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Information microscope: <http://www.lcam-fnwi.nl/facilities/flimscope/>

For data acquisition first record an EB reference solution. For all steps use left monitor except for step 10 and 12.

- 1 Move stage and focus microscope to focus inside an Erythrosin B solution (1 mg/ml).
- 2 Select OFP cube in left Nikon tab menu (regardless of LED & exc. filter used)
- 3 Make sure Nikon microscope is directing light towards Eye (oculars). Press "EYE" button in the front of microscope if not.
- 4 Press "open shutter" on LIFA 10007 left menu.
- 5 Now faint yellow/orange EB fluorescence should be visible (z pos approx 2100 um).
- 6 Press "close shutter" on LIFA 10007 left menu.
- 7 Open manual (orange) shutter in front of LI camera (left port microscope).
- 8 Press L100 on Nikon microscope to direct the light towards the LIFA detector.
- 9 Set exposure time to 50 ms in Li2CAM 54543145.
- 10 Press "FLIM" button in acquisition menu. Now a live phase image is shown in the right monitor. Vary the phase (slider in LIFA 10007 menu) to get max intensity (maximum close to 300 degrees). Adjust focus in microscope and/or exposure time if necessary. Too low counts gives a too dark image/ too much counts give red areas. Press "IDLE" when ready.
- 11 Press on "Reference" button which records reference.
- 12 (OPTIONAL) Check in right monitor if you have oversaturated pixels (red). Move the phase bar below the image to see the brightest phase image. If necessary go back to step 9 with altered exposure time.
- 13 Save reference as file (using file menu or shortcut with disk containing "R"). Use the .fli standard output.
- 14 In case of need for highly accurate data press averaging $\geq 2x$ (top left acquisition pane) and repeat from step 10 (takes proportionally more time and light exposure).
- 15 Press "EYE" on Nikon microscope to direct the light to the oculars.

For sample acquisition all steps on left monitor except for step 9, 11,15 (&16):

- 1 Select correct cube in left Nikon tab menu (only once).
- 2 Make sure Nikon microscope is directing light towards Eye (oculars). Press "EYE" button on microscope if not.
- 3 Move stage and focus microscope to approximate correct location.
- 4 Press "open shutter" on LIFA 10007 left menu.
- 5 Now localize your object (cells or solution) (z pos approx. 2100 um).
- 6 Press "close shutter" on LIFA 10007 left menu.
- 7 Press L100 on Nikon microscope to direct the light towards the LIFA detector.
- 8 Set exposure time to 100 ms (or other value depending on intensity) in Li2CAM 54543145.
- 9 Press "FLIM" button in acquisition menu. Now a live phase image is shown in the right monitor. Vary the phase (slider in LIFA 10007 menu) to get max intensity. Adjust focus in microscope and/or exposure time if necessary. Too low counts gives a too dark image/ too much counts give red areas. Press "IDLE" when ready.
- 10 Press on "Sample" button, which records the sample.
- 11 (Optional) Check in right window if you have enough/too much counts. Use the middle tab which contains the phase images. Too low counts gives too dark image, too much counts gives red images. Move the phase bar below the image to see the brightest phase image. If not ok alter the exