

Basic information on FLIM with Leica Stellaris 8:

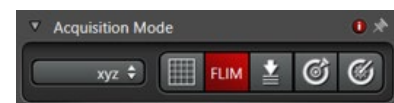
1. The Leica white light laser (WLL) pulses at 80MHz (80,000,000 pulses per second). This means the longest lifetime we can measure is 12.5ns (1 second/80 million = 12.5 ns).
2. The detectors record the time between when the pulse originates and when a photon arrives at the detector. The difference between the 2 times is the fluorescence lifetime. A pulsed laser (WLL) and photon counting detectors with precise timing are required to measure this.
3. The 405nm laser is not pulsed so you cannot use it for FLIM or the semi-quantitative Tau modes.
4. TauSense, TauContrast, TauGating, TauScan, TauSeparation are semi-quantitative only. You can use any of the detectors (HyDS or HyDX) in Tau modes.
5. For quantitative FLIM imaging and analysis, you must open the FLIM module and use the HyDX detectors. These detectors are photon counting and have the short response times needed to measure FLIM. They also have a low dynamic range, which means they can't handle very bright signals.
6. Keep in mind that lifetimes are dependent on the microenvironment. Companies report fluorescence dye lifetime when calculated in PBS or water, not in a cell. So the reported lifetime could be different.

Semi-Quantitative Tau Modes:

1. The modules described in this section are all semi-quantitative and use average photon arrival times. To calculate fluorescent lifetimes, you'll need to open the FLIM module.
2. TauContrast: Simple to turn on/off. When you turn it On and scan in Live mode you will see the intensity image and the TauContrast image, which shows average lifetimes. It uses a LUT (look-up-table) where blue = short lifetimes (shortest arrival times) and red = longer lifetimes. It won't take any longer to image and works inside Navigator (tile-scanning module), too.
3. For the all other Tau modes, turn off TauContrast.
4. TauGating: autofluorescence usually has short lifetimes. If you set a gate to cut out 0.3 to 0.5ns, you can usually get rid of reflection and autofluorescence. You can make several gates by clicking on the + button. Now when you scan you'll get each bin (gate) in a separate image.
7. GateScan: you can set different "start gates" while keeping the end the same, so you can see exactly when autofluorescence disappears. You'll get a series of gates that start in different places but all end at the same lifetime. You can restrict/compress either side.
5. TauScan: is a precursor to Tau Separation. It shows the predominant average arrival times in a graph/Gaussian curve.
6. TauSeparation: is the most useful. It does the same as TauScan but now makes each channel its own color. You can place "ticks" under the peak of each curve. It will take multiple images and assign them colors for the lifetimes. You can delete one channel and get rid of it if you want (to remove autofluorescence or something). This one is useful when trying to separate out 5+ colors. For example, Alexa 555 has a very short lifetime (0.3ns) whereas Alexa 546 and 568 have 3-4ns lifetimes. You can separate these dyes by lifetimes rather than by wavelengths (or can use alongside intensity/emission-based separation).

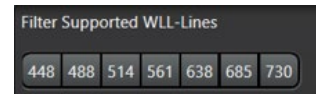
FLIM Acquisition

1. To access the FLIM software, click on FLIM in Acquisition. The FLIM analysis software will open on the 2nd monitor.



2. When FLIM is activated you will be collecting/saving lifetime information for each pixel, which means large file sizes. Images are 16 bit, though you will never fill up the whole bit depth.

3. When setting the emission window, set it > 10-12nm away from the laser line and use the closest laser line/notch filter to the excitation line you need.



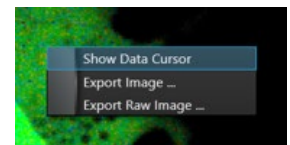
4. You must use the HyDX detectors for FLIM. They can be used in photon counting mode or digital mode. In digital mode, you will have access to gain, which will only affect the intensity image (helpful for morphological studies, etc., where a good intensity image is required). Whichever you choose, the FLIM image will be the same.

5. Speed: 600 Hz is a good scan speed for FLIM. You can use bi-directional scan mode but will need to calibrate it in the FLIM software (under FLIM calibration, Tools, Scanner Phase). To avoid this, use uni-directional.

