Tau-gating for SP8 Stellaris

What the microscope does

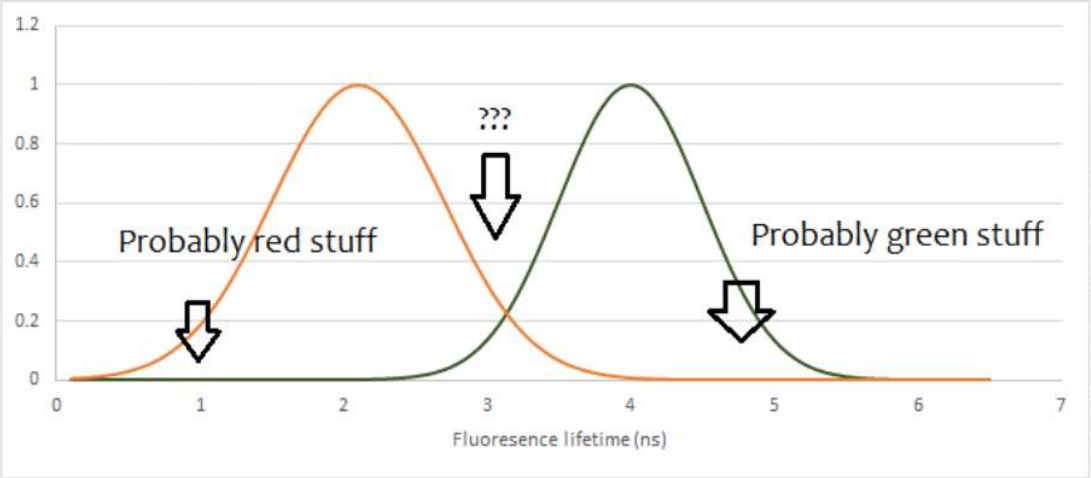
Leica STELLARIS 8 FALCON is equipped with sensitive Hyd detectors that, when used with pulsed white light laser, can measure the delay between the excitation pulse and detection allowing us to probe how long fluorochrome remains excited. This technique is not without its drawbacks however, as accurate measurements necessitate the system to disregard majority of the excitation signal, making lifetime measurements exceedingly painful in dim or poorly stained samples. This technology, however, allows us to filter incoming photons based on lifetime, in theory removing unwanted signal.

Theoretical background

Should the fluorescence lifetime of the unwanted signal differ sufficiently from the signal of interest, we can 'filter' it out by telling the system to ignore photons with a lifetime below or above certain threshold. As fluorescence lifetime is denoted by tau (τ), this method is known as tau-gating. Importantly it is fairly common that the autofluorescence has a characteristic fluorescence lifetime that is different from the lifetime of the stain or dye used. This means that well placed filter, given an otherwise bright sample, can be used to acquire an image without background signal.

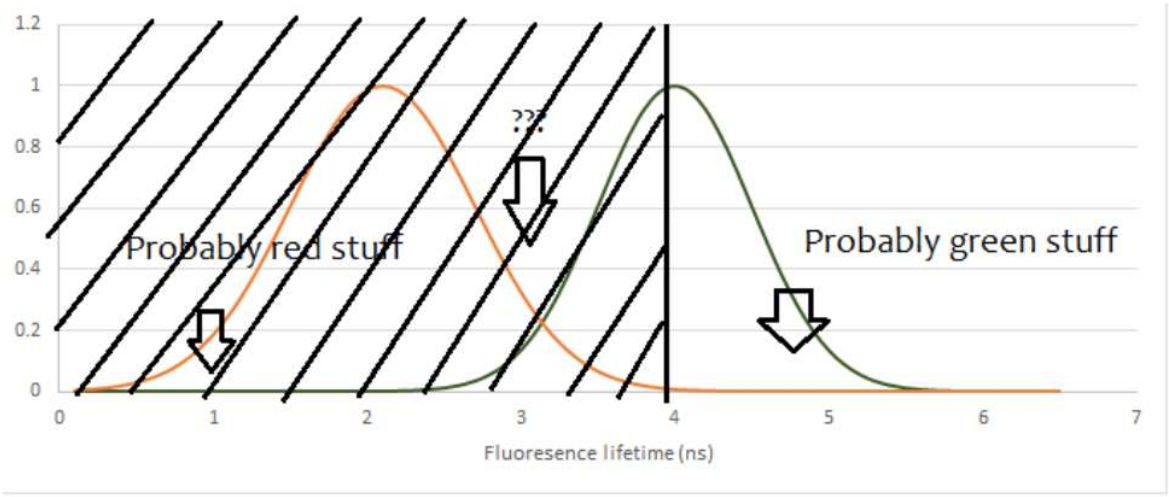
It should be noted that tau-gating cannot be used to eliminate unspecific staining because the stain used, regardless of its localization, has the same fluorescence lifetime everywhere.

