leica Stellaris 8 Falcon · PicoQuant



5th edition
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Contents

Prefatory note

Welcome to the CBIS manual for users of our STELLARIS 8 FALCON FLIM with additional guidance for PicoQuant instruments. This manual has the following unique features:

- It covers advanced procedures and alerts the user to hardware and software issues not covered in the manufacturers' documentation, including the use of a variety of third-party hardware and software tools, refined over many hours of actual testing by the author on instruments from different manufacturers with the help of researchers and facility managers. It also contains practical tips and tricks known to master practitioners of the art of FLIM that can't be found in regular manuals. Matters of interoperability, standardization and scientific reproducibility are also addressed.
- It incorporates quick explanations of more general FLIM principles and guidelines where they provide context and insight for better understanding of instrument operation and experimental design. It also cites literature to assist the user in deeper study.

The aims of this manual are twofold:

- To cater to advanced FLIM researchers who want the most out of the STELLARIS and beyond.
- To be a one-stop resource that synthesizes and reconciles conflicting information from scattered sources into a coherent whole and serves as a hands-on crash course for the FLIM novice.

If you wish to contribute to the development of detailed protocols for ensuring scientific rigor in every aspect of FLIM, consider joining QUAREP-LiMi Working Group 15 FLIM (`https://quarep.org/working-groups/wg-15-flim/`).

I am grateful to the vendors, software developers, facility managers and researchers from around the world whose assistance and discussion greatly improved this manual directly or indirectly—Adam Cliffe, Taryn Guinan, Luis Alvarez, Melanie Tan, Veronique Teo, Tong Yan, Alessandro Rossetta, Daniel Aik, Zbigniew Baster, Thorsten Wohland, Wu Yue, Kamal Kant Sharma, Zhou Shiyue, Lee Shu Ying, Leong Kim Whye, Radek Macháň, Liu Jun, Lisandro Falomir, Andre Gomes, Jenu Varghese Chacko, Pan Xiaotao, Steffen Dietzel, Alex Hunt, Alexander Vallmitjana, Li Yushan, Li Tianyi, Yeo Zhen Yuan, Ma Xiaoxiao.

The latest version of this manual can be downloaded from `https://www.dbs.nus.edu.sg/2021/09/14/cbis-lm-core-leica-sp8-stellaris-upright/` by clicking the `CBIS advanced FLIM manual` link.

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1 Fluorescence lifetime

1.1 Applications of FLIM

In fluorescence lifetime imaging, the image is a map of fluorescence lifetime (usually denoted by τ in literature and software) rather than fluorescence intensity. Unlike steady-state intensity obtained in a regular confocal image, lifetime is unaffected by label concentration artifacts caused by specimen preparation.

Structures or states that are indistinguishable in a normal fluorescence intensity image can be distinguished in a fluorescence lifetime image if they are in different environmental, biochemical, physiological or mechanobiological states that influence the fluorophore lifetime.

Can be used to separate fluorescent labels from autofluorescence if lifetimes are different (e.g. Deore *et al.* [1]), especially in plant biology. Even lifetimes of natural autofluorescence itself can also be useful. Donaldson [2] reviews FLIM of autofluorescence in plants.

Fluorescence lifetime can also be used to improve STED resolution with less STED power.

Can be used to follow metabolic trajectory over time by measuring autofluorescence lifetime of NADH which increases with binding ratio which is affected by drugs or pesticides like rotenone. Easier to do with multiphoton because NADH excitation wavelength is so short that it is unavailable on most bioimaging instruments and quickly kills cells anyway.

Better for measuring FRET efficiency than other methods:

- unaffected by photobleaching, cross-talk, fluorophore concentration, nonuniform illumination
- faster than measuring photobleaching
- only donor needs to be measured

Fret efficiency is a highly sensitive indicator of distance between, and relative orientation of dipoles of, donor and acceptor. Good for precision biosensors [3].

The emission wavelength-dependent average fluorescence lifetime spectrum (§3.2.2) can also be used to distinguish materials. Thomas *et al.* [4] demonstrated the differentiation of species of bacterial spores and pollen in the forensic/military application of detecting the use of biological weapons.

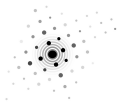
Fluorescence lifetime correlation spectroscopy: use lifetime to separate fluorescence correlation spectroscopy channels.

time-resolved fluorescence anisotropy. unlike steady-state anisotropy, can measure how fast molecules are rotating (to study microviscosity or molecular characteristics/interactions) and separate static and dynamic depolarization. See Avilov *et al.* [5] for an application example.

1.2 FLIM in time and frequency domains

Time-domain FLIM, traditionally TCSPC (time-correlated single photon counting) [6].

In contrast, frequency-domain FLIM (not available on STELLARIS) uses sine-modulated excitation and measures lifetime through phase shift and demodulation. It is camera-based and can be combined with specialized imaging techniques like TIRF [7]. The longer the lifetime, the larger the phase shift (delay) and the greater the demodulation (reduction of amplitude of the sine curve).[1] It is faster than time-domain FLIM but needs strong signal and may be unsuitable for dim and sensitive live specimens. See Lakowicz [8] for theory and instrumentation.



[1] This principle is used in phasor analysis (§8) in time-domain FLIM.

2 Experiment design & specimen preparation

The CBIS STELLARIS is an upright microscope. Prepare your specimens accordingly.

avoid weak fluorescence as it will produce lousy FLIM. For anisotropy (§11.4) you need even brighter specimens.

If using Hoechst for FLIM, we recommend using the UV-converted green-emitting form, as the shortest available FLIM excitation on our instrument is 440 nm.

The lifetime of your label should be no longer than the time scale of the dynamic process you're studying.

Environmental/physiological factors affecting lifetime:

- pH (e.g. CO_2)
- temperature (big factor but also depends on specimen properties)
- oxygen levels
- osmotic pressure
- viscosity of solvent

Sample preparation factors affecting lifetime:

- mounting medium (refractive index)
- depth of specimen (affects optical path length)
- dye settling on the slide or adhering to the coverslip

Instrumental factors affecting lifetime and/or its *measurement* (may be able to compensate with IRF; see §5):

- laser pulse rate [9]
- width and shape of laser pulse
- excitation or emission detection wavelength (depends on specimen properties)
- excitation polarization
- multiphoton excitation?
- presence of STED laser (shortens lifetime)
- use of adaptive optics (sample depth-dependent)

Things that don't affect lifetime:

- concentration (unless quenching in organic solvent at high concentration)
- excitation intensity (if pulse width and shape remain unchanged)
- photobleaching (but bleached specimen may have undergone other changes that affect lifetime)

In most cases it's best to have a control specimen and study relative lifetimes for more robust conclusions. Absolute lifetimes are usually not meaningful as they are highly sensitive to measurement conditions and also depend on the calibration of the instrument and the laser pulse rate [9]. If reporting absolute lifetimes you must report the measurement conditions and ideally have checked the accuracy of the instrument using a bright reference specimen with a stable known monoexponential lifetime, such as Atto 488 ($\tau = 4.2$ ns). FLIM LABS makes a 2.5 ns monoexponential calibration slide. If you need a reference specimen with multiple mono- and multi-exponential lifetimes to test across a measurement range, PolyAn Fluorescence Lifetime Beads is an option.

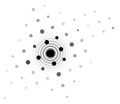
For FRET, best to have three specimens: donor only (control), donor-acceptor, and autofluorescence (control). Prepare specimens with cells at same developmental stage and in identical environment with identical specimen preparation protocol. If donor alone not possible, try depleting acceptor.

can do z-stack, time lapse, spectral binning etc. for FLIM but large file size.

For time lapse, if you are unsure of the temporal scale of the dynamics, acquire at a higher frame rate and you can bin time later. For long time lapse, watch out for focus drift as the STELLARIS has no focus drift correction.

If you have a large number of experiments and just want to separate signal by lifetime, including separating autofluorescence, first do a FLIM and then plug the lifetimes obtained into TauSeparation or TauGating (to exclude signals) which produce smaller file size.

For testing and demonstrating FLIM, a *Convallaria* slide is a convenient, good and durable specimen. Please contact us for a demo.



3 STELLARIS 8 FALCON FLIM system

3.1 System specifications

0.2 ns laser pulses fixed at 78 MHz so lifetimes being measured should be longer than 0.2 ns and shorter than pulse interval of about 12.5 ns, preferably no longer than 3 ns otherwise tail of decay curve may extend into the next pulse interval [10].

Start/end of pixel dwell time of confocal scan probably not synchronized with start/end of pulse interval as the pulse interval is much shorter than pixel dwell time.

The 405 nm laser is not pulsed and can't be used for FLIM.

Time resolution of detector: 10 GHz binary sampler with fixed 97 ps resolution.

Counts up to two photons per laser pulse. Different from earlier-generation technology which continues to be used by some manufacturers which count only the first photon within each pulse, which tends to underestimate distribution of arrival times if photon arrival rate is too high.

The laser is linearly polarized by the AOBS. Measured absolute lifetimes may be different from those from an instrument whose laser polarization has been scrambled, even if other environmental conditions are controlled.

3.2 Instrument setup

if possible, turn on microscope and laser at least 30 min to one hour in advance to let them stabilize, otherwise you may get unreliable data and encounter focus drift especially at high magnification. The laser must be turned on in the software as well as by the physical switch. Note that our STELLARIS takes longer to stabilize than some of our other instruments.