6. Format and pixel size: when possible, use a smaller frame size (512 x 512 or 1024 x 1024). You can go up to 4096 x 4096 frame size if needed (that's the maximum) but the pixels will be very small and you'll need to do a lot more accumulations to get enough counts per pixel (i.e., smaller pixels have fewer counts). If you are looking at FRET inside of cells, you may need to increase resolution beyond 512 x 512.

Note: you can do pixel binning under analysis if you can't get enough counts. This way you can take a high resolution image but still use it for FLIM analysis.

7. When you take an image in FLIM mode, you can right click on the FLIM image (in the software on the 2nd monitor) and click "Show Data Cursor". It will now tell you the # of counts/pixel as you hover over each pixel.



8. **Make sure only one photon is arriving at the detector at the same time!!!** To determine the amount of laser power and line repetition (accumulation) you'll need to collect enough counts, start a live image, select Intensity. Using the Pixel Intensity Histogram (which is in log scale), increase the laser intensity until you see a red line appear. Stop at that point. This is where the flux of photons is too high, which means more than one photon is arriving at the detector at the same time with each pulse. You cannot have too many photons arriving at the same time. You must **keep it under 1 photon/pulse!!!** If you collect > 1 per pulse, you must deal with it during the analysis under Specialist settings.

a. Specialist settings: 3 types of photon filters.

i. "Standard (high speed)" is recommended; it will discard the information containing >1 photon/count. This is the least-biased way to deal with the "error" of collecting the extra photons in the first place.

ii. "All photons" will keep everything. This results in a pulse pile-up. It will skew the data to shorter lifetimes. Not recommended.

iii. "1st photons" will only keep the first photons that arrive first; this will skew the data to shorter lifetimes and create bias. Not recommended.

9. **The # of counts (photons) per pixel is important!!!** For FLIM analysis, if you are expecting one contributing lifetime (mono-exponential), then the minimum # of counts should be 100 per pixel. If you are using a multi-exponential fluorescent protein, like Cerulean (bi-exponential), you will need more counts. For each additional exponent (contributing lifetime), you need to add at least another 100 counts per pixel at a minimum. Some conservative researchers say you need a fold difference; like 1,000 counts. This is very difficult to get. In any case, err on the side of more counts.

Most biological applications are multi-exponential. For example, a dye can be in different compartments within the cell and the lifetime might change depending on environment; this may result in several lifetimes.

If you don't have enough counts per pixel, use line repetition (equivalent to accumulation). The maximum line repetition is 16 but you can also use frame repetitions. If the sample is alive, use line repetition to minimize aberrations. Note: when you collect a FLIM image with repetitions, you must click on "Start".

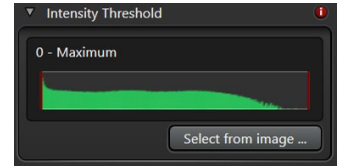
Note: the image on the left monitor may look like it's saturated because it's displaying in 8 bits and the FLIM image is 16 bits (this happens only with line-repetition, not frame-repetition).

FLIM Analysis

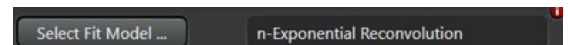
Once you take an image with enough counts and with < 1 photon per pulse, there are two ways to analyze/interpret the data: 1) Curve fitting (fit the observed curve to a theoretical curve), and 2) Phasor Plot, which is a way to plot all the lifetimes in an image on one graph.

1. Curve Fitting:

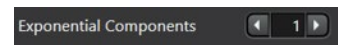
- You can exclude parts of the image that you don't want to analyze by drawing an ROI around the region you want to measure. This may not work if you don't have enough photons for an accurate measurement.
- You can also exclude low count pixels using "Select from Image". It will open the FLIM Intensity Threshold window where you can use a threshold to exclude low values from the dataset.



- To calculate FLIM, use the n-Exponential Reconvolution default fit model (not the other one that only uses the "tail" of the curve).

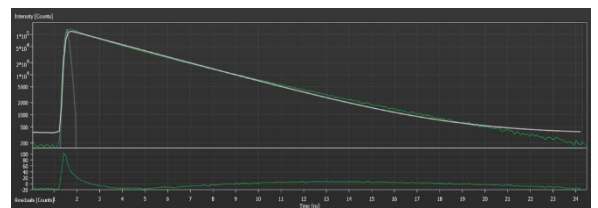


- To calculate, select 1 exponent and click "Fit" to try fitting the data.



Note that when Chi squared (χ^2) = 1 = this means the fit is best.

