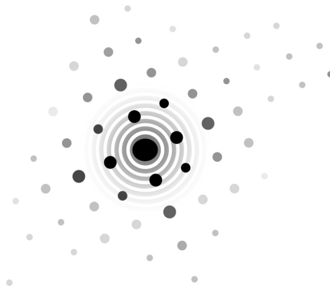
CBIS FLIM MANUAL
LEICA STELLARIS 8 FALCON



updated May 23, 2026

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Prefatory note

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

- It covers advanced procedures beyond the manufacturer's documentation, including the use of a variety of third-party hardware and software tools, refined over many hours of actual testing by the author. Matters of interoperability, standardization and scientific reproducibility are also addressed.
- It incorporates explanations of more general FLIM principles and guidelines where they provide context and insight for better understanding 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 to max out the STELLARIS and its data.
- To be a one-stop resource that synthesizes confusing information from scattered sources 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 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 and Radek Macháň for their advice, discussion or specimen tests that improved this manual.

Please note that I created this document without using AI. If you see AI-generated content that looks similar, AI has plagiarized my work.

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 Applications of FLIM

In fluorescence lifetime imaging, the image is a map of fluorescence lifetime 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 by lifetime differences (e.g. Deore *et al.* 2022). Even lifetimes of natural autofluorescence itself can also be useful. Donaldson (2020) reviews FLIM of autofluorescence in plants.

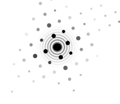
Fluorescence lifetime can also be used to improve STED resolution with less STED power.

Can be used to follow metabolic trajectory over time by labelling with NADH and measuring its autofluorescence lifetime which increases with binding ratio.

Better for measuring FRET efficiency than steady-state methods as it can tell what's really going on at the molecular level.

The emission wavelength-dependent average fluorescence lifetime spectrum can also be used to distinguish materials. Thomas *et al.* (2006) demonstrated the differentiation of species of bacterial spores and pollen in the forensic/military application of detecting the use of biological weapons.

time-resolved fluorescence anisotropy: see Vogel *et al.* (2010) for in-depth introductory treatment. unlike steady-state anisotropy, can measure how fast molecules are rotating (to study microviscosity or molecular characteristics/interactions) and separate static and dynamic depolarization.



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 (§11.3) 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.

lifetimes can be influenced by pH (e.g. CO_2), temperature (big factor), oxygen levels, osmotic pressure, mounting medium (refractive index), viscosity of solvent, polarization of excitation light etc. but not by excitation or emission wavelength, excitation intensity or photobleaching and not by concentration unless quenching in organic solvent at high concentration

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 accuracy of the instrument. If reporting absolute lifetimes you must report the measurement conditions and ideally have checked the accuracy of the instrument using a reference specimen with a stable known lifetime. 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.

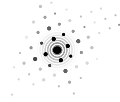
For FRET, best to have three specimens: donor only, donor-acceptor, and autofluorescence. Prepare specimens with cells at same developmental stage and in identical environment with identical specimen preparation protocol. If donor alone not possible, try depleting acceptor.

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, 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

Time-domain FLIM, but unlike traditional TCSPC (time-correlated single photon counting), can count all photons, not only the first photon.

In contrast, frequency-domain FLIM (not available on STELLARIS) uses sine-modulated excitation and measures lifetime through phase shift and demodulation. The longer the lifetime, the larger the phase shift and the greater the demodulation (reduction of amplitude of the sine curve).

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 12.5 ns, preferably no longer than 3 ns otherwise tail of decay curve may extend into the next pulse interval (Leung *et al.* 2011).

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

Time resolution of detector: 10 GHz binary sampler with 97 ps resolution. Better than earlier-generation systems which count only the first photon within each pulse, which tends to underestimate distribution of arrival times

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 at least 30 min to one hour in advance to let it stabilize thermally, otherwise you may get unreliable data and encounter focus drift especially at high magnification.

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.* (2026) for further explanation. When acquiring a 3D *z*-stack, click the down arrow under `Stack Direction` to get correct chirality; if the up 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 IRF (§5) is modeled for X detectors although S detectors are better for blue-green region while X detectors are better for orange-red. Our S detectors are also older. STELLARIS Power detectors have a response closer to linear than traditional detectors as excitation intensity increases. X detectors are more sensitive overall but have lower dynamic range than S detectors. If you flood an X detector with too many photons at once, it will beep and shut off. but you can accumulate photon counts instead.