Check that the objective DIC prism and the polarizer mounted in the slider above the objective are out of the light path. Check that the objective is clean.

If your analysis is sensitive to spatial orientation:
The eyepiece view rotates the specimen 180° from how you see it with the naked eye when looking down at it on stage while standing in front of the microscope. The 2D scan in LAS X flips the image so it appears as if you are viewing the specimen from under the stage. See LAPRAZ *et al.* [11] for further explanation. When acquiring a 3D z-stack, to reproduce correct chirality, click the down arrow under `Stack Direction` if not tiling and click the up arrow if tiling; if the wrong arrow is highlighted, LAS X reconstructs the stack in reverse order giving wrong chirality (tested on 19 May 2026 in LAS X version 4.9.0.30221).

In LAS X, click FLIM button at top left to bring up FLIM Settings box and a separate FLIM window

Assign FLIM channel(s) to HyD X detector(s) where possible, as the calculated IRF (§5.1) is modeled for X detectors. Under otherwise identical acquisition configurations, our X1 detector yields considerably higher photon counts than X3 which is positioned farther down the sequential chain of detectors. S detectors[2] have been found to give lifetimes shifted by 1 ns, although experimental IRF (§5.2) might correct for that (not tested).

Note: HyD X detectors start to show comet-like signals (tail in direction of scanning) in the images after being severely overexposed (much longer than nanosecond response). Comets may also be due to avalanche events being aberrantly triggered. (SERGIO LOPEZ/PETRO KHOROSHYY/G. ESTEBAN FERNANDEZ/IVAN NOVOTNY, Confocal Microscopy List)

First aid methods for comets:

- mount a bright fluorescence (e.g. Chroma slide) or reflectance specimen (e.g. blank part of your microscope slide), start live view, open the pinhole if necessary and slightly oversaturate detector so it beeps but does not trigger safety cutoff. Keep live view on for 30 s to a minute.
- take detector out and light it with an LED flash light.
- if due to aberrant triggering of avalanche events, reset/recalibrate the threshold for avalanche triggering.

[2] S detectors are better for blue-green region while X detectors are better for orange-red. X detectors are more sensitive overall but have lower dynamic range than S detectors. Our S detectors are older than the X detectors. These detectors have a response closer to linear than traditional detectors as excitation intensity increases.

doesn't matter what mode detector is in as it doesn't affect FLIM which always counts photons. just set it to photon counting mode.

You can use multiple detectors for one channel for the following purposes:

- collect more photons faster without exceeding one photon per pulse per detector, especially if you need high speed.
- collect photons at different emission wavelengths (try 20 nm interval) to do global analysis for recovering more reliable multi-exponential lifetimes (Lakowicz [8] p. 144). Overall collection range should span emission spectra of all fluorescent components.

First toggle `ON` the desired detectors and set their detection ranges.

Then activate Specialist Settings in one or both of the following places in order to select individual detectors or combinations of detectors from which to display FLIM data:

1. left panel of main LAS X window. Allows you to see the FLIM results during live view or acquisition. Activating this before acquisition will automatically activate nº 2.

2. Specialist Settings box in left panel of FLIM window: tick `Manually Assign Detectors`. Allows you to see the FLIM results after acquisition. You can do this even if you don't do nº 1.

use frame mode instead of line mode to be able to assign overlapping detector ranges in sequential channels

limit emission collection wavelength range to emission spectrum of fluorophore. Keep the range fixed for control and treatment specimens.

make sure no crosstalk

If you need to suppress effects of rotational diffusion and/or fluorescence anisotropy on measured intensity decays (Lakowicz [8] p. 139), set the emission polarizer to the so-called magic angle of 54.7° relative to excitation laser polarization. Click the `Show Fluorifier Settings` button at upper right of the acquisition window and select the polarizer. On our STELLARIS, this polarizer is parallel to the excitation polarization when set to 90°, so instead of setting it to 54.7° you should set it to 35.3° to get the magic angle.

Make sure 405 nm laser is not active in a FLIM channel. The randomly timed photons coming from it will skew lifetime measurements (see Fig. 8.2 for effects on phasor plots).

Field of view: You may want a large field of view to capture many cells in one image and segment them later, but make sure it doesn't amount to statistical pseudoreplication. You may even wish to do tiling. Or you may want to fill the view with one object at high magnification and exclude as much unwanted structure or background as possible. Plan your strategy. Choose the appropriate objective and then adjust the zoom if necessary.

Pixel dimensions: As a general guide, set 512×512. Can use higher if you need resolution but need enough photons.

Use fewer (larger) pixels if:

- you have a large homogeneous object in the field of view. There is no need to sample it with more than one pixel essentially.
- you need more photons per pixel at a high frame rate to capture dynamics.

3.2.1 Time-resolved fluorescence anisotropy

If possible, use an objective of NA between 0.8 and 0.9 [12], as lens curvature in high-NA objectives will depolarize both excitation and emission and reduce the accuracy of anisotropy measurements.

There is no polarization beamsplitter. The emission polarizer has to be rotated sequentially to 0° and 90° for getting the two anisotropy images. Click the `Show Fluorifier Settings` button at upper right of the acquisition window, select the polarizer and set the rotation angle. The rotation cannot be automated.

To measure the G factor (Lakowicz [8] p. 361), use a fast-moving small molecule with excitation and emission characteristics similar to that of your anisotropy sample. On the STELLARIS, G cannot be directly measured due to the hardware configuration which can't change emission polarization orientation while keeping relative orientation to excitation polarization fixed. You may be able to account for this indirectly by calibrating with a sample of known anisotropy. Otherwise, you can consider assuming $G = 1$ as the transmissivity of the emission filters is probably independent of polarization orientation.

Since acquisition is not simultaneous, there may be some loss of precision due to variation in the timing electronics, specimen drift or environmental fluctuations.

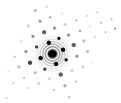
Before anisotropy imaging, check what angle of the emission polarizer is actually the darkest by adjusting it in reflectance mode live view using the reflection from a microscope slide or coverslip. Then use this angle and the angle perpendicular to it for anisotropy imaging.

Because of low NA and cross-polarization, your specimen has to be very bright.

3.2.2 Lifetime spectrometry

Emission wavelength-dependent fluorescence lifetime spectrum

select one of the lambda modes such as `xy`λ. laser will be auto set to constant power if excitation wavelength changes. in FLIM mode, the actual number of photons is counted so no need to correct for wavelength-dependent quantum efficiency of detector.



4 Live FLIM optimization

- place black cover over stage
- turn off lights
- click `LIVE`
- fine-tune focus. If specimen has no structure, such as calibration solution, focus by observing the live decay curve and finding the position that gives the max photon count (highest peak in the curve).
- if objective has correction collar, adjust it to get max photon count.

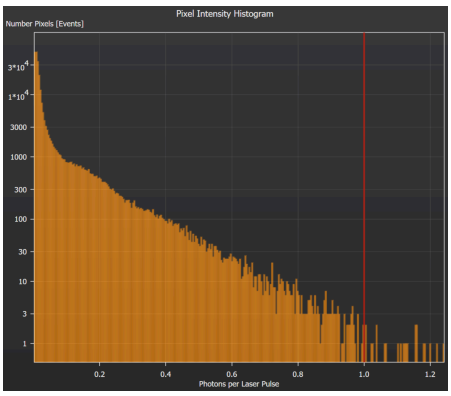


Figure 4.1. Photons per pulse.

- Inspect the pixel intensity histogram (Fig. 4.1).
- Adjust laser power to obtain the desired photon count rate (photons per pulse). In PicoQuant SymPhoTime, count rate is given in cps (counts per second). To convert to photons per pulse, simply divide cps by laser pulse rate in Hz.
- In traditional TCSPC, photon count rate should be 0.01 to 0.1 photon per pulse to avoid pile-up caused by more than one photon arriving within a pulse interval and not being recorded, causing underestimation of lifetime. 0.1 photon per pulse still gives a little pile-up; for greater precision use 0.01 (`https://www.tcspc.com/doku.php/howto:avoid_pile_up_effect_in_flim_measurements`).
- The STELLARIS can count up to two photons per pulse and LAS X can correct for the pile-up. If you plan to do all your analysis in LAS X, Leica says you can increase laser power until histogram fills up space to the left of red vertical line (one photon per pulse) with a bit of it going past the red line, which places the histogram peak at around 0.37 photons per pulse. This can be advantageous as it takes less time to accumulate

data. But more photons per pulse increases risk of two near-simultaneous photon arrivals being counted as one (besides pile-up) and photostresses the specimen more.
- if flimming live cells or photosensitive specimens you may have to lower the power below the Leica guideline. You can try 0.5 photons per pulse.
- if you plan to analyze the data using external software, you must acquire at the traditional low count rate as Leica only exports all photons for external analysis despite allowing you to select and analyze only the first photons in LAS X.
- if using two detectors to cover emission range, adjust the collection ranges of the detectors until their histograms are similar.

must have enough photons per ROI for good statistics. to check number of photons:

- inspect peak of decay curve (whole image, single frame).
- right click on Intensity image → `show data cursor` to see counts for individual pixels as you mouse over.

single exponential fit: minimum 1000 photons for each region of interest
double exponential fit: minimum 10000 photons for each region of interest
phasor (§8): minimum 100 photons per pixel

to get enough photons:

- larger pinhole
- high laser power
- slow scan speed (speed shown in lines per second)
- larger pixel pitch
- accumulate multiple scans

Note that although higher photon count gives higher signal:noise ratio, the background also tends to be higher.

