LEICA STELLARIS 8
FLIM USER GUIDE

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We are grateful to Adam Cliffe (Leica), Luis Alvarez (Leica) and Daniel Aik for their inputs.

The latest version of this guide can be downloaded from
https://www.dbs.nus.edu.sg/2021/09/14/cbis-lm-core-leica-sp8-stellaris-upright/

FALCON application note: https://www.nature.com/articles/d42473-019-00261-x.pdf

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EXPERIMENT DESIGN & SAMPLE PREP

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.

avoid weak fluorescence as it will produce lousy FLIM

lifetimes can be influenced by pH (e.g. CO2), temperature (big factor), mounting medium (refractive index), polarization of excitation light etc. but not by excitation wavelength and not by concentration unless quenching in organic solvent at high concentration

You should have a control to compare with. Absolute lifetimes are usually not meaningful as they are highly sensitive to measurement conditions. If reporting absolute lifetimes you must report the measurement conditions.

can do z-stack, time lapse etc. for FLIM but large file size
If you are unsure of the temporal scale of the dynamics, acquire at a higher frame rate and you can bin time later.

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SYSTEM SPECS

Laser pulsing fixed at 78 MHz so lifetimes being studied should be below interval of 12.5 ns
405 nm laser not pulsed and can’t be used for FLIM

10 GHz binary sampler. 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.

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SOFTWARE SETUP

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

Stellaris Power detectors have a response closer to linear as excitation intensity increases. Nevertheless be careful not to saturate the fluorophore.

Assign FLIM channel(s) to HyD X detector(s) where possible
S detectors are better for blue-green region while X detectors are better for orange-red
However, our S detectors are older

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.

Several methods have been suggested for treating the comets:
- wait (reportedly did not help)
- image any standard bright fluorescent sample for about an hour and the comets gradually disappear
- using convalaria slide, saturate it until it beeps for 15-20 seconds (reported to be successful)
- take detector out and light it with a flash light
- if due to aberrant triggering of avalanche events, reset/recalibrate the threshold for avalanche triggering

(Sergio Lopez/Petro Khoroshyy/G. Esteban Fernandez, Confocal Microscopy List)

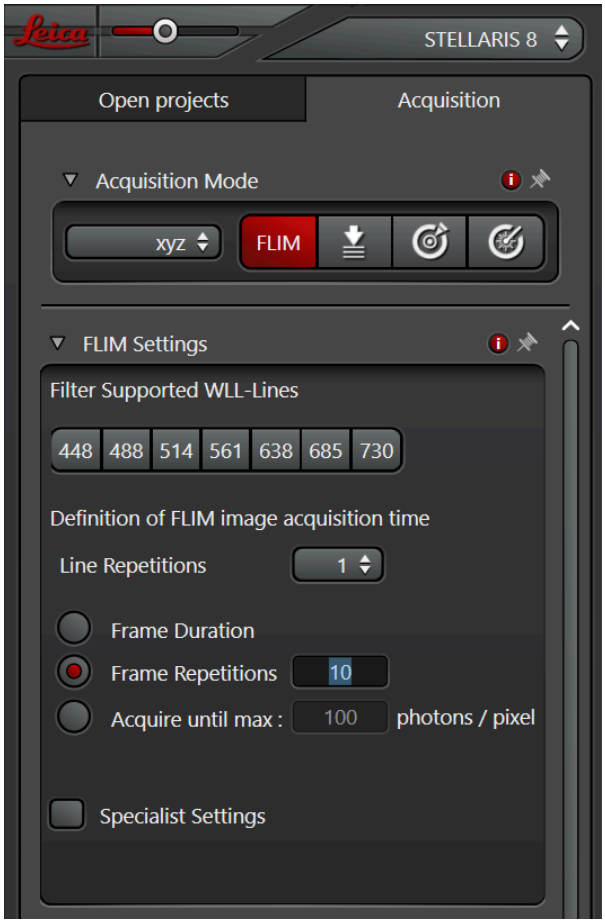
doesn't matter what mode detector is in as it doesn't affect FLIM
can set it to photon counting mode

if signal weak or need high speed, assign two detectors to cover the emission range and further configure in specialist settings tab