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:

- mount a bright fluorescence (e.g. *Convallaria*) or reflectance specimen, 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.

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 2006 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 2006 p. 139), click the `Show Fluorifier Settings` button at upper right of the acquisition window and set the emission polarizer to the so-called magic angle of 54.7° (relative to the polarized excitation laser). But a very strong signal is required as the polarizer greatly attenuates the signal when rotated to this angle.

Make sure 405 nm laser is not active in a FLIM channel. The random photons coming from it will add noise.

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. Or you may want to fill the view with one object 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.

In FLIM Settings box at upper left of main window: set accumulations by line (better for live cells whose contents may move) or frame (easier to separate data by frame later)

3.2.1 Time-resolved fluorescence anisotropy

If possible, use an objective of NA between 0.8 and 0.9 (Vogel *et al.* 2010), as lens curvature in high-NA objectives will depolarize both excitation and emission and reduce the accuracy of anisotropy measurements.

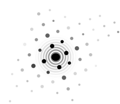
There is no polarization beamsplitter. The emission polarizer has to be rotated sequentially to 0° and 90° for getting the two anisotropy images. Click the `Show Fluorifier Settings` button at upper right of the acquisition window, select the polarizer and set the rotation angle. The rotation cannot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

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. It must not move in between the two sequential images.

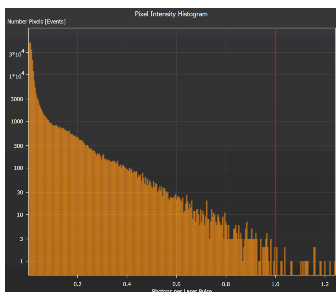
3.2.2 Emission wavelength-dependent fluorescence lifetime spectrum

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

- insert black cover over stage
- turn off lights
- click `LIVE`
- fine-tune focus



Inspect the pixel intensity histogram:

- Vertical red line denotes ideal 1 photon per pulse
- ideally, adjust laser power until histogram fills up space to the left of red line with only a bit of it going past the red line
- fewer photons per pulse will take too long to accumulate data, more photons per pulse increases risk of two near-simultaneous photon arrivals being counted as one and causing unnecessary photostress to specimen
- however, if flimming live cells you may have to lower the power below this guideline. You can try 0.5 photons per pulse.
- 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 object 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

to get enough photons:

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

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.

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 probably because the machine can't just scan the thresholded parts of the field of view.

decay curve and/or IRF (§5) may have stray/multiple peaks caused by stray reflections in the optics. these peaks will skew the fit. to minimize the peaks:

- check that AOBS is set to fluorescence and not reflectance
- use notch filters (multiple notch filters cannot be used simultaneously)
- increase lower limit of detection range further from excitation, especially for specimens attached to coverslip.

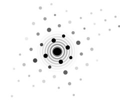
Curve fitting (§7) not available in live FLIM.

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.

To run experiment, click `Start` button at bottom centre of main window. Once experiment is finished, 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`.

You can also use LAS X to simulate FLIM data. Click Tools button at upper right of FLIM window and select `FLIM Simulator`.



5 Instrument response function (IRF)

Analogous to PSF but in time dimension. IRF is combined effect of hardware components such as lasers (especially pulse shape), optics, electronics, detector etc. IRF of instrument with PMT detector may have an afterpulse (second smaller peak). Not a problem if afterpulse is very low relative to data. As with PSF, a way to quantify the IRF is by its FWHM.

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

calculated IRF (by the software) is shown in the displayed graph and used by default.

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

5.1 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.

IRF specimen:

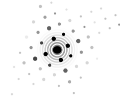
- species with extremely short lifetime such as gold nanorods, or fluorophores quenched with KI (potassium iodide) prepared fresh. Use the same emission collection range as your FLIM specimen to ensure that there is no difference in path length in the prism-based spectral detection optics.
- Scattering specimen with zero lifetime such as LUDOX colloidal silica. Use the same emission collection range as your FLIM specimen but temporarily change excitation wavelength to lie within the emission collection range. Using scattering specimens for IRF may be problematic as you may get reflection peaks from various parts of the optical train.

Collect FLIM data from the IRF specimen. The count rate should be much lower than that for fluorescence; for explanation see `https://www.tcspc.com/doku.php/glossary:differential_count_rate`

Then, with this data open 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. You can also revert to the calculated IRF or remove your experimental IRF.

5.2 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 FLIMfit (§11.2) for analysis.



6 FLIM data preprocessing

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

Specialist Settings box in left panel of FLIM window:

- standard: if two photons arrive, ignore both to get high speed, but be careful of bleaching with excessive laser power.
- all photons: count all (up to two photons only, according to STELLARIS 8 specifications) with slight decrease in accuracy but may be needed for fast live imaging.
- first photons: emulate legacy TCSPC systems

raw data contains count of all photons (up to two) regardless of option selected.

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

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

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 is to right click the image in LAS X, 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. You have to continue analysis 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 time or z-slices 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.
- bin pixels to get higher photon count per pixel at the expense of spatial resolution
- bin lifetime [to be clarified]

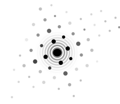
Time Gate box: adjust range to see map and analysis of only the desired photons. Leica expert recommendation is to use all photons for analysis and not time gate. IRF will still be used for fitting even if it is outside a time gate.

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) 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

IRF peak should correspond to peak of decay curve

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

if there is still a reflection peak in the curve, tick `Reflection Filter` in FLIM box in left panel of FLIM window.

in left panel, adjust intensity threshold to exclude background (click `Select from image` to see preview)

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 use `n-Exponential Tail Fit`.

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.

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 residuals shown in the graph.

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

Explanation of parameters:

- `Amplitude`: fractional amount of fluorophore responsible for each exponential component. Intensity-weighted mean lifetime more robust than amplitude-weighted (Fišerová & Kubala 2012).
- `Tail offset` = background intensity
- `IRF Background`: background of IRF relative to background of decay.
- `IRF Shift`: time offset required to align peaks of IRF and measured decay curve. Peaks 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.

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. value decrease by half or more indicates fit is considerably better. but beware of overfitting.

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. Be careful when interpreting this kind of visualization as it can be misleading (see Koch 2026). right click to Export diagram data.

`Free` button at top: select custom parameters to display intensity and colour in image.

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 lifetime change over time.

If other models needed, you have to export data and fit using your own code, such as in Python (§11.6). For example, you may want to fit a lifetime distribution model (Lakowicz 2006 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.

7.1 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.

7.2 Global analysis

Gets more reliable lifetime values. See Lakowicz (2006) 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.

7.3 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

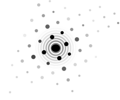
Decay diversity map reveals fluorophore populations with their average photon arrival time (by pixel) on the x-axis and standard deviation in lifetimes (how multi-exponential they are) on the y-axis. unfortunately, right click export image saves only an image without axis information.

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).

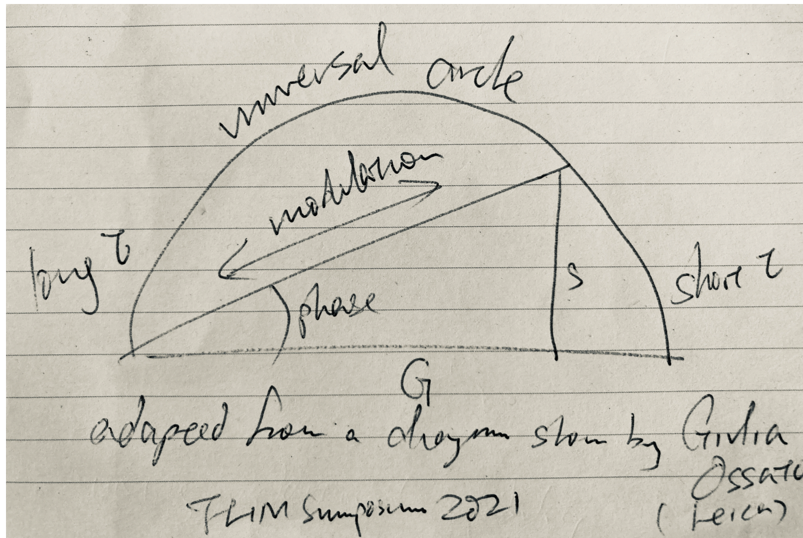
`Shift` parameter: offset of photon arrival time between two patterns

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



8 Phasor analysis

8.1 Phasor plot



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.

also used for hyperspectral image analysis

Advantages of phasor plots:

- model-free (no parameter uncertainty), shows raw data.
- 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

Phasor plot should be calibrated so it isn't affected by the IRF. Usually you can let it autocalibrate using the pulsed laser frequency.