Note that if you do a large number of accumulations at high laser power, the specimen may heat up and yield changing lifetimes as lifetime is temperature-sensitive, and may also suffer photodamage. You can check for this by fitting earlier and later frames separately, or running a time lapse at maximal frame rate and using the `Mean ROI` feature (§7.3) to plot changes in lifetime. In the lifetime image you may also see spots of very fast decay [13].

if signal is weak, select one of the wavelengths with notch filter if possible (FLIM Settings box at top left of main window) as notch filter suppresses excitation background better. Multiple notch filters cannot be used simultaneously.

adjust colour palette range on left to optimize visual differentiation in FLIM image

doesn't matter for FLIM if confocal image is saturated. But if doing FRET, don't bleach the acceptor!

You can adjust time gate.

The software has a bug that lets you adjust the intensity threshold sliders, but ignores your input and simply "autoscales from memory". This is perhaps because the machine can't update the display fast enough to show the thresholded pixels of the field of view changing with every scan.

decay curve may have a peak at around 8 ns caused by stray reflections or scattering in the optics or by the electronics. This will skew the fit. Try the following to minimize the peak. If you can't get rid of the peak during acquisition, you will have to do fitting in external software (§11) as the the post-acquisition software cutoff filter in LAS X is misaligned.

- increase lower limit of detection range further from excitation ($>$ 20 nm gap), especially for very thin specimens attached to coverslip.
- check that AOBS is set to fluorescence and not reflectance (less likely to help)
- use a notch filter. multiple notch filters cannot be used simultaneously. (less likely to help)
- changing pinhole size will not help.

Curve fitting (§7) not available in live FLIM.

right click Fast FLIM image → show data cursor to see pixel values as you mouse over. You have to keep clicking to update the value.

You can analyze the live phasor plot (see §8). Leica suggests selecting the Preview filter which uses a Gaussian filter to improve SNR in live mode.

In `FLIM Settings` box at upper left of main window, you can specify how to decide when to stop acquisition:

- set accumulations by line if you are imaging live cells whose contents may move. But if you need to subset data later, this will make subsetting harder.
- to ensure sufficient photon count for a single specimen, set to acquire until specified nº of photons per pixel. Note that line accumulations take precedence over the specified photon count; lines will be accumulated until the specified number of lines is reached even if the specified photon count is exceeded.
- to keep excitation dosage constant across specimens, set frame accumulation. It is better if you have previously determined the optimal number of frames by acquiring until the required photon count and seeing how many frames it took.
- set total duration

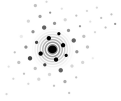
To run experiment, click `Start` button at bottom centre of main window. You can click `Stop` any time and the photons accumulated to that point will be recorded.

Once experiment is finished, visually check that the plotted decay and calculated IRF are not corrupted (see Fig. 5.1 for examples of corrupted IRF).

all subsequent processing can be done on any computer with LAS X software with FLIM/FCS module. To see microscope settings right click on dataset in Projects panel and select `Properties`.

If you wish to simulate FLIM data for testing or demonstration, you can use one of the following:

- LAS X simulates data for use in LAS X. Click `Tools` button at upper right of FLIM window and select `FLIM Simulator`.
- FLIMKit (§11.2) simulates complete dataset including instrument response function (§5) and allows you to simulate artifacts such as background, photon pile-up and reflection peaks.



5 Instrument response function (IRF)

Analogous to PSF but in time dimension. Depends on the shape of the laser pulse and how it is recorded by the instrument [8]. Combined effect of hardware components such as lasers (especially pulse shape), optics, electronics, detector etc.

IRF width determines minimal measurable lifetime. As with PSF, a way to quantify the IRF width is by its FWHM.

IRF of instrument with PMT detector may have a count rate-dependent afterpulse (second smaller peak). Not a problem if afterpulse is very low relative to data.

5.1 Calculated IRF

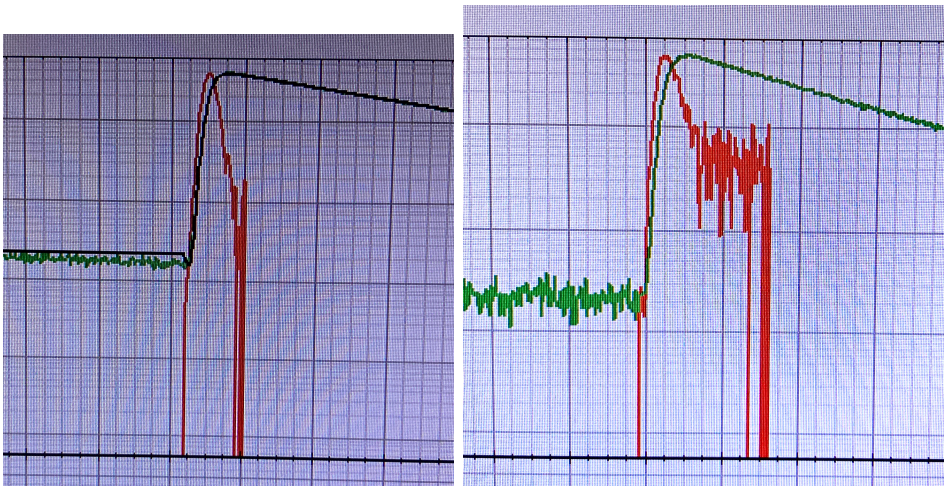


Figure 5.1. Corrupted calculated IRF (red) with noise artifacts from an earlier-generation PicoQuant instrument.

calculated IRF (by the software) is shown in the displayed graph and used by default. In general, calculated IRFs in the latest instruments are based on detector characteristics as well as the derivative of the rising edge of the measured decay.

At least in the STELLARIS, the calculated IRF doesn't account for different pulse width and shape at different laser power. It almost always falls off before 2 ns regardless of laser power, while the actual IRFs vary with laser power (Fig. 5.2).

Nevertheless, calculated IRFs are good enough for most purposes unless you have very short lifetimes or second harmonic generation [6]. Check that there is no stray sharp peak as high as the main peak, or weird plateaux or humps after and lower than the main peak (Fig. 5.1), or other deformities like dents or kinks. When the photon count is very low, the IRF curve may remain high and get truncated mid-way through the pulse interval. All these phenomena indicate corrupted calculated IRFs and fit will be skewed. If this keeps happening try the remedies listed on page 13.

You can try compiling a machine IRF if you use FLIMKit for analysis (see §11.2). collect 15–20 pairs of FLIM data and corresponding calculated IRF on different days with same acquisition configuration but different samples used in a given project.

Measured decay = convolution of IRF and actual decay law (assuming no photon pile-up requiring dead time correction).

5.2 Experimental IRF

If your experiment is sensitive enough to require a measured IRF, you should measure it before every imaging session after the microscope has stabilized. You should have already optimized the instrument configuration for your FLIM specimen, as you should use many of the same settings when measuring IRF.

for principles of IRF measurement, see PicoQuant's article at `https://www.tcspc.com/doku.php/howto:how_to_measure_the_instrument_response_function_irf`

IRF specimen:

- species with extremely short lifetime such as gold nanorods, or fluorophores quenched with saturated KI (potassium iodide) prepared fresh. Store in the dark and not for too long as KI undergoes photochemical decomposition. Use the same emission collection range as your FLIM specimen

to ensure that there is no difference in path length in the prism-based spectral detection optics.

- Raman scattering of water. Allows emission filters to be used but water must be very pure to avoid background fluorescence contamination.
- Non-fluorescent reflecting or scattering specimen with zero lifetime such as glass slide, dilute milk or LUDOX colloidal silica. If the glass slide is too bright, try the frosted end of the slide if it has one, or other frosted glass surface. On the STELLARIS you have to switch to reflectance mode, which uses a different optical configuration. Optical path length and background signal may differ from that for your FLIM specimen and slightly shift the IRF. Shift has to be corrected later.
- urea (second harmonic generation) for multiphoton instruments.

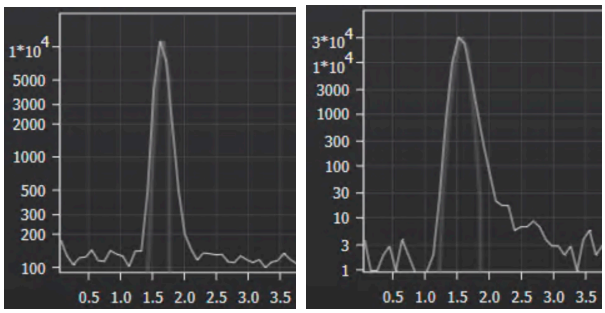


Figure 5.2. Different widths and shapes of measured IRFs at laser power 0.1% (left) and 100% (right) on the STELLARIS.