However fluorescent lifetimes are always distributions, even if literature tends to report the average values. This means that the closer the two lifetimes are to each other, the harder it is to tell in which distribution given measured photon belongs. This makes tau-gating somewhat challenging operation and means we often have to make a compromise between maintaining the real signal and removing the unwanted signal.

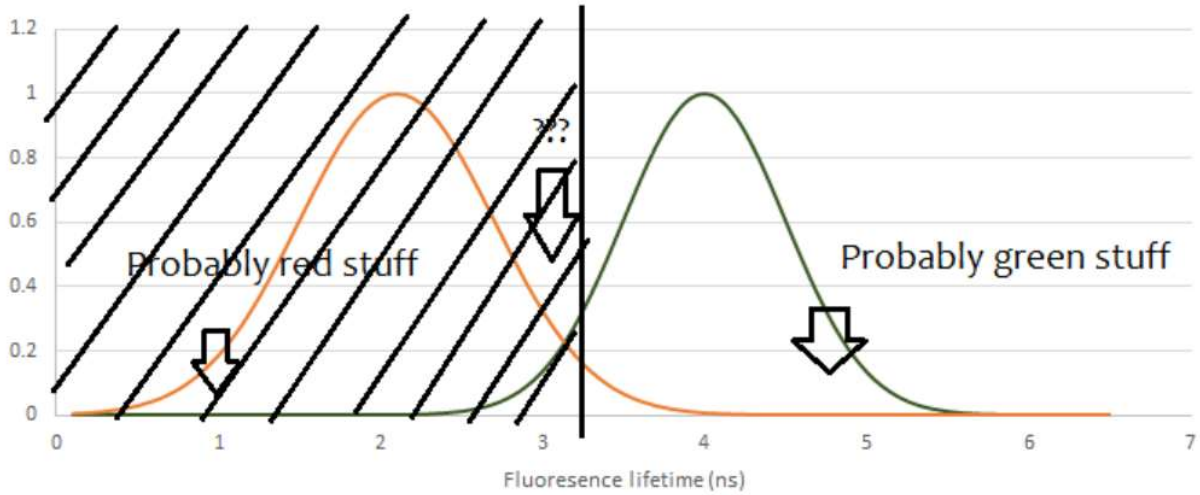


Fluorochrome on the left has the average lifetime of 2.1 ns while the fluorochrome on the right has the average lifetime of 4 ns. However, any photons arriving roughly 3 ns after excitation could belong to either population.

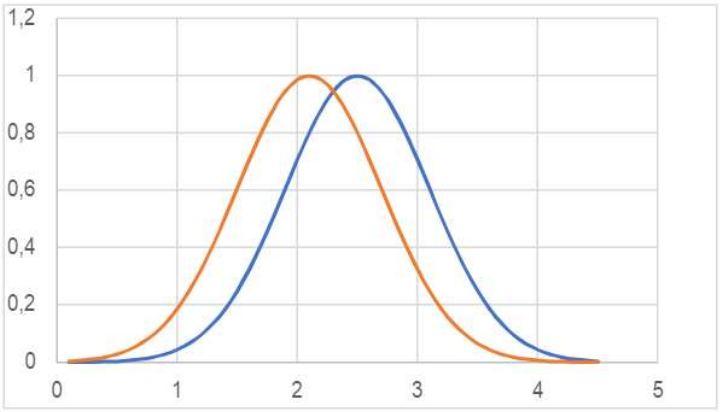
So, say that our background has lifetime of 2.1 ns and our fluorochrome (say Alexa 488) has a lifetime of 4.0 ns. How do we filter out the unwanted signal?