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

make sure no crosstalk

set image size around 512 x 512
if you need high frame rate, try larger pixels to get enough photons per pixel



In FLIM Settings box at upper left of main window:
- set repetitions line (better for live cells whose contents may move) or frame (easier to separate data by frame later)
- if using two detectors to cover emission range, check Specialist Settings and click “all detectors” - will sum up everything

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LIVE OPTIMIZATION

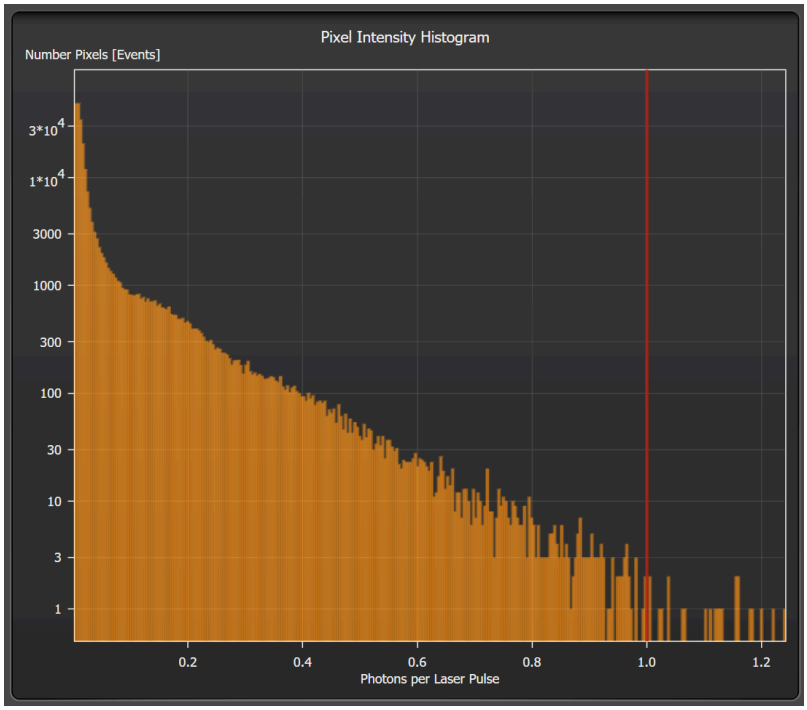
turn off lights
click LIVE
fine-tune focus

must have enough photons for good statistics
single exponential fit: minimum 1000 photons
double exponential fit: minimum 10000 photons
[where to check count?]

to get enough photons:
- high laser power
- slow scan speed
- larger pixels
- accumulate many times
- use two detectors to cover emission range (only when really necessary)

doesn't matter for FLIM if confocal image is saturated

if signal is weak:
- select one of the wavelengths with notch filter if possible (list of available wavelengths in FLIM Settings box in main window). Multiple notch filters cannot be used simultaneously.
- try opening pinhole slightly



Inspect pixel intensity histogram
- Red line denotes ideal 1 photon per pulse
- 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 sample
- however, if flimming live cells you may have to lower the power below this guideline
- if using two detectors to cover emission range, adjust the collection ranges of the detectors until their histograms are similar

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

To run experiment, click Start button at bottom centre of main window.

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DATA PROCESSING & INTERPRETATION

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 subsequent experiments.