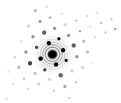
Collect FLIM data from the IRF specimen. Laser power should be the same as for the FLIM specimen because different power gives different pulse shape and timing affecting the IRF (Fig. 5.2). The count rate should be much lower than that for the FLIM specimen because the photons for the IRF are all arriving within a much shorter interval near the beginning of the pulse interval and not spread throughout the pulse interval[3]. To get low-enough count rate at the given laser power you may have to dilute the IRF specimen. However, if you're already using a very low count rate in the order of 0.01 photons per pulse for your FLIM specimen, you can probably get away with the same count rate for the IRF.

Ideally, accumulate photons for the IRF until the peak is about the same height as that of the FLIM decay, although software can automatically scale and normalize it later.

[3] For further explanation see `https://www.tcspc.com/doku.php/glossary:differential_count_rate`

5.3 Estimate IRF from known lifetime

Estimate IRF instead of direct measurement. use a blue or green Chroma slide (suggested by FLIMfit developers) or other specimen with stable known mono-exponential lifetime and recover the IRF using an algorithm such as BIRFI. This can't be done in LAS X so after acquiring your FLIM data and IRF estimation data, export both from LAS X as `ptu` and load them in external software (§11) for analysis.



6 FLIM data preprocessing

The FLIM feature set in LAS X is quite sophisticated by industry standards, although some features are buggy or not working at the time of writing and others are in paid modules not available at CBIS. See §11 for alternative software if LAS X can't do what you need.

Besides the microscope workstation, you can use LAS X on CBIS' `confoll3` offline workstation (Block S1A 03-07) for analysis.

`Specialist Settings` box in left panel of FLIM window, choose how the raw data is filtered for analysis:

- `standard`: for decay curve fitting, uses only pulse intervals in which only one photon arrives. For `FLIM Image Fit`, uses all pulse intervals and does dead time correction for pile-up. This setting allows you to increase count rate for faster acquisition while avoiding pile-up in the decay curve. But be careful of bleaching with excessive laser power.
- `all photons`: raw data with up to two photons recorded per pulse interval. Slight decrease in fitting accuracy but may be needed for fast live imaging.
- `first photons`: emulates behaviour of legacy TCSPC systems. Use with care, making sure no pile-up during acquisition, otherwise fitting will give wrong result.

Exported raw data (§10.2) contains `all photons` regardless of option selected in `Specialist Settings`.

6.1 Fast FLIM image

average arrival time in each pixel (similar to Tau Contrast). IRF peak "lifetime" is subtracted from the measured arrival time, which may yield negative lifetimes especially in the background pixels if the background is devoid of fluorescence and dominated by small amounts of reflection or scattering. useful for preliminary assessment and comparison.

click `Fast FLIM` button at top centre of FLIM window. adjust brightness range and colour palette for optimal visualization. Note that the image shows both intensity and lifetime, so brighter doesn't necessarily mean longer lifetime. look

at the pixel colours and histogram to judge variability of lifetimes. scroll mouse wheel to zoom. right click → `show data cursor` to see pixel values as you mouse over.

for more discussion see `https://forum.image.sc/t/leica-stellaris-to-flimj/106989`

6.2 Regions of interest (ROI)

object-based analysis for identifying e.g. different cell types or states

6.2.1 Manual segmentation

You should do this for preliminary data exploration only.

In the ROI box under FLIM tab on left side of FLIM window, click one of the shape buttons to draw on image. you can also select a single pixel (using the `+` button) or use the magic wand button to click inside an object to automatically demarcate it.

if you add multiple shapes they will be added to the same ROI. to create a different ROI click the `New` button. Click `Rename` button in `ROI` panel to rename the ROI before you get confused.

ROIs are replicated in all data dimensions unless you subset the data (§6.3). ROIs are added per dimension to the table at the bottom of the FLIM window.

check decay curve of ROI to see that there are enough photons

Tick `Invert` to analyze the area outside the ROIs.

To save the ROIs: select the required ROIs, right click in the `ROI` panel and choose a suitable save option.

6.2.2 Automatic segmentation

If your analysis requires autosegmentation, consider using FLIMKit (§11.2) which integrates cellpose.

Autosegmentation in LAS X is performed by the Image Analysis module, which segments using either thresholding or AI and assigns a unique ID to each object. However, it seems to be able to perform only phasor analysis on the segmented objects and can't fit decay curves. CBIS does not currently have this module, but it is available on an offline workstation at the Multiphoton unit in the NUSMed Microscopy Cluster (`https://research.nus.edu.sg/research-facilities/project/microscopy-cluster/`) near CBIS. Interested users please contact them for details.

The alternative option if you wish to stick to LAS X is to right click the image, export raw image and segment it in another software such as Fiji or cellpose, and load the mask back into LAS X. LAS X can only load binary external masks (Save the mask as 8-bit with pixel values 0 and 255), not 16-bit integer masks with object ID. right click inside the LAS X `ROI` panel and select `Load Binary Mask` to load the mask. If you wish to load a second mask, click `New` in the `ROI` panel before loading, otherwise the second mask will overwrite the first. You cannot load multiple mask files in one step. If you have an external 16-bit integer mask with object ID, you can't load it in LAS X. Export your data (§10.2) and analyze in external software (§11).

6.3 Post-acquisition optimization

You can change various settings to see what it does to the data. The software will recompute the results from the raw data so you don't have to reacquire. This will also allow you to determine optimal settings for future experiments.

FLIM box on the left of FLIM window:

- subset repetitions to see if fewer reps are enough.
- subset and/or split time, z-slices or tiles as required.
- bin z if your object of interest straddles multiple z slices and you wish to analyze it as a single entity with more photons.

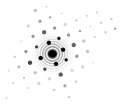
- bin time (for time lapse) to see if fewer time points are still enough to capture dynamics and/or to sum more photons.
- `Pixel Binning` to get higher photon count per pixel at the expense of spatial resolution
- `Lifetime Binning` to get higher photon count per time bin at the expense of temporal resolution

For multidimensional data, analysis is automatically performed over all dimensions unless you subset. However, 3D visualization has to be done in external software (§11).

Subset data in the FLIM box under FLIM tab in FLIM window:

- check `SPLIT` to perform fitting for individual time points and/or slices and/or emission bins
- click `Crop` button to crop data in XY or subset in T/Z
- subsets will appear as separate items in table at bottom of FLIM window
- `Shift`/`CTRL`-click to select multiple entries to show their decay curves simultaneously
- Click `Fit All` (§7.3) to batch fit everything (batch process).

To batch change analysis parameters: Tools button at upper right of FLIM window → `FLIM Parameters`



7 Decay curve fitting

Gives more precise lifetimes than phasors if you need absolute values or are calibrating the instrument. The short-term instrument variation in fitted lifetime of the STELLARIS is about 0.1 ns as observed in repeat measurements of Atto 488.

7.1 Inspect measured decay

decay curve should decay to nearly 0 for accurate analysis. will overshoot if lifetime is too long. time gating will not fix this problem. be careful when inspecting displayed plot as the y-axis does not start from 0.

Monoexponential looks straight

Although the pulse interval is 12.5 ns, Leica data may extend to 13 or 14 ns if `all photons` is selected in `Specialist Settings`. Part of the reason could be that as one pulse interval ends, the next pulse is fired with a pulse length of 0.2 ns, plus some dead time after that, during which any photons arriving at the detector would be from the previous pulse. The instrument therefore assigns around 1 ns of photon arrivals during the beginning of the new pulse interval to the previous pulse interval, thereby obtaining more information about the decay. This is the only explanation I can think of that fixes the pulse interval and temporal resolution at the specified values while allowing the total number of time bins to exceed 133. However, the photon count falls off a cliff after 12.5 ns and this part of the decay will make the overall fit very bad if included in the fitting range. The mechanics of the phenomenon and the rationale for storing time bins beyond 133 are not fully understood as the manufacturer does not provide documentation.

If you need to correct for time-varying background autofluorescence, use FLIMKit (§11.2) or FLIMfit (§11.3).

in left panel:

- If there is still a peak artifact in the curve around 8 ns, tick `Reflection Filter` in FLIM box in left panel of FLIM window. If the cutoff range is misaligned, you're out of luck as it can't be adjusted. Either eliminate

the peak at acquisition (p. 13) or analyze your data in software with adjustable cutoff, such as FLIMKit (§11.2).
- Time Gate box: does same thing as `Fitting Range` (§7.2).
- adjust intensity threshold to exclude background (click `Select from image` to see preview)

7.2 Fitting controls

lifetime fitting controls (right panel of FLIM window):

fit function: use `n-Exponential Reconvolution` unless your lifetimes are much longer than the IRF width and there is significant overshoot, in which case that model will underestimate lifetime and `n-Exponential Tail Fit` (which ignores the IRF) should be used instead.

If other models needed, you have to export data and fit elsewhere. For example, you may want to fit a lifetime distribution model (Lakowicz [8] p. 143) rather than a multi-exponential model if your specimen has a continuum of lifetimes, in which case phasor analysis (§8) can also be useful. Know your specimen to decide on the most appropriate model. FLIMKit (§11.2) can fit lifetime distribution models.

`Fitting Range`: Adjust range to see map and analysis of only the desired photons. Leica's advice is to leave it as it is for better fit in LAS X. The software appears to automatically exclude expected irregularities at the beginning and end of the pulse interval. IRF will still be used for fitting `n-Exponential Reconvolution` even if the IRF is outside the range. If `n-Exponential Tail Fit` model is selected, the range is automatically adjusted and a `Lifetime Offset` parameter appears in the table below. Nevertheless, you should check and if necessary fine-tune the range in this case.