- Check the Decay Curve to see the correspondence between the white theoretical curve and the experimental. The bottom portion of the graph shows the residuals. Whatever the curve can't fit is displayed here.



The decay curve will start with a sharp peak ~ 2 seconds into the pulse. You'll have a lot of counts here and over time you get less and less. The shape of curve is indicative of how long the lifetime is. Longer lifetimes will have a flatter curve. No matter what, you want the curve to be smooth. If there is too much noise, the calculation won't be accurate.

Note: if you see a small peak/bump on the far-right of the decay curve that shows up $\sim 8-8.5$ ns, you can click on "reflection filter" to exclude this data (which is due to reflection in the system) from the analysis.

- Take note of the χ^2 value and check the residuals: the curve should look like a flat line with noise when it fits well, possibly with a slight "blip" at the beginning. If it doesn't and if χ^2 is high, try fitting "2" exponential components and repeat the process. You should be looking for a large fold-change (maybe 4-5 fold change) in the χ^2 value. If you try "3" exponents and the value doesn't change much, go back to 2 again.

- For FLIM-FRET, you will need to copy/record the value shown under Mean τ , Amplitude Weighted (takes into account the whole sample and weighs it based on the height of the peak) or sometimes Mean τ , Intensity Weighted (the sum of all intensities under the curve). Use whichever is closer to the actual lifetime of interest.

Mean τ , Intensity Weighted	2.969 \pm 0.012	ns
Mean τ , Amplitude Weighted	2.573 \pm 0.027	ns
χ^2	1.896	

- In the FLIM Image Fit window, you can select the parameters to fit to the image. The default is to use the average values calculated in the χ^2 table (meaning the boxes next to the lifetimes are unchecked). If you want the software to fit every single pixel, check the mark next to each lifetime: this will take much longer. In the same window, you can select "Intensity" to get rid of data with low counts using a threshold.

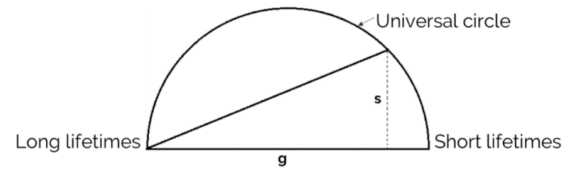
Parameters to fit	
Parameter	Fit
Lifetime (τ) 1	<input type="checkbox"/>
Lifetime (τ) 2	<input type="checkbox"/>
Amplitude 1	<input checked="" type="checkbox"/>
Amplitude 2	<input checked="" type="checkbox"/>
Tail Offset	<input checked="" type="checkbox"/>

- i. If you have multiple lifetimes, you'll need to report them. Use the Mean τ , Amplitude Weighted or Mean τ , Intensity Weighted. The values will be slightly different from each other.

2. Phasor Plot

a. Most lifetimes will converge in a bright spot.

b. For a mono-exponential decay, the lifetimes will be placed directly on the semicircle. Multi-exponential lifetimes show up under the curve. Longer lifetimes are on the left side of the graph/curve and shorter lifetimes are on the right.



- c. No component separation is available using Phasor. If you have autofluorescence, it will be included in the plot and make the data more difficult to interpret.
- d. You can place the cursor on the bright spot and it will highlight those pixels in the corresponding image. You can add multiple cursors.
- e. If you have 2 populations, you can press “separate” and it will be divided into 2 channels.
- f. FLIM-FRET publications often show phasor plots.

FLIM-FRET

1. For FLIM-FRET, you will need to calculate the lifetime of the donor alone (as described in the prior FLIM section). You must image a “donor only” sample and a sample with the donor in the presence of the acceptor. In each case, you only need to actually image the donor.

The idea is to figure out the lifetime of the donor, then use FLIM/FRET software to analyze donor-acceptor image using the lifetime of the donor compared to the donor-acceptor to calculate the % FRET efficiency.

The time spent in the excited state (the lifetime) is a competing process. In the donor only sample, the lifetime will be normal, but in presence of acceptor, the lifetime will be shorter. In other words, FRET causes transfer of energy, which will shorten the lifetime of the donor. The amount it shortens the lifetime is proportional to the amount of FRET happening. FRET efficiency is calculated based on this.

2. You will need to collect data for the 1) donor alone, and 2) donor-acceptor sample. You don't need to take an acceptor-only image.

