FLIM readout, from optomicroquant course

As explained by Hänsch, Sebastian , Weidtkamp-Peters, Stefanie , **June-July 2019**

Data processing steps using symphotime:

- Open your work space
- Open first tab in analysis column
- Select Lifetime FRET option
- Select channel to analyze (from 780, you can get 4 channels, parallel/perpendicular and gfp/rfp).
- TCSPC binning - do we need this high time resolution (1 ps range...)? select 8-Chnl binning!
- -> Calculate fast flim, for an initial view of the lifetime data in pixels
- r/click image: "show data reader" = mouse-over shows counts now. can be useful

Pointing SymPhoTime to the right ROI, IRF and data to-be-fit

- Make an ROI either manual, or based on threshold (that you set depending on measured background, mouse over image to get info).
- Mouse-over can get an idea of how many signals are in the background.
- Note: program now uses calculated IRF (but isn't too exact). we need to tell it to use ours later
- -> use fitting model: multi-exponential donor! multiple lifetime components (populations of molecules!) might be hiding in the gfp signals.
- -> set "Decay" to ROI0 (selects our defined ROI)
  -> set IRF from calculated to measured (import, take Erythrosin KI function)
- Use a sensible threshold - limit analysis to thresholded area!
- Everything below model parameters (parameter-data set screen) is already some results. Some fit.
- -> click initial fit, to make the software make a first guess
- Click yes on popup of change amplitude etc.?
- initial fit, see black line appear in histogram
- then normal "fit" button to get real fit

Evaluating and storing results

- How good is my fit?
  Chi-squared, how big is your error? 1.000 is the best (but you will never reach), eg. something 1.050 is quite a good value.
- Second graph, under histogram = residues at each time point! Should be as flat as possible. Sometimes early lifetimes have more residuals. Warning: if there is a "wave" pattern in the residuals, this indicates more components to fit might be needed. Demonstrated by adding a component when not necessary, and then re-evaluating the residuals graph.
- Data: especially interesting is
  -Tau average Intensity ("intensity weighted lifetime", how intense are the pixels in terms of photons, and what is its contribution to each lifetime component, is weighted based on that). But, intensity of donor decreases, and acceptor increases, when FRET happens - so donor gets unfairly penalized!
  -Tau average Amplitude ("amplitude weighted lifetime" - imagine, two components are located somewhere in the graph (right of peak). they are then fitting straight lines in parallel to the graph at those positions. The y-axis intercept, is the amplitude. This is weighted)
- Now to extract results. EG. copying from sympthotime to excel (export to clipboard).
- We also might want to show a picture with FRET effect-size as a pixel. To reveal whether there may be lower/higher lifetime regions. Students can use this in their report. However, for this you officially need the donor lifetime as known. Can use with a "guessed" value.
- Evaluate: intensity weighted compared to amplitude weighted, see the difference. eg. 2.1 ns in intensity, 1.9 in amplitude weighted... in the end, something like 250 ps is a good, clear FRET effect. Will be underrepresented in intensity based.

Batch processing approach:

- Close the software
- Reopen in batch mode? or just reopen? BatchLTfret (lifetime fret)
- In batch, you can also use threshold (lower, to get above background, and higher, to avoid pileup (eg. counts above the 5% of repetition rate (of 30 MHz)
- ROI and thresholding, get a good one. calculate all and save, goes through them in batch mode.
- We can there also enter the IRF, and apply it to all the to-be-measured batch files! "IRF to all"
- Here, make sure that you perform an initial fit on an image with counts that roughly match the number of counts in the IRF measurement; and ideally a cell that has counts in the average range of the dataset. Then "fit" and next, "fit to all".
- Fit all starts with all parameters, and generates an overview table!
- It might happen, that starting from a different image, some other images will not fit. That could be because the IRF peak is at a hugely different count (orders of magnitude different) compared to the peak of the sample.

More things to do with students:

- Compare fret flim to fret APB!
- Compare different groups fits (started using a different image) for the same sample set.
- Compare over/under right amount of fitting parameters (see waves of residuals).
- Compare batch vs. single processing.
- Remember the localization "dimension". Where is each interactor? What changes in that, upon treatment?