FLIM startup, from optomicroquant course

Gather materials

- Rhodamine110 solution, slower lifetime: used for comparing the signals between parallel and perpendicular detectors, and for calibrating the system at the start (comparing to historical values)
- Erythrosin solution + KI (saturated), has an extremely short lifetime, used for measuring our IRF
- 40x water objective, dedicated to spectroscopy (remove DIC filters from it!)
- Number 1.5 coverslips (system is calibrated for that)
- Make sure the right filters have been mounted in the detector boxes.
- Laser power meter, to determine laser dosage at the objective lens: should not exceed 1 µW!
- Black-paper cover for over the sample area, blocking out environmental light.

ZEN first, set up a simple FCS measurement

- Prepare a coverslip with a droplet of rhodamine, and find focus using the edge of the drop (that is a sharp line, then go into the drop a bit and start measuring)
- Perform a continuous FCS measurement existing pre-set settings: 488 nm, point-scan, cw laser (argon)
- this happens at 500 KHz repetition (of detector), but is with CW laser so you can't draw lifetime conclusions
- Get diffusion time. During the course, we saw 32 µs for rhodamine.
- Reveal count rate: how many events per molecule do we detect in the setup?
- Correction collar: Maximize this number by adjusting the objective correction collar. That is the optimal correction.
- Pinhole alignment: Can be simply used here to automatically optimize X and Y position of pinhole based on intensity.

SymPhoTime first, start observing photons

- Make sure the relevant PC is also switched on...
- Create a new workspace ("file" menu), make sure it is saved in the right location
- Select "point" for a point-measurement. We are running a parked laser using ZEN already.
- The software now collects data all the time. You can open the individual detectors (there are 4) count rates window, to make them bigger (double click)
- Inspect detector performance: how do the count rates compare? For the GFP detectors (parallel, perpendicular) we find (240000, 230000) counts.

ZEN and SymPhoTime, set up for acquiring FLIM data, checking channels

- Use pre-set settings to pick the 485 pulsed laser (diode), at 60% intensity, point measurement. Then click start!
- At this point, you can measure laser power. The attenuator knobs (course, fine) on the laser combiner box, can be used to correct the laser power to 1 µW or less.
- Regardless of power metering, using the laser power attenuators should lead to a visible change in counts in the live displays on symphotime. Confirm with the students.
- Set up a first 10-second measurement in SymPhoTime, have a cursory look at the correlation curves of GFP and RFP-channel signals. By now, make sure to have familiarized yourself with which channel is which (1, 2, 3, 4 correspond to GFPpar GFPperp, RFPpar RFPperp)
- Compare intensities between channels. Are they close? Yes, any small differences that appear can be corrected in analysis steps. Trying to adjust the beam splitter usually makes it worse anyway.

Get a Instrument Response Function using Erythrosin

- Goal: visualise how the laser pulse looks like after passing through all the optics.
- Prepare a droplet of Erythrosin+KI solution on top of a coverslip, mount sample onto microscope.
- Start experiment in ZEN, start detection in SymPhoTime, for a 10-sec experiment
- Look at count rates! Compare parallel to perpendicular signals. We found (parallel, perpendicular) (180000, 85000).
- The parallel channel has a lot more counts. This makes sense, because the molecules don't have time to rotate after being excited.
- Show the IRF, save it as a measured IRF (label it "erythrosin")

Background measurement using just water

- Prepare a coverslip with a drop of water - focus (find the edge, move into the sample)
- See counts in the relevant channels (evaluate for both green/red). Take a 60 second measurement.
- Inspect the "macrotime" graph (lower right) where counts/time unit are recorded. Confirm that individual photons are detected.

Get the first real measurement

- Get the real sample ready for mounting (eg. imaging chamber out of the incubator)
- Select pre-set settings that include 488/561, continuous, focus on the well, select a nice cell that has both constructs
- Zoom to fill the screen with the sample, instead of having a majority of black pixels
- Then select different pre-sets in ZEN, 485 only, 256x256 px, 40 frames time course
- SymPhoTime: confirm that the settings match the new pre-set settings in ZEN
- Start in SymPhoTime
- Start in ZEN (sends a trigger to SymPhoTime, to start the measurement)
- Evaluate the detection rate. Ideally, we count number of photons/second at 5% (160000/sec) of the repetition rate (32 MHz)
- At minimum, we pick up at least 1000/second.

Routine loop: go find another cell

- In ZEN, switch back to "standard" GFP/RFP settings, and find another cell that has comparable, good expression levels.
- Focus and zoom the cells, go back to pre-set measurement settings for flim (eg. 485 etc.)
- Start in SymPhoTime, start in ZEN, wait for acquisition.
- Interpret, and evaluate data. Besides judging expression level from images, count rate can also be used to compare between cells.

- Record data in existing template word file.
- Always include a donor-only control!