In this case we would set the 'filter' somewhere bit below 4. However, doing this also removes 50% of our Alexa 488 signal. A more reasonable cut-off might be something like 3.2 ns. However this would allow some of the background to end in the final image. There are no hard and fast rules regarding the choice of threshold and the final threshold will always depend on the sample.



Notably, the two fluorescent signals have similar lifetimes, filtering the unwanted signal out becomes practically impossible.

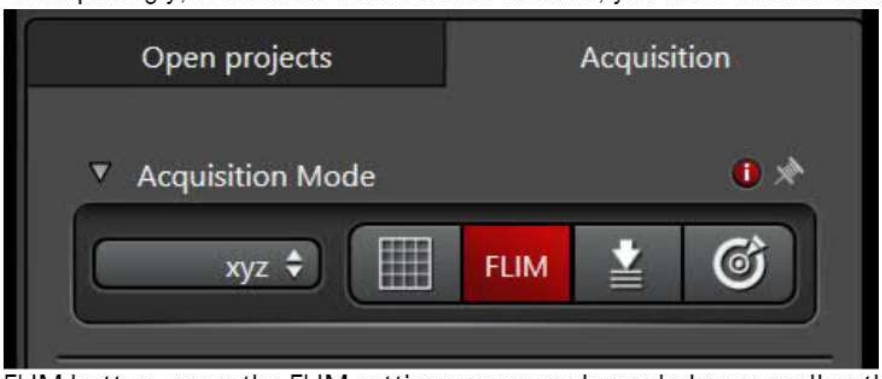


Practical guide

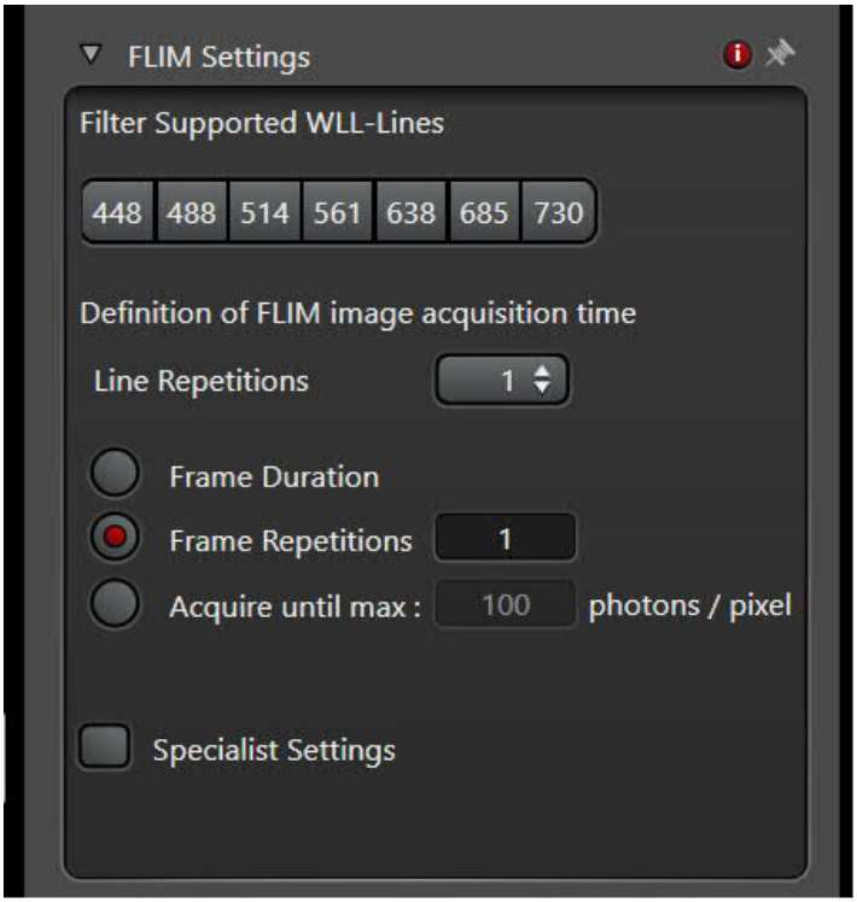
Practical guide is divided into two parts. In the first part we'll go through how to measure lifetime of a given region of interest with Leica SP8 Stellaris. In the latter part, we'll see how this information can be used to determine a 'tau gate' that disregards unwanted photons.