For standardization with other FLIM systems, you can calibrate the phasor plot using a specimen of stable known monoexponential lifetime and manually enter the lifetime. The calibration solution should have very good signal. FLIM LABS makes a green calibration slide of lifetime 2.5 ns.

To calibrate the phasor plot, click the `Calibrate` button in the phasor panel. Click the autocalibration check mark so it disappears. Click `Find`.

8.2 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.* (2019)
- 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

scattering/background/autofluorescence can produce diffuse clusters inside or outside semicircle. cluster near lower right: tends to be autofluorescence. things like stray 405 laser left on can also produce patterns outside semicircle

8.3 Phasor visualization controls

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.

Adjust threshold to reduce background artifacts if necessary.

Phasor filters:
- best to use wavelet filter (uses Anscombe transformation) which increases "contrast" to make blobs more distinct when there aren't quite enough photons. Better than median filter and pixel binning combined. If wavelet not good enough, add pixel binning.
- can also use median filter and increase value to concentrate the blobs, but you lose resolution.

In the panel to the right of the phasor plot, select items 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

8.4 Phasor analysis tools

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 (§11.4).

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 (§11.4).

Ratio tools:

- 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 (§11.4).
- mouse over line or circle to move, drag corners to change shape or direction

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

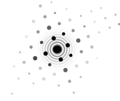
To get the G (real x axis) and S (imaginary y axis) values of the phasor plot corresponding to the added circles, right click in the upper right panel and select `Show G and S`. To get the modulus lifetime, select `Show Modulation`.

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 (§11.4).

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

- 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 trajectory

acceptor reduces donor lifetime

9.2.1 Decay curve method

Not recommended. especially tricky if donor is multiexponential (influences FRET)

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

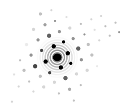
FRET efficiency is the ratio of (pure donor lifetime − FRET lifetime) to pure donor lifetime. One should use amplitude-weighted average lifetime to calculate FRET efficiency.

9.2.2 Phasor method

tick all three specimens (donor, donor-acceptor and autofluorescence) in phasor control panel to the right of phasor plot panel to show phasors simultaneously

elongated quenching trajectory towards lower right. 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.



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.

Don't open the `lif` file directly in Fiji. It will open but in mostly wrong and unusable formatting.

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, such as in FLIMfit (§11.2), 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`. Best

to have used single photon counting regime to avoid having to perform dead time correction for pileup. 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 (§11.4).

Export raw phasor G and S coordinates to open in GSLab (§11.4) for further phasor analysis and to generate high-quality phasor plot:

- It doesn't matter how the display options (harmonic, filter etc.) are set in LAS X as you're exporting raw coordinates.
- 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.

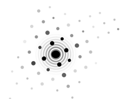
- The general steps are to right click one of the images under the appropriate tab (`Intensity`, `Fast FLIM` etc.), select `Export Raw Image`, click the `TIFF` (one file for each dimension) or `ImageJ TIFF` (single multilayer `tiff` file) tab in the export popup, uncheck `Save Palette Image as RGB` and 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.

10.3 Import external data

if you want to use LAS X to analyze your data or to test whether LAS X gives the same results.

for decay curve fitting, you also have to open the `ptu` of your IRF in LAS X and load it as an IRF.

one nice feature of LAS X is the wavelet filter for phasor plots, but GSLab (§11.4) also has this filter.



11 Processing in external software

11.1 Visualization in Fiji

Raw photon arrivals/counts

In Fiji, open the `ptu` file exported from LAS X using the `PTU Reader` plugin.
download: `https://github.com/UU-cellbiology/PTU_Reader/wiki`
user guide: `https://github.com/UU-cellbiology/PTU_Reader/wiki/How-to-use-plugin`

`Plugins` → `PTU Reader` can be scripted

images open showing photon counts, fast FLIM lifetimes (corrected for IRF) and stack of pixel-wise raw photon arrival times.

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.

metadata is displayed in the log window.

It is possible to use the `FLIMJ` plugin to fit decay curves, but the plugin fails to load on some computers and you may have difficulty processing large datasets. I suggest using FLIMfit (§11.2) if you need decay curve analysis outside LAS X.