Specify how many exponents. Fitting not recommended if more than two exponents as it is unreliable especially without prior controls, due to parameter correlation of amplitude and lifetime; use phasor instead (§8).

If you have a control specimen with known lifetime(s), manually enter the lifetime(s) and uncheck the corresponding fit box(es). Similarly enter values for other parameters if required.

To use an experimental IRF, open the `lif` file in the projects tab, right click the blank lower part of the right-hand lifetime fitting panel and select `Load Instrument Response Function`. Select your IRF specimen data and click `OK`. In the upper part of the right-hand lifetime fitting panel, there will be a drop-down button to select the IRF. Fit the IRF shift (especially if your IRF was obtained by reflection/scattering) and background by ticking the `Fit` boxes next to `IRF Shift` and `IRF Background`. You can also revert to the calculated IRF or remove your experimental IRF. Beware: loading an experimental IRF for multiple selected decays is buggy in LAS X.

7.3 Fitting output

click `fit`, or `fit all` (batch process) if you have multiple channels/ROIs etc. This will fit constant lifetime(s) across each image/ROI.

visually check fitted curve and residuals shown in the graph. This is the first important step in assessing the result.

Right click the graph for display options such as legend and data cursor.

The fitted lifetimes and other parameters are shown at upper right and in the table at the bottom of the window.

Explanation of parameters:

- `Amplitude`: fractional amount of fluorophore responsible for each exponential component. Intensity-weighted mean lifetime more robust than amplitude-weighted [14].
- `Tail offset` = background intensity
- `IRF Background`: background of IRF relative to background of decay.
- `IRF Shift`: time offset required to align IRF and measured decay curve. May be misaligned if experimental IRF was measured separately and instrument time counting wasn't exactly the same between IRF measurement and specimen measurement. For system-calculated IRF, shift is 0.

`Free` button at top: select custom parameters to display intensity and colour in image. Doesn't work (reported to Leica).

To see how lifetime changes over a time series (not applicable if you did not do time lapse):
- toggle `Mean ROI` button at top left of FLIM window.
- draw ROI(s).
- the graph shows ROI mean photon count change over time.

7.4 Assessing goodness of fit

7.4.1 χ^2 statistic

LAS X gives χ^2 (chi2/chi-squared) values in reduced form (divided by degrees of freedom i.e. nº of datapoints, in this case the number of 97 ps time bins, minus nº of fitted parameters). Good reduced χ^2 value close to 1, usually no larger than 1.5. When you have very few photons you can also get a χ^2 very close to 1, but dont trust the fit too much. χ^2 decrease by half or more indicates fit is considerably better. but beware of overfitting. χ^2 considerably smaller than 1 indicates possible overfitting.

There are two ways of calculating χ^2, Neyman and Pearson. We don't know which one is given by LAS X. Neyman is biased towards larger values with lower photon count, while Pearson is unbiased. If you need this differentiation, use FLIMKit (§11.2) for fitting. Ji *et al.* [15] formulated a combined Neyman-Pearson χ^2.

On the STELLARIS, higher photon count sometimes gives worse χ^2. This may be due to more electronic artifacts (such as the reflection bump at 8 ns) in the decay curve at high photon count and high laser power. This χ^2 anomaly is more pronounced if fitting range includes decay rise and before [why?]. Another possible cause is incomplete decay (because of high pulse frequency of 78 MHz) and wrong model used for that. Try fitting with `n-Exponential Tail Fit`. For Atto 488 lifetime 4.2 ns, not much difference seen in χ^2 or fitted lifetime between the two models if `n-Exponential Reconvolution` is used with fitting range excluding decay peak. STELLARIS χ^2 sweet spot for photon count appears to be when decay peaks at around 10000 photons.

An alternative to χ^2 is to compute the temporal autocorrelation function of the residuals (Lakowicz [8] p. 132). If the model is good and there is no systematic error, there would be no autocorrelation as the residuals are randomly

distributed around zero. You have to export the data (§10.2) and do the auto-correlation outside LAS X.

More rigorous statistical evaluation is needed if you are trying to characterize subtle differences or clusters across many cells or ROIs.

7.4.2 Parameter uncertainty

To display parameter confidence intervals in the table, right click inside the table and select `Show Confidence Interval` [what percent]

Confidence intervals for fitted parameters (by bootstrapping). right click the blank lower part of the right-hand lifetime fitting panel and click `Error Correlation Plot`. choose parameters and set desired confidence interval. you can also inspect the correlations between parameters. Right click to Export diagram data.

According to Lakowicz [8], confidence intervals obtained by common methods usually underestimate parameter uncertainty. To get a real idea of parameter uncertainty, it is best to do support plane analysis: vary each parameter from the initial fitted value, refit the model with that parameter held fixed at the new value, repeat with many different values of different parameters, and see how the χ^2 (chi2/chi-squared) surface changes. Obviously impractical to do manually, possible to script it using FLIMKit (§11.2) or the `FLIMJ` plugin (§11.1.2) in Fiji or write your own programme.

See Lakowicz [8] p. 134 for more in-depth coverage of parameter uncertainty.

7.5 Pixel-wise fit

I call it Slow FLIM.

not appropriate for FRET because of changing lifetimes.

each pixel should have enough photons for a good fit. You may want to bin data to get enough photons.

If you have a control specimen, you should have already fitted a decay curve to it.

click `FLIM Image Fit` button in right panel of FLIM window.

use intensity preview to adjust photon count threshold until background is removed.

- Untick lifetimes if you fitted a control specimen earlier, or if you have obtained lifetimes from an overall fit of a homogeneous ROI and just want to analyze the proportion of each component in each pixel.
- Tick lifetimes if lifetime varies spatially.

batch process: where applicable, tick boxes will appear at lower left where you can select fit all channels or fit all images

click `Fast Fit` or `Precise Fit`. not much difference in results usually, according to Leica.

Additional analysis Buttons at top

buttons that work only after pixel-wise fit:

- `Components`: see separate intensity (photon count) map for each exponential component.
- `Grayscale`: gives same information as components, in greyscale.
- `Lifetime`: shows intensity as well as lifetime differences by pixel, useful only if you checked lifetimes for image fit.
- χ^2: map of χ^2 (chi2/chi-squared) values (more details below).

for any image/map, right click → show data cursor to see pixel values as you mouse over.

7.6 Global analysis

Gets more reliable lifetime values. See Lakowicz [8] p. 144 for detailed explanation. You have to have collected different emission bands using multiple detectors under specialist settings.

In the fitting table in the right-hand panel in the FLIM window, for each lifetime row, click the cell under the `Global` column and check `Link Channels` in the popup. This will force it to fit the same lifetime value for each emission band, as lifetime should be independent of emission wavelength. Don't link anything for the other parameters such as amplitude, as amplitude varies with emission wavelength.

Click `Fit All` to perform global analysis.

7.7 Pattern fit

If you have a mix of molecules with multiexponential components. better than decay curve fitting but not quite as intuitive and efficient as phasor (§8).

Click `Pattern Fit` at top left of FLIM window

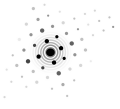
Decay diversity map reveals fluorophore populations with their average photon arrival time (by pixel) on the x-axis and standard deviation in lifetimes (how multi-exponential they are) on the y-axis. unfortunately, right click export image saves only an image without axis information.

In decay diversity map, draw ROI on a blob and click `Add ROI` to define a pattern. Repeat for all blobs.

Click `FLIM Image Fit` (while in `Pattern Fit` mode).

`Shift` parameter: offset of photon arrival time between two patterns

The separated patterns are displayed in the `Components` tab.



8 Phasor analysis

8.1 Phasor plot

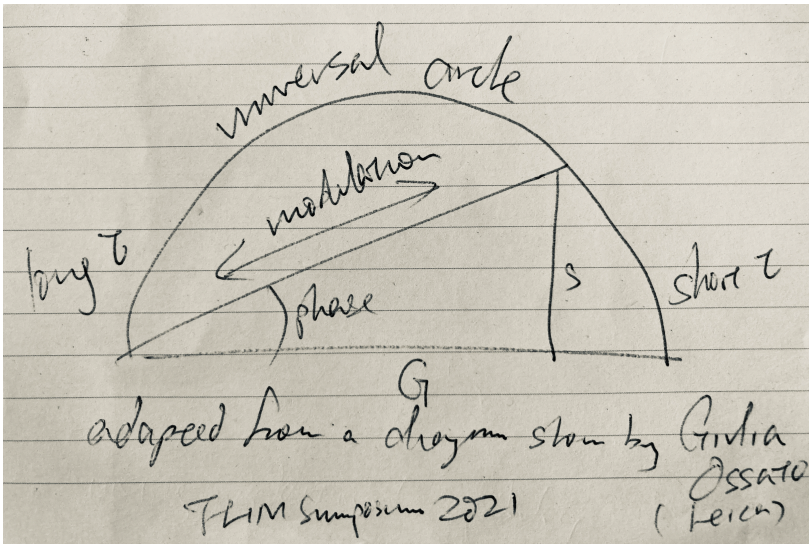


Figure 8.1. Explanation of phasor plot.

obtained by Fourier transform of time-domain decay curve into frequency-domain phase and modulation information in terms of G (x-axis) and S (y-axis) coordinates (Fig. 8.1).

also used for hyperspectral image analysis

Advantages of phasor plots:

- model-free (no parameter uncertainty), shows raw data.
- does not depend on IRF.
- needs fewer photons. 100 photons per pixel sufficient although couple of thousand photons per pixel is better
- very sensitive to small differences in lifetime
- good for specimens with continuous environmental variation that produces lifetime distribution rather than discrete lifetimes

To switch to phasor analysis mode, click `Phasor` at top left of FLIM window

8.2 Phasor calibration

Phasor plot should be calibrated so it isn't affected by the IRF. Leica says you can usually let it autocalibrate using the pulsed laser frequency. With Atto 488 the autocalibrated phasor lifetime was found to be about 0.5 ns shorter than the lifetime obtained from decay curve fitting.