Measuring lifetime

Unsurprisingly, to measure fluorescence lifetime, you need to turn on the FLIM module.

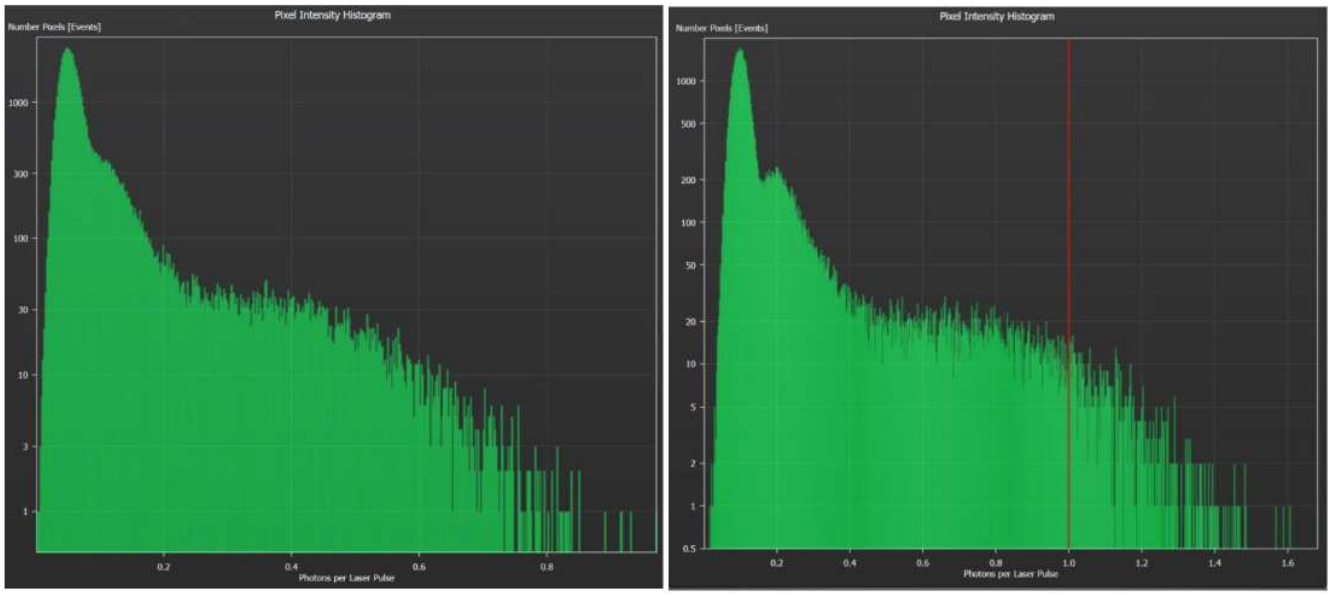


FLIM button opens the FLIM settings menu as shown below as well as the FLIM measurements extension on the right screen.



When you turn the 'live' on when FLIM button is active, the microscope adjusts the detection automatically and starts to measure the influx of photons. At this point, you need to adjust the laser power to ensure that you get sufficient amount of light from the sample, while making sure you do not get too

much light into the detectors as this distorts the measured values. Notably, you need to look at 'Pixel intensity histogram' on the bottom left of the FLIM screen and adjust the laser power so that the 'photons per pulse' is preferably above 0.2 and definitely below 1.0. A photon flux above 1.0 will yield unreliable results, while low yields (below 0.2 or so) mean that your measurements will take far longer than you would likely want and that you risk bleaching your sample.