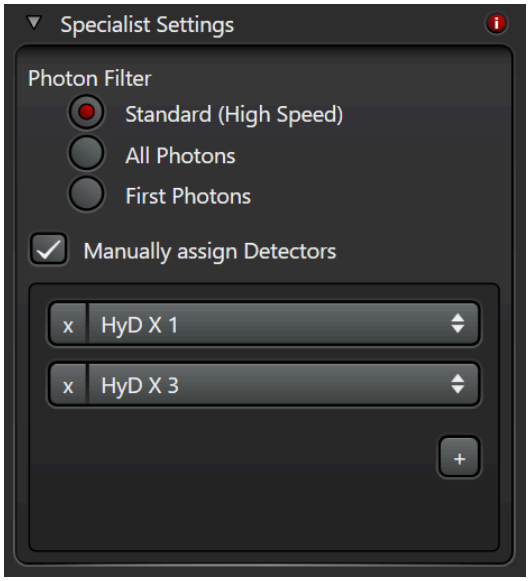
Under FLIM tab on the left of FLIM window:

FLIM box:

subset repetitions or z-slices to see if fewer reps/slices are enough
bin time (for time lapse) to see if fewer time points are still enough to capture dynamics
bin pixels to get higher photon count per pixel at the expense of spatial resolution

Time Gate box:

adjust range to see map and analysis of only the desired photons



Specialist Settings box:

standard: if two photons arrive, ignore both (be careful of bleaching with excessive laser power)
all photons: count all
first photons: emulate legacy systems

if using two detectors to cover emission range, check manually assign detectors

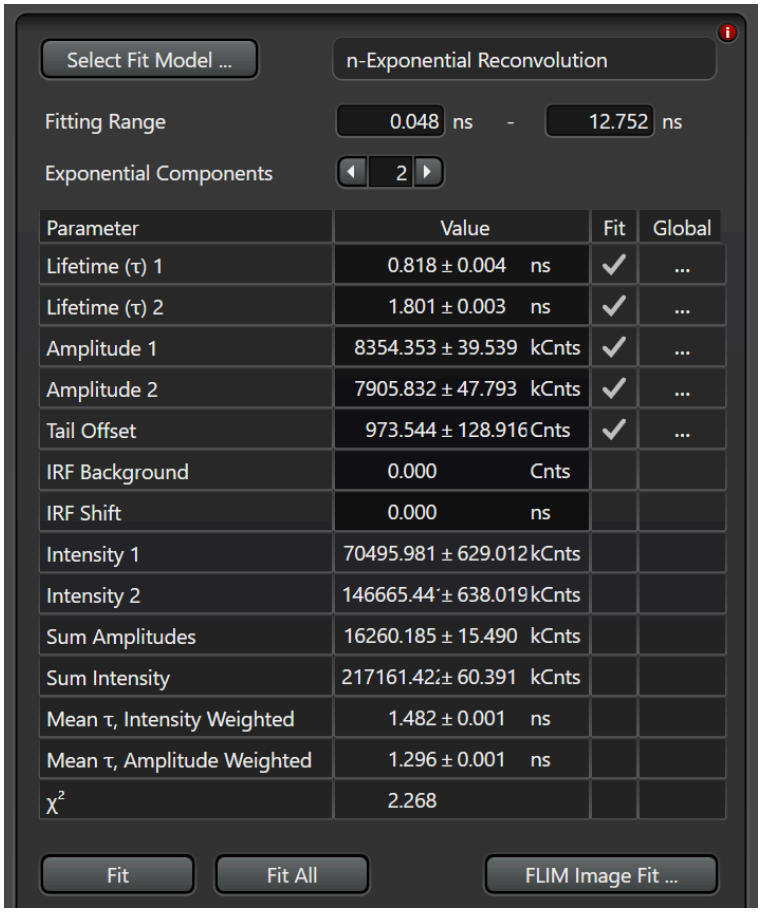
FAST FLIM
average arrival time over entire image (similar to Tau Contrast)
click Fast FLIM button at top centre of FLIM window
look at the pixel colours and histogram to judge variability of lifetimes
right click --> show data cursor to see pixel values as you mouse over

DECAY CURVE

calculated IRF (instrument response function) is shown.
you can also obtain experimental IRF using species with extremely short lifetime such as KI
IRF peak should correspond to peak of decay curve

decay curve should decay to nearly 0
but will overshoot if lifetime is long

decay curve and/or IRF may have stray/multiple peaks caused by stray reflections in the optics.
these peaks will skew the fit.
to minimize the peaks, use notch filters and increase lower limit of detection range further from excitation.