For accurate absolute lifetimes or standardization with other FLIM systems, you can calibrate the phasor plot using a bright specimen of stable known monoexponential lifetime. FLIM LABS makes a green calibration slide of lifetime 2.5 ns. Acquire using same instrument configuration as your FLIM sample.

Select both the FLIM and calibration datasets in the `Projects` tab and click the `Calibrate` button in the phasor panel. Toggle off the `Auto-Calibrated` check mark. Enter lifetime of calibration sample. Click `Find`. Click the appropriate `Apply` button.

To reset to auto calibration, turn the `Auto-Calibrated` check mark back on. Click `Reset` to ensure future acquisitions use auto calibration.

8.3 Phasor interpretation

fluorescence decay of each pixel is plotted as a point. red on the LAS X phasor plot means lots of pixels in the FLIM image have the lifetime characteristics denoted by that location in the plot, blue few

lower right of semicircle (so-called universal circle) denotes 0 ns lifetime (which can be caused by reflection). lifetime increases faster and faster as you move along semicircle. For 78 MHz laser pulse, 12.5 ns lies along semicircle somewhere near upper left of plot.

- blob on semicircle: monoexponentially decaying dye
- three blobs in straight line: middle blob is a mixture of the other two lifetimes, ratio being proportional to the relative distances to the two outer blobs. Perfectly straight line is rare in practice. This is the so-called law of linear addition.

- three or more blobs on semicircle forming polygon: three or more lifetime components, with a blob inside polygon being mixture of the components. see Ranjit *et al.* [16]
- blobs inside semicircle e.g. CFP: more than one lifetime (either multi-exponential species or mixture), deeper in circle = more lifetimes
- elliptical/elongated blobs or smear: FRET or lifetime varying with environmental diversity
- Cluster near lower right tends to be autofluorescence although autofluorescence can appear elsewhere too. Cluster at lower right corner has zero lifetime and indicates stray reflection or scattering.

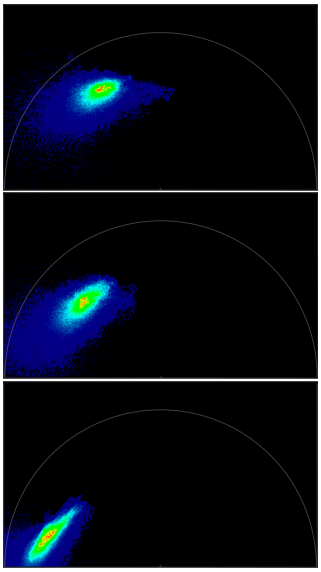


Figure 8.2. Effects of continuous-wave 405 nm laser accidentally left on. Top, off; middle, 1% power, bottom, 10% power.

If the continuous-wave 405 nm laser is accidentally left on, it will cause haze towards lower left or even shift the entire blob towards lower left (Fig. 8.2). Stray unmodulated room or monitor light may have similar effects but much less pronounced.

You may see pixels outside universal circle. These pixels have disproportionately less demodulation for the corresponding phase shift than would be expected for pure monoexponential component, indicating that something else is going on other than direct fluorescence e.g. excited-state products (sensitized emission) or FRET causing delayed excitation of acceptor. Scattering/background can also produce diffuse noise that extends outside circle.

8.4 Phasor visualization controls

You may wish to first define an ROI (§6.2) or select a single pixel to show in phasor plot.

Phasor box on left of window:

To visualize very short lifetimes more easily, you can set a higher `Harmonic` to shift the blobs towards the centre of the phasor plot. Ticking the `Multiple` checkbox will make additional phasor pattern analysis tool buttons appear that use multiple harmonics to better separate components.

If photon count per pixel is very low, you may see a curved row of bright spots at upper right and/or a faint square grid across the plot caused by single-photon pixels and wide temporal histogram bins. Adjust `Threshold` to suppress these artifacts.

Phasor filters:
- different filters can show qualitatively different things so I recommend examining your data with different filters.
- wavelet filter (uses Anscombe transformation) [17] increases "contrast" to make blobs more distinct when there aren't quite enough photons. Wavelet filter gives more phasor contrast than median filter and pixel binning combined, and if wavelet not good enough, add pixel binning. Wavelet filter can also be used to optimize STED acquisition (not available on our STELLARIS). However, there are different types of wavelets and I have found that wavelet filter gives quite different patterns between LAS X and GSLab (§11.5) when per-pixel photon count is low. Use with caution.
- Median filter: Leica says this should be used for analysis. You can increase value to concentrate the blobs, but you lose resolution.
- Preview filter: recommended for live FLIM (§4).

LAS X appears to threshold then filter, as pixels that have been thresholded out sometimes reappear.

In the panel to the right of the phasor plot, select channels or ROI to plot simultaneously. Can also hold `CTRL` and click to select multiple datasets in project tab, then select them at lower right to show in phasor

8.5 Phasor analysis tools

Consider GSLab (§11.5) for more advanced phasor analysis and publication-quality graphics, unless doing metabolic trajectory or FRET (§9).

circle icon:

- drag circle onto the different blobs and see which parts of the image above the phasor plot are highlighted. You can save the highlight mask or convert it to ROI by right-clicking on the image.
- scroll mouse wheel to change size of circle
- lifetime at centre of circle is shown at upper right of FLIM window
- if you know the lifetimes of the components, click to make circles on the component blobs and drag the circles until the lifetimes are indicated correctly

to do the above automatically by machine learning, use GSLab (§11.5).

to unmix, add two or more circles to the phasor plot and click `separate` at right. This accounts for all the photons in the blobs, not only those along a straight line joining the blobs. tick Show Image Overlay if you wish to see the intensity image at the same time. If you need pixel-wise ratios for further analysis, use GSLab (§11.5).

- icon of circle with line through it: draw straight line and move small circle to find ratio (shown at top right of FLIM window).
- icon of triangle with circle in it: draw triangle and move small circle. If you need more than three sides, use GSLab (§11.5).
- mouse over line or circle to move, drag corners to change shape or direction

- appears when `Multiple` checkbox next to `Harmonic` is checked.
- allows you to specify number of monoexponential components and fix or fit lifetimes and ratios of your choice.

- line(s) joining component lifetimes on universal circle, lengths of line segments correspond to ratio(s)

multicoloured line:
draw along smear and see which parts of image above are coloured correspondingly
adjust transect width by scrolling mouse wheel.

multicoloured angled line:
can be used for non-straight smears also
draw along smear, clicking on blobs along the way to display lifetimes

click cross button of analysis item at upper right of FLIM window to delete it from phasor plot

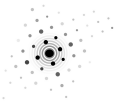
To get the G (real x axis) and S (imaginary y axis) values of the phasor plot corresponding to the added circles, right click in the upper right panel and select `Show G and S`. To get the modulus lifetime, select `Show Modulation`. See Michalet [18] for explanation and use of phase lifetime and modulus lifetime.

SD error bars:
right click on phasor and select `Show Mean and Standard Deviation`.
cross appears on plot centered on means.
Mean and SD values of G (x direction) and S (y direction) shown in right panel

click colour bar or colour triangle button to add colour key.

To contrast the image by phase or modulation lifetime or distance from a known molecular species, use GSLab (§11.5).

To batch process phasor unmixing (separation) for multiple images and/or multiple channels, add circles to all the required phasor plots and click the `Batch` button at right instead of the `Separate` button. When viewing the results, select the desired channels using the channel buttons to the right of the images, select the desired time point and z slice using the sliders.



9 Metabolic/FRET analysis

9.1 Metabolic trajectory

binding ratio of NADH

decay curve fitting (§7) can be used but phasor is more intuitive.

- switch to phasor mode.
- select `Intensity` tab at top of window.
- click `Draw Metabolic Scale` button. click on a blob in the phasor plot. binding ratio isopleths (10% to 100%) appear on the phasor plot.
- In the upper right panel, binding ratio of clicked blob is displayed. you can also manually enter the unbound autofluorescence lifetime (default 0.4 ns) according to your control specimen.
- the intensity image is now colour-coded according to binding ratio.

9.2 FRET efficiency

acceptor reduces donor lifetime due to energy transfer to acceptor

9.2.1 Decay curve method

Tricky if donor is multiexponential or if there's autofluorescence background.

- Click `FRET` button at upper left of FLIM window
- fitting model: choose monoexponential donor (does double-exponential fitting) or multiexponential donor (including autofluorescence).
- Click `Fit`.
- FRET efficiency displayed in upper right panel.

FRET efficiency is the ratio of (pure donor lifetime − FRET lifetime) to pure donor lifetime. One should use amplitude-weighted average lifetime to calculate FRET efficiency.

9.2.2 Phasor method

For published examples of this approach see Szmacinski *et al.* [19] and Lou *et al.* [20].

tick all three specimens (donor, donor-acceptor and autofluorescence) in phasor control panel to the right of phasor plot panel to show phasors simultaneously. If there is autofluorescence, quenching trajectory will converge to autofluorescence blob rather than to 0 lifetime (in the case of 100% FRET efficiency and no background fluorescence).

- Draw FRET trajectory: click this button at bottom left of phasor panel.
- Click on autofluorescence blob and drag to donor-only blob
- Click and drag white triangle in phasor plot to adjust FRET trajectory curve until it passes through the FRET smear
- Drag circle to desired location along FRET trajectory. FRET efficiency displayed in upper right panel
- Inspect colour-coded image to see where FRET is happening.
- To delete FRET trajectory from phasor plot, click cross button in upper right panel.

Decay curve fitting approach (§9.2.1) can be done alongside phasor approach to get additional information like average lifetime.

10 Saving & export/import

10.1 Save in Leica format

Save entire project by clicking save icon in projects tab of either main or FLIM window. You can delete components within a project or copy/paste them to existing or new projects.

more photons will give larger file size probably because more time bins within each laser pulse have non-zero photon arrivals recorded.

at bottom of FLIM tab on left of FLIM window are buttons to save results and images within Leica project file:

- click `Save Results` to save analysis.
- click `Save Phasor` to save phasor plot.
- click `save image` to save a confocal image with high bit depth.

10.2 Export for external processing

LAS X can do most of the FLIM analysis but can't do things like custom decay models, further statistical analysis such as multivariate classification of different physiological states, publication-quality custom plots etc. For these you have to export the data/results from LAS X and do further processing in external software (§11).

If you only need individual or subset of accumulated frames, z-slices, time points or channels, subset in LAS X before export. Channels are subsetted by toggling the channel buttons to the right of the images, while the other dimensions are subsetted in the left panel of the FLIM window.

For serious external analysis, export data (and measured IRF if applicable) as `ptu` (PicoQuant format) containing raw photon arrival times and counts. at top right of FLIM window: `File` → `Export Raw Data`. Exports accumulation of all repetitions even if you have subsetted them in LAS X. Exports all photons regardless of whether standard, all photons or first photons is selected in `Specialist Settings`. Best to have used single photon counting regime to avoid having to

perform dead time correction for pile-up. The `ptu` file may contain more than 133 time bins as explained in §7.1. The `ptu` file does not store pixel dwell time.

To export pixel intensity or fast FLIM histogram:
- select the `Intensity` or `Fast FLIM` button at the top to show the desired histogram.
- right click the histogram → `Export diagram data`

To export measured and fitted decay curves and IRF data points: right click the graph → `Export diagram data`

Fitted params: right click on table in FLIM window and select `Export table content` to export as `csv`

Export phasor analysis results in upper right panel: right click inside panel and select `Export Data` to export as `ptu`.

Export phasor plot image: right click on plot and click `Export Image`. Embeds user annotations like circles and colour key but no axis annotations and overall low quality unsuitable for publication. To get a proper phasor plot, export raw phasor coordinates and use GSLab (§11.5).

Export raw phasor G and S coordinates. Can load in GSLab (§11.5) for further phasor analysis and to generate high-quality phasor plot, but if you need multicomponent unmixing using higher harmonics in GSLab, export `ptu` instead.

- Turn off any filters and thresholding.
- Phasor mode must be selected at upper left of FLIM window.
- Right click on intensity image and select Export Raw Image.
- In the popup, click the `TIFF` tab and tick `Save Phasor GS`.
- Select `Fixed Range` and `per Grey Level` and type 1 in the number field.
- Click ok.
- Three `tiff` files are generated for each phasor plot of each image in the active dataset. Put them in a folder.
- can't batch export.

export FLIM images or masks generated from FLIM analysis:

- if you don't subset the data, it will export one image for each time point/z-slice/channel. This is useful if you wish to make 3D visualizations or animations which have to be done in other software.

- The general steps are to right click one of the images under the appropriate tab (`Intensity`, `Fast FLIM` etc.), select `Export Raw Image`, click the `TIFF` (one file for each dimension) or `ImageJ TIFF` (single multilayer `tiff` file) tab in the export popup, uncheck `Save Palette Image as RGB` and tweak the settings.
- to export Intensity images with pixel values scaled to 16 bits, select `Auto Range` under the `TIFF` tab in the export popup.
- to export photon count map, select `Fixed Range` and `per Grey Level` and enter `1` in the number field.
- to export lifetime or χ^2 maps, select `Fixed Range` and `per Grey Level` and enter `0.001` in the number field. The pixel values of the exported map are 1000 $\times$ the actual values so you have to divide by 1000 in subsequent analysis.
- for large-format display, you can right click the image and select `Export Image` (instead of `Export Raw Image`) for an interpolated image of about 8000x8000 pixels. This will preserve crisp borders of squares representing the pixels in a small FLIM image when enlarged for display.

11 Processing in external software

11.1 Decay curves in Fiji

If you're established in the Fiji ecosystem, it has functions for visualization and standard decay curve fitting. However, don't open the `lif` file directly in Fiji. It will open but in mostly wrong and unusable formatting.

11.1.1 Opening ptu files

you can open `ptu` files using the `PTU Reader` plugin and do basic visualizations of the maps of photon counts and fast FLIM lifetimes (IRF-corrected) and the stack of pixel-wise raw photon arrival times.

plugin download: `https://github.com/UU-cellbiology/PTU_Reader/wiki`
plugin user guide: `https://github.com/UU-cellbiology/PTU_Reader/wiki/How-to-use-plugin`

`Plugins` → `PTU Reader`
can be scripted

The slices in the stack of arrival times represent the temporal resolution of 97 ps. Multiply the index (start from 0) of a given slice with 97 ps to obtain the arrival time represented by said slice. You may see arrival times later than 12.5 ns—see §7.1 for explanation.

metadata is displayed in the log window.

For 3D animation, use the `3Dscript` plugin.

11.1.2 FLIMJ plugin

Gao *et al.* [21]. Standard decay curve fitting. Scriptable (`https://github.com/flimlib/flimj-ops`). May have difficulty processing large datasets. I suggest using FLIMKit (§11.2) or FLIMfit (§11.3) if you need more advanced decay curve analysis outside LAS X.

Installation and user guide: `https://imagej.net/plugins/flimj`

11.2 Decay curves in FLIMKit

Note that FLIMKit is still under development. Some features may not work fully.

For standard decay curve fitting, LAS X is adequate and has more advanced parameter uncertainty analysis.

Although FLIMKit can do phasor analysis, I recommend GSLab (§11.5) as the latter has more comprehensive features.

Useful features of FLIMKit:

- reflection filter with adjustable range
- able to fit lifetime distribution model
- able to perform time-resolved fluorescence anisotropy analysis (§11.4)
- able to reconstruct tiled data from LAS X that gets untiled on export
- time-varying background correction for autofluorescence
- able to use machine IRF (see §5.1) which according to the developer is much more robust than calculated IRF although not as good as measured IRF
- cellpose segmentation integrated
- command line interface for advanced use and batch processing
- export `GeoJSON` of ROI for spatial analysis in software like QuPath
- simulate FLIM data (including IRF) in both `ptu` and `sdt` formats

Installation/documentation: `https://github.com/FLIMKit`

Installation of anisotropy plugin: `https://github.com/FLIMKit/flimkit-anisotropy`

11.3 Decay curves in FLIMfit

FLIMfit is another alternative if:
- you need more control over preprocessing (e.g. smoothing out noise) and decay curve fitting (e.g. fitting algorithm)

- you need time-varying background correction for autofluorescence, but FLIMKit (§11.2) can also do it
- you have time-resolved anisotropy data (§11.4) in `sdt` format (see §11.4 for analysis).

However, note the following caveats:
- FLIMfit is no longer maintained.
- FLIMfit calculates that there should be 133 time bins in STELLARIS data based on the bin width and pulse interval, and throws away data beyond 133 bins (see §7.1 for explanation). You lose some of the raw Leica information (whose utility is as yet unknown) but it shouldn't affect the fitting.
- If you used a PicoQuant instrument, FLIMfit may not be able to open the `ptu` depending on the encoding used.

Therefore I recommend trying FLIMfit only if other software options don't work for you.

Warren *et al.* [22]. Documentation/installation: `https://www.flimfit.org/`

On a Mac, when installing MATLAB runtime it will ask you to append something to your `DYLD_LIBRARY_PATH`. If you don't know what to do, see `https://discussions.apple.com/thread/8018254`

Note: FLIMfit flips the 2D image in the `ptu` file generated from our STELLARIS so the image displayed in FLIMfit is topologically identical to the eyepiece view as described in §3.2.

to view measured decay curve, click a pixel in the image or draw an ROI using one of the shape tools at top left. Click the image to remove an ROI.

perform segmentation in FLIMfit or load external mask:
- `Segmentation` → `Segmentation Manager`
- To load external mask, click `File` in the `Segmentation Manager` window and select one of the load options.