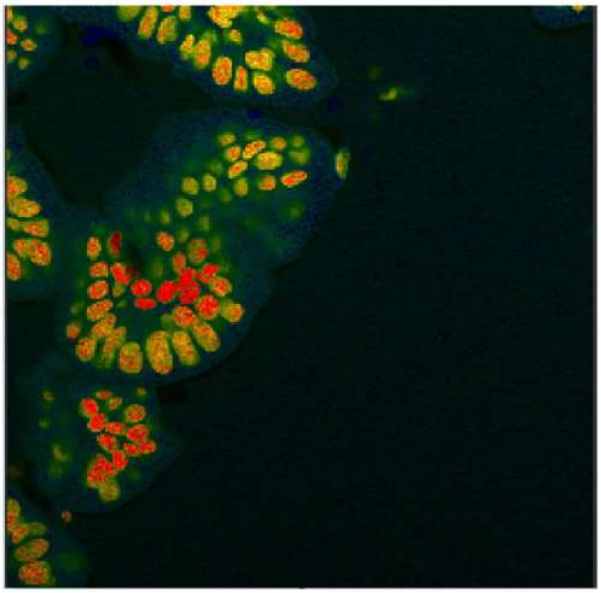


Pixel intensity histogram. The red line at 1.0 photons per pulse is the upper limit the system can analyse reliably. Ideally the graph would look like something on the left. You can limit the number of photons measured per pulse by reducing laser power or by zooming out.

Once you have established appropriate settings, you need to determine how long you are going to measure signal from your sample. This is done by the 'Definition of FLIM image acquisition time' settings shown on the previous page.

Using the 'Acquire until max:' setting and demanding something like 200-300 photons / pixel seems to give reasonable results most of the time – and more importantly ensures that your images have more or less same intensities.

You could alternatively use 'Frame Repetitions', setting it to 10 or so, but this setting collects less light from dim samples and more light from bright samples as the collection time is constant. In contrast, 'Acquire until max: photons per pixel' setting will continue recording data until the given criteria is met.



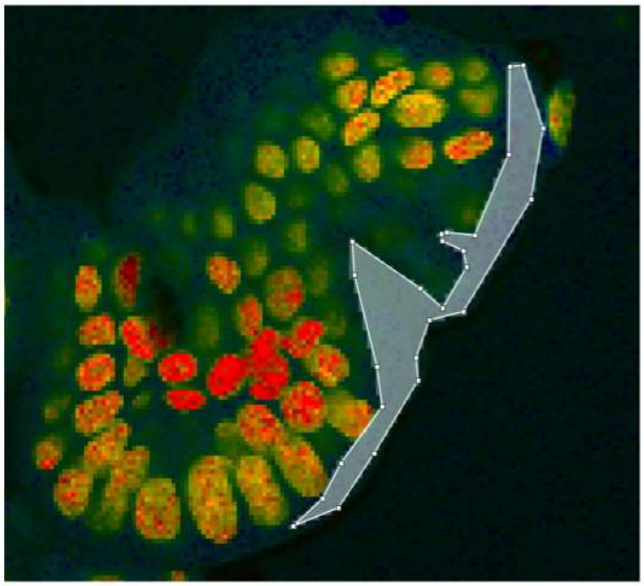
Once you have set your FLIM acquisition time, you can press 'Start' to record your FLIM image that should look something like this on the right screen.