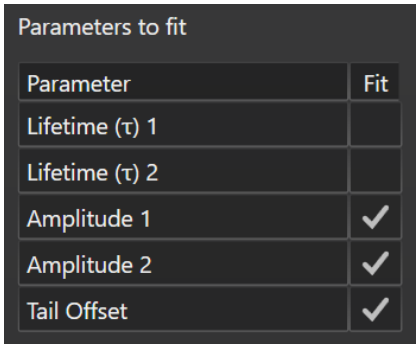
fitting (right panel of FLIM window):
fit function: use “n-Exponential Reconvolution” by default, or “n-Exponential Tail Fit” to accommodate overshoot
specify how many exponents - not recommended if more than two exponents as it is unreliable; use phasor instead
click fit
visually check residuals

FLIM IMAGE FIT - fits pixel by pixel

not appropriate for FRET because of changing lifetimes

click FLIM Image Fit button in right panel of FLIM window

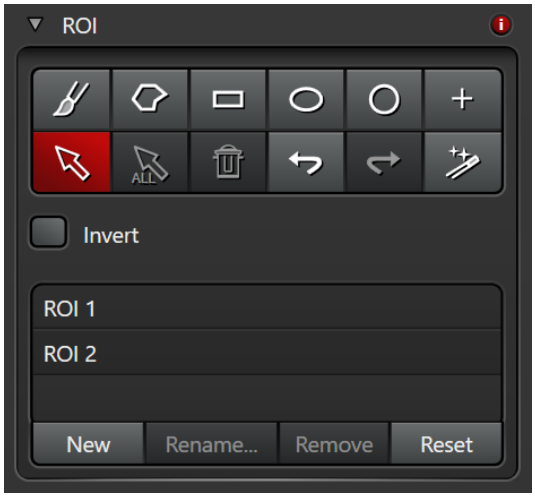
set threshold of no. of photons (too few photons = rubbish results):
use intensity preview to adjust until background is removed



if you only need proportions, uncheck lifetimes (already known from whole-image fit)
if lifetime varies spatially, check lifetimes

click Fast Fit or Precise Fit - not much difference usually

click “components” at top to see separate



ROI to exclude background from media or to exclude defective/dead cells
ROI box under FLIM tab in FLIM window

click one of the shape buttons to draw on image
if you draw multiple shapes they will be added to the same ROI
to create another ROI click the New button
when drawing shapes, make sure the correct ROI is selected
rename the ROI before you get confused

check decay curve to see that there are enough photons

“Fit all” to fit all ROIs [returns overall fit or individual fits?]

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PHASOR PLOT (obtained via Fourier transform)

needs fewer photons - 100 photons per pixel sufficient although couple of thousand photons per pixel is better
very sensitive to small differences in lifetime
can distinguish cells in different states e.g. Hoechst lifetime depends on compactness of DNA but can't separate if there's no spatial separation of lifetime ratio
can use to see FRET response but don't bleach the acceptor!