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

11.2 Decay curves in FLIMfit

LAS X fits decay curves just fine in most situations. Use FLIMfit if:
- you need more control over preprocessing (e.g. smoothing out noise) and decay curve fitting (e.g. fitting algorithm)
- you no longer have access to LAS X

Warren *et al.* 2013. 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`

launch FLIMfit

`File` → `Load FLIM Data`

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 emission collection range from the FLIM specimen, fit and correct for possible optical path length difference as described at `https://vimeo.com/210234360`

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.

Subtract background from measured decay: Go to the `IRF` tab, copy the value in the `Background` field, then go to the `Background` tab, select `Single Value` for `Background` and paste the copied value into the `Background Value` field.

Curve fitting:
- In the `Advanced` tab, select the fitting algorithm. Choose `Maximum Likelihood` if you have a low photon count.
- click `Fit Dataset` 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.
- `Images`: select parameters to map at top right.

11.3 Time-resolved fluorescence anisotropy

background subtraction from each decay curve

calculate anisotropy values to get anisotropy decay curve. 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 time 0 will be 0.4 (for one-photon excitation) if fluorophores are static and have no depolarization.

anisotropy decay over time indicates dynamic depolarization processes at nanosecond time scale

11.4 Phasors in GSLab

Vallmitjana *et al.* 2025, Ranjit *et al.* 2026

Features not available in LAS X:

- machine learning-based (currently only Gaussian mixture model) clustering for automated image segmentation using the phasor space. However you still have to manually specify how many blobs. FLIM Symposium talk: `https://www.youtube.com/watch?v=6RH9v6F7AvM`
- Quantitative pixel-wise unmixing of multiple fluorescent species using both first and second harmonics for higher precision in unmixing more components. FLIM Symposium talk: `https://www.youtube.com/watch?v=ZPUQVIDgvxc`
- Contrast by phase, modulation and distance etc.
- Graphical display of ratio polygon of more than three sides.
- Colour palette of phasor plot has higher contrast and discerns finer gradations.
- loading of external 16-bit integer masks with object ID.

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

download/installation/manual/tutorials: `https://alexvallmitjana.github.io/GSLab/`

GSLab requires MATLAB. NUS staff/students have to install their own NUS-licensed MATLAB on their own computers as NUS does not provide MATLAB licenses for facility workstations. Please contact NUS IT if your installation is blocked by their security protocols.

besides the MATLAB toolboxes listed in the GSLab installation website, also install the wavelet toolbox to get the wavelet filter for phasor plot.

Load data:

- launch MATLAB and type GSLab in the prompt.
- right click in the empty space under `File` tab → `Load` → `Leica FALCON 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.

Image Thresholding can be done within GSLab but more sophisticated image segmentation has to be done outside and loaded in.

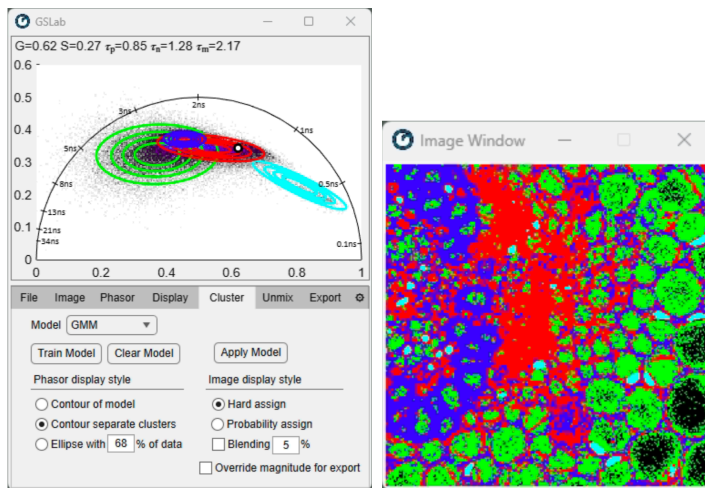
- In the Phasor tab, choose the desired filter.
- you can mouse over the image and the corresponding position of the pixel in the phasor plot will be shown as a white dot. LAS X doesn't do this.
- you can add and resize circles on the phasor plot by clicking and scrolling mouse as in LAS X. Remove circles by clicking the X button at upper right.

For different contrast methods, go to 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.