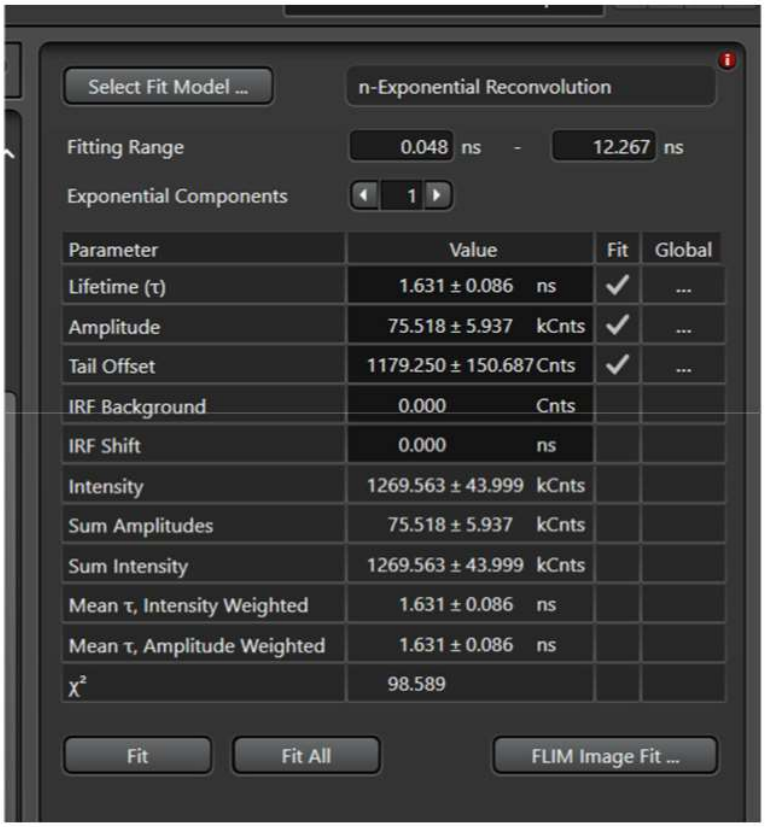
Now we need to estimate the lifetime in the stained parts of the sample (cells) and outside them (the background).

This is done using ROI tools found under the FLIM tab on the right screen.

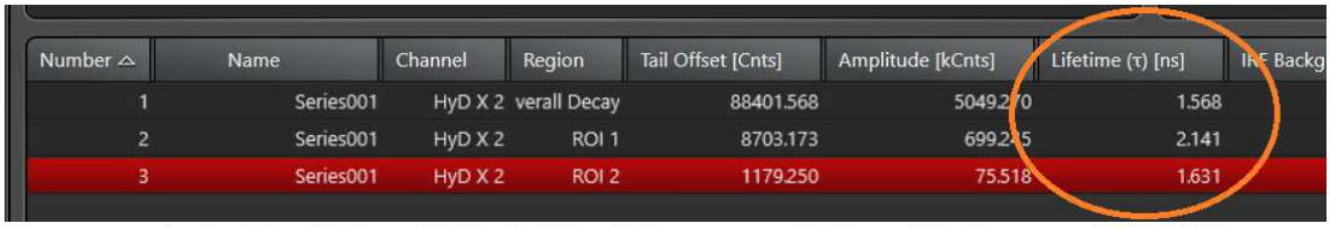
To define a ROI, press 'New' and then draw a region that encompasses your 'true' signal.

To define a ROI, press 'New' and then draw a region that encompasses your unwanted signal.

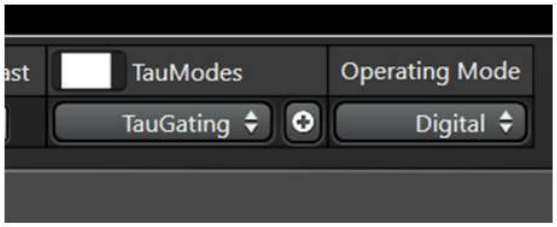




Now look at the right side of the FLIM screen. Click 'Fit All'. This will estimate the average lifetime in the whole image as well as in each ROI defined. Now you can read the results on the bottom of the FLIM screen.

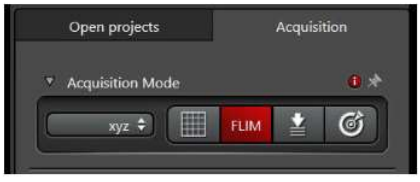


Now you can look at the Lifetime [ ]. The first line is the average lifetime of the image and this a little use for us. The second line is ROI1 – our real signal. The third line is ROI2 – our background. We can read that the real signal has a lifetime about 2.1 ns and that the background has a lifetime about 1.6 ns.



So now we can adjust the gating. This setting can be found the detector settings. Perhaps unsurprisingly you want to choose the TauGating mode. Pressing the (+) icon next to the modes opens the following window.

Now you need to adjust the window. The white bar is the 'gate' we let through. So in this case a gate from 1.9 ns to maybe 5.0 ns would be reasonable.



Finally, turn of the FLIM mode. We don't actually want to take FLIM images as we perform the gating.