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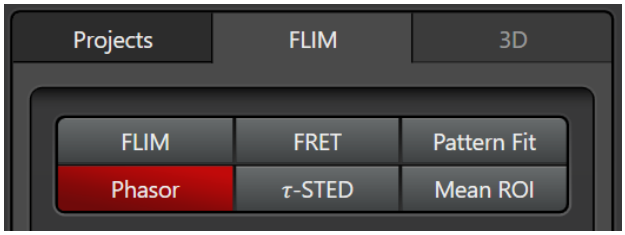
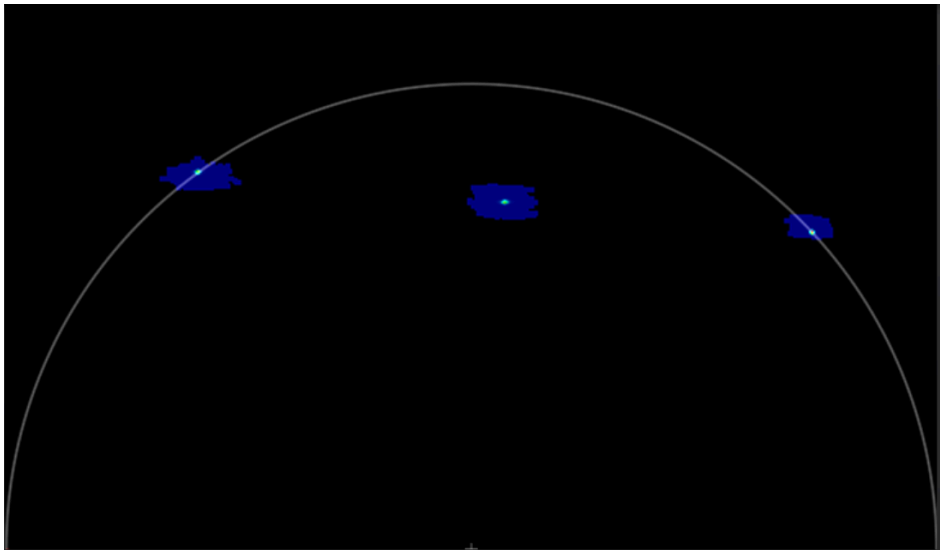


click Phasor at top of FLIM tab in FLIM window

To calibrate phasor plot, click the Calibrate button.
Usually you can let it autocalibrate using the pulsed laser frequency.
For cross-checking with results from older FLIM systems, you can use a calibration solution of known lifetime close to the lifetime of your sample and manually enter the lifetime.



lower left of semicircle denotes 12.5 ns (end of laser pulse), lower right denotes 0 ns
you can scale the plot by going to the Phasor box on the left and changing the harmonic from 1 to a larger number

each pixel is plotted. red means lots of pixels, blue few

To make blobs more distinct when there aren't quite enough photons, go to Phasor box on left of window:

1) best to use wavelet filter (uses Anscombe transformation) which increases "contrast". Adjust threshold to reduce background if necessary. Better than median filter and pixel binning combined. If wavelet not good enough, add pixel binning.

2) can also use median filter and increase value to concentrate the blobs, but you lose resolution.

hold CTRL and click to select multiple datasets in project tab, then check them at lower right to show in phasor

check ROIs at lower right to plot by ROI

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Visual interpretation:

monoexponentially decaying dye: all blobs on semicircle
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.
blobs inside semicircle e.g. CFP: more than one lifetime, deeper in circle = more lifetimes
three blobs forming triangle: three lifetimes, with a blob inside triangle being mixture of the three

elliptical/elongated blobs or smear: FRET or lifetime varying with environmental diversity