Automatic segmentation using phasor space using machine learning:

- 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 (currently only GMM).
- click Train Model.



Bi-harmonic pixel-wise unmixing: refer to GSLab manual and tutorial.

11.5 Further statistics

[under construction]

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

11.6 Custom decay models in Python

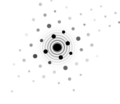
[under construction]

e.g. if you need custom decay models.

Install Anaconda, which will install Python.

```
pip install flimview ptufile liffile tifffile numpy matplotlib scipy
```

Write your code in a code editor. Save it as `xxx.py` Open terminal window (if using Windows, open it from inside Anaconda) and type `python xxx.py` to run.



12 References

BECKER, W. 2026. *The bh TCSPC handbook* 11th ed. `https://www.becker-hickl.com/`

DEORE, P., WANIGASURIYA, I., CHING, S. J. T. M., BRUMLEY, D. R., VAN OPPEN, M. J. H., BLACKALL, L. L. & HINDE, E. 2022. Fluorescence lifetime imaging microscopy (FLIM): a non-traditional approach to study host-microbial symbioses. *Microbiology Australia* 43(1):22–27.

DONALDSON, L. 2020. Autofluorescence in plants. *Molecules* 25:2393.

FIŠEROVÁ, E. & KUBALA, M. 2012. Mean fluorescence lifetime and its error. *J. Lumin.* 132(8):2059–2064.

KOCH, L. 2026. Plotting correlated data. *Journal of Data Science, Statistics, and Visualisation* 6(2):170.

LAKOWICZ, J. R. 2006. *Principles of Fluorescence Spectroscopy* 3rd ed. Springer.

LAPRAZ, F., BOUTRES, C., MONTERROSO, B. & NOSELLI, S. 2026. Mirror-image inversion in commonly used compound microscopes. *J. Cell Sci.* 139:jcs264545.

LEUNG, R. W. K., YEH, S.-C. A. & FANG Q. 2011. Effects of incomplete decay in fluorescence lifetime estimation. *Biomed. Opt. Express* 2(9):2517–2531.

PERIASAMY, A. & CLEGG, R. M. eds. 2010. *FLIM Microscopy in Biology and Medicine.* CRC Press.

RANJIT, S., DATTA, R., DVORNIKOV, A. & GRATTON, E. 2019. Multicomponent analysis of phasor plot in a single pixel to calculate changes of metabolic trajectory in biological systems. *J. Phys. Chem. A* 123:9865–9873.

RANJIT, S., TORRADO, B., VALLMITJANA, A., DURKIN, A. F., DVORNIKOV, A., GANESAN, A., KELLY, K. M. & BALU, M. 2026. Label-free human skin imaging with enhanced molecular contrast via time-resolved fluorescence and advanced phasor analysis. *Commun. Biol.* 9:149.

RUPSA DATTA, TIFFANY M. HEASTER, JOE T. SHARICK, AMANI A. GILLETTE & MELISSA C. SKALA 2020. Fluorescence lifetime imaging microscopy: fundamen-

tals and advances in instrumentation, analysis, and applications. *J. Biomed. Opt.* 25(7):071203.

Thomas, A., Sands, D., Baum, D., To, L. & Rubel, G. O. 2006. Emission wavelength dependence of fluorescence lifetimes of bacteriological spores and pollens. *Appl. Opt.* 45(25):6634–6639.

Vallmitjana, A., Torrado, B., Durkin, A. F., Dvornikov, A., Rajil, N., Ranjit, S. & Balu, M. 2025. GSLab: open-source platform for advanced phasor analysis in fluorescence microscopy. *Bioinformatics* 41(4):btaf162. https://academic.oup.com/bioinformatics/article/41/4/btaf162/8113903

Vogel, S. S., Thaler, C., Blank, P. S. & Koushik, S. V. 2010. Time-resolved fluorescence anisotropy. In *FLIM Microscopy in Biology and Medicine* eds. Periasamy, A. & Clegg, R. M. pp. 245–288.

Warren, S. C., Margineanu, A., Alibhai, D., Kelly, D. J., Talbot, C., Alexandrov, Y., Munro, I., Katan, M., Dunsby, C. & French, P. M. W. 2013. Rapid global fitting of large fluorescence lifetime imaging microscopy datasets. *PLOS One* 8(8):e70687. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0070687