3. In the FLIM-FRET workflow, you will first image/calculate the lifetime of the Donor Only Control sample and note the Mean τ , Amplitude Weighted (or Intensity Weighted) value. You will also need to note if you the used mono-exponential or a multi-exponential curve fitting model.

4. Then you will collect the donor-acceptor sample image (as described in the FLIM section); making sure you have >100 counts/pixel (for mono-exponential, and more for multi-exponential and <1 photon/pulse.

5. Click on FRET and select from the 2 fit models depending on whether the donor was 1) mono-exponential or 2) multi-exponential.

6. Enter in the exponential components and the Unquenched Donor Lifetime (copy the Mean Tau, Amplitude Weighted (or Intensity Weighted) from donor-only sample and paste into Unquenched Donor Lifetime)).

7. Click “Fit” and it will calculate the FRET efficiency.

8. FRET Image Fit: recommend working with averaged values here because some pixels may not have enough signal/counts to work properly. Use caution if you



Select Fit Model ...		Multi-Exponential Donor		
Fitting Range	0.048 ns	-	24.194 ns	
Exponential Components	◀ 2 ▶			
Parameter	Value	Fit	Global	
Forster Distance	1.000 nm			
Unquenched Donor Lifetime	2.573 ns			
Lifetime (τ) 1	0.807 ± 0.104 ns	✓		...
Lifetime (τ) 2	2.657 ± 0.080 ns	✓		...
Amplitude 1	81.429 ± 3.286 kCnts	✓		...
Amplitude 2	54.105 ± 4.556 kCnts	✓		...
Tail Offset	75.469 ± 9.712 Cnts	✓		...
IRF Background	0.000 Cnts			
IRF Shift	0.000 ns			
FLIM FRET Efficiency	39.931 ± 2.661 %			
Donor-Acceptor Distance	1.070 ± 0.020 nm			
Intensity 1	677.762 ± 72.127 kCnts			
Intensity 2	1482.301 ± 84.427 kCnts			
Sum Amplitudes	135.534 ± 6.679 kCnts			
Sum Intensity	2160.063 ± 20.490 kCnts			
Mean τ, Intensity Weighted	2.077 ± 0.026 ns			
Mean τ, Amplitude Weighted	1.546 ± 0.068 ns			
χ²	5.231			



decide to do it pixel by pixel. Better to define multiple ROI's if you want to do a more granular analysis.

More notes:

1. In Phasor Plot for FLIM/FRET: each pixel will be graphed based on lifetime.
2. Note, you can control-click on any image to pull up information in the right bottom table.
3. Tool: Draw FRET Trajectory: can look at FRET efficiency along that curve. This graph is sometimes reported in papers.
4. Z-stacks. There is a "split" button. To analyze each image plane on its own, select Split.
5. You can use 3D to show volume but you can't analyze voxels. "Split" just looks at the individual image.
6. Note: "Fit all" is used if you have a z-stack (or time lapse) and want to fit the whole dataset. If you select Fit All, it will fit all z-planes (or time points) and will give you information about each focal plane in the data table below. If you select Fit, it will give you an average value for the whole dataset.
7. Time lapse: use Mean ROI—will show graph of lifetime/intensity changes over time.

Exporting Data

1. To export lifetime data, you can right-click and export the data table.
2. The FLIM Image Fit will use lifetime, amplitude, intensity to create image with lifetimes per pixel. You can (for example) select intensity counts and mean Tau amplitude.
3. To export data, select "Free" and keep the default: fixed range and 1.000 (Intensity) and 0.001 for the lifetime (should be in picoseconds). This will give you 2 images: an intensity image and a lifetime image. If you mouse over the pixels in the lifetime image, it will say "intensity" at the bottom, but the values are actually lifetime values. You can also export color-coded images.
4. Use "Free" to create color-coded lifetime images for presentation. For example, select Intensity and mean lifetime or the lifetime of the 1st or 2nd component, or select events and mean amplitude weighted.
5. When you want to export 2 dyes that have overlapping spectra that you separate using lifetimes, use Export: Fixed Range. Intensity 1.000. This will give you standard intensity images.
6. Color coded FAST FLIM image. This displays the data before it's calculated for each pixel (I think). If want to look at the fitted data set, select FLIM Image Fit, change the threshold and either select the lifetime of image and click Precise or Fast fit. Or, if this takes forever (which it did) you can uncheck both lifetimes and have it calculate it fast based on the average lifetimes (rather than the lifetime of each pixel).