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

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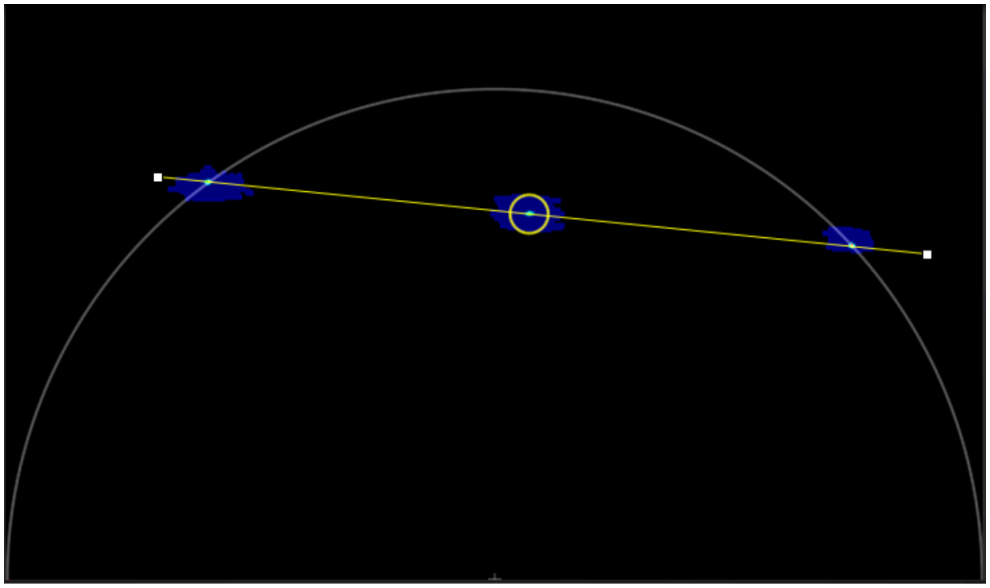
GUI analysis tools

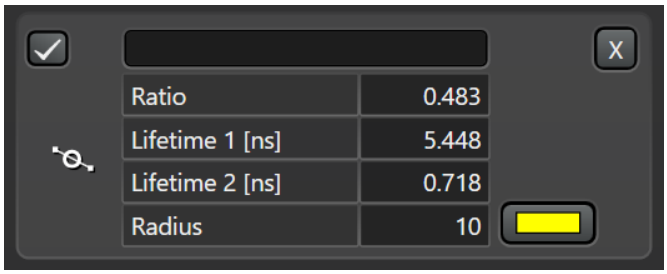
icon of circle with line through it: draw straight line and move small circle
icon of triangle with circle in it: draw triangle and move small circle
mouse over line or circle to move, drag corners to change shape or direction
scroll mouse wheel to change size of circle
[how to delete?]



drag circle onto the different blobs and see which parts of the sample image above the phasor plot are highlighted

to unmix using phasor, click to get circles and click “separate” at right. This redistributes photons not only along the straight line joining the blobs but for all the photons

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
then draw line and circle to find ratio [?]



can see ratios at top right

multicoloured line:
draw along smear and see which parts of image above are coloured correspondingly
you can adjust width of the transect

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

Draw FRET trajectory: click this button at bottom left of phasor box
[how]

click colour bar or colour triangle button to add colour key

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MULTIDIMENSIONAL DATA

In FLIM box under FLIM tab in FLIM window:
- check SPLIT for T and/or Z to perform fitting for individual time points and/or slices
- click Crop button to crop data in XY or subset in T/Z

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SAVE & EXPORT

save entire project by clicking save icon in projects tab of either main or FLIM window

at bottom of FLIM tab on left of FLIM window:
- click Save Results to save analysis in project file in Leica format
- click save image to save in the project file in Leica format a confocal image with really big dynamic range

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To export whole or parts of project to common formats for external processing and analysis and display:

in Project tab on left of FLIM window, right click on the project or one of its components and select Export.
in the popup, choose:
- Text File to export raw FLIM data [what format exactly does it save]
- ptu? (PicoQuant format) for analysis in Fiji
- Image

- Movie File: autoscale the confocal image brightness/contrast in LAS X before exporting, as it will be hard to adjust later

FLIM images: right click on image to export
Fitted params: right click on table in FLIM window to export as excel
Phasor: right click on plot and click Export Image

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FLIM ANALYSIS IN FIJI

If you don't have access to LAS X

Install plugins:
- https://github.com/UU-cellbiology/PTU_Reader
- FLIMJ update site

open ptu file and visualize photon arrivals:
Plugins --> PTU Reader

FLIM analysis:
select the appropriate window opened by PTU Reader
Analyze --> Lifetime --> FLIMJ
Time bin 0.1 ns (10 GHz sampling)?
See https://imagej.net/plugins/flimj/ for tutorial