Under Data tab:

- set smoothing to reduce noise.
- `Rep. Rate` should have been autoset to `78` (MHz).
- Adjust `Integrated Min.` to exclude background (thresholded red in image)

- Set `Time Min./Time Max.` to `200/12000` (ps) to exclude laser pulse duration at beginning and any artifacts at the end.
- The decay curve may not update automatically when you make these changes. Refresh the curve by re-clicking the pixel or redrawing the ROI.

Measured experimental IRF:

- load the `ptu` exported from LAS X.
- draw ROI of representative region in image.
- right click on the decay curve and Export Data.
- if you measured the IRF using a different detection configuration from the FLIM specimen, fit and correct for possible IRF shift caused by optical path length difference as described at `https://vimeo.com/210234360` (fast forward to 03:14)

Data from monoexponential dye for IRF estimation:

- load the `ptu` exported from LAS X.
- draw ROI of representative region in image.
- `Tools` → `Estimate IRF`. Check residuals graph at bottom and χ^2 at upper right (should be close to 1).
- Click `Save IRF` at bottom right to save as `ptu`.

To load Leica-calculated IRF or saved `ptu` of measured/estimated IRF:

- extract the two columns (time and count) of the IRF from the exported Leica decay curve data, multiply the time column by 1000 to convert to picoseconds and save the data as a separate `ptu`.
- In FLIMfit, `IRF` → `Load IRF` and select the `ptu`.
- The IRF should appear on the decay graph as a red graph.

Curve fitting:
- In the `Lifetime` tab, set `Fit IRF Shift` to `Fitted` to ensure that any IRF shift is corrected.
- In the `Advanced` tab, select the fitting algorithm. Choose `Maximum Likelihood` if you have a low photon count.
- click the appropriate `Fit` button at bottom left.

Tabs at top:
- `Parameters`: see fitted parameters and χ^2 (chi2/chi-squared) value which should be close to 1 for a good fit. The parameter `t0` is the fitted IRF shift.

To export parameters, go to `File` → `Export Fit Results Table`
- `Images`: select parameters to map at top right.

For time-varying background autofluorescence correction, see `https://vimeo.com/210192564`

11.4 Time-resolved fluorescence anisotropy

Analyze according to Lakowicz [8] section 11.2.2 on p. 388.

since same detector used for both images, no need to correct for difference in detector sensitivity

fit anisotropy decay curve.

max theoretical anisotropy value at peak of anisotropy decay curve will be 0.4 (for one-photon excitation) if fluorophores are static and have no depolarization, and 0.57 for two-photon excitation.

rotational, segmental and local correlations at progressively smaller spatiotemporal scales.

anisotropy decay over time indicates dynamic depolarization processes. Interpret carefully as depolarization has multiple possible causes.

11.5 Phasors in GSLab

Good not only for STELLARIS data but also for data from other instruments with no or only basic phasor analysis capabilities. GSLab has the rare ability to load file formats from all major manufacturers. However, it doesn't do FRET or metabolic trajectory analysis.

Features not available in LAS X:

- machine learning-based clustering for automated image segmentation using the phasor space. However you have to manually specify how many blobs. `https://www.youtube.com/watch?v=6RH9v6F7AvM`

- Quantitative pixel-wise unmixing of multiple fluorescent species using multiple harmonics for higher precision in unmixing more components.
- Contrast by phase, modulation and distance etc.
- Graphical display of fraction polygon of more than three sides.
- Colour palette of phasor plot has higher contrast and discerns finer gradations.
- Proper plot labels and annotations.
- loading of external 16-bit integer masks with object ID.
- auto detection and numbering of objects in loaded 8-bit binary masks.
- other advanced visualization and analysis settings.

publications: Vallmitjana *et al.* [23], Ranjit *et al.* [24]
download/installation/manual/tutorials: `https://alexvallmitjana.github.io/GSLab/`

If you are installing the package version of GSLab that runs on a separate installation of MATLAB: besides the MATLAB toolboxes listed in the GSLab installation website, also install the wavelet toolbox to get the wavelet filter for phasor plot.

If you are installing the standalone Windows executable, nothing may seem to be happening at first but wait a few minutes as it is probably running in the background.

GSLab cannot be scripted or batch processed although you can open multiple phasors and analyze them together.

- launch MATLAB and type GSLab in the prompt.
- In the cogwheel tab you can find the update button in the lower right corner. If the button has a red number, you can click it to update to that version number.
- To load data, right click in the empty space under `File` tab → `Load` → `Leica FALCON SnG` (first harmonic only) or `PicoQuant ptu` (can compute higher harmonics for multicomponent unmixing).
- If loading `SnG`, choose folder containing the `tiff` files with phasor G and S coordinates exported from LAS X. Multiple sets of `tiff` files for multiple phasors can be placed in the same folder for batch loading. Phasor calibration is not needed.
- If loading `ptu`, also load `ptu` of experimental IRF or monoexponential reference lifetime dataset for phasor calibration. After loading the calibration dataset, right click it and select `Calibration File`. Enter lifetime and check `Apply`. Files from some PicoQuant instruments may fail to load and

you will see error messages relating to Python in the MATLAB command window. If this happens, try installing Python 3.11 and then installing the `ptufile` and `xarray` modules in Python, and then try loading your `ptu` in GSLab again.
- Before starting analysis, go to the `Export` tab and set `Output Folder`.

In the `Image` tab you can smooth the image using `Gaussian Filter` and threshold out pixels with low photon count to suppress phasor background and artifacts. More sophisticated image segmentation has to be done outside and the mask loaded in.

To load an external mask, click `Load mask` in the `Image` tab. Tick `Apply mk` to apply the mask to the data. If the mask contains multiple unnumbered objects, GSLab can number them and export object-wise data (see §11.5.3).

In the `Phasor` tab, click `Plot HR` button to display and analyze original non-downsampled plot (slower). Choose the desired filter. Gaussian is faster while median is recommended for presentation.

You can mouse over the image and the corresponding position of the pixel in the phasor plot will be shown as a white dot. LAS X doesn't do this. You can add and resize circles on the phasor plot by clicking and scrolling mouse as in LAS X. Remove circles by clicking the × button at upper right.

For different contrast methods, go to `Measured magnitude` section at bottom of `Phasor` tab:

- select `Phase`, `Mod`(ulation) etc.
- for distance from known molecular species, select `CustomRadial`
- adjust `Color range` and `Centering` (on position of known species) where necessary.
- the phasor plot background and the images will be colour-coded accordingly.
- to turn off the colours, go to `Display` tab and uncheck `Magnitude background color`.

11.5.1 Automatic phasor segmentation

Using machine learning (Fig. 11.1):

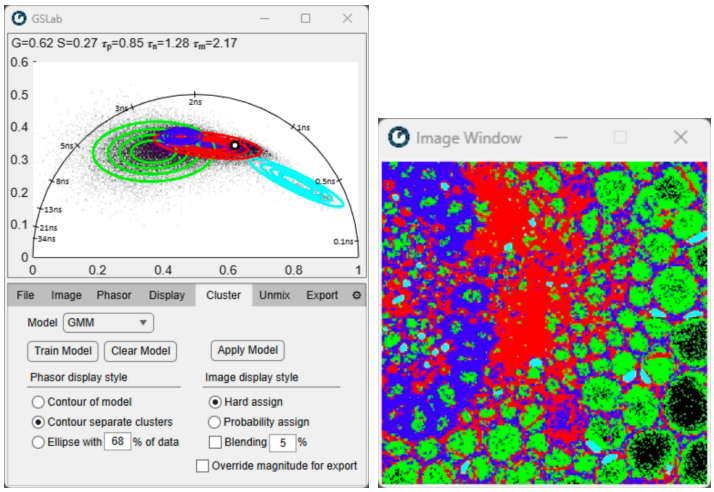


Figure 11.1. Phasor segmentation by machine learning in GSLab.

- first examine the phasor plot and decide how many biologically meaningful blobs you want to fit the model to.
- if you want n blobs, click n random locations within the phasor plot to create n circles. It doesn't matter where you click as machine learning will take care of everything.
- click the `Cluster` tab.
- select model (`GMM` recommended).
- click `Train Model`.
- if you can't see the mapped colour in the dark parts of the image, go to the `Display` tab and uncheck `Soft Masks`.

11.5.2 Multi-harmonic pixel-wise unmixing

In cogwheel tab, set `Max harmonics` to 2 or higher if necessary.

In `Unmix` tab:

- Click + button to add desired number of pure components to universal circle and enter their lifetime values.
- Tick `Export fraction csv` to get pixel-wise fractions of each component for further statistical analysis. Negative fractions may occur in noisy data where some pixels are outside the boundary demarcated by the pure components.

- If you tick `Export ratio csv`, enter nonzero values for the two components.
- Click one of the `Compute` buttons.

11.5.3 Export results

Go to the `Export` tab.

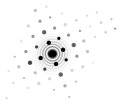
Select what kinds of images, phasors and data you want for presentation and further analysis.

To number objects in an unnumbered mask and get object-wise data, tick `Masked obj data as`.

Finally, click one of the `Export` buttons to export everything you selected.

11.6 Further statistics

After getting FLIM analysis results, perform further statistical analysis using whatever tools you are already familiar with. Contact us for advice on your specific needs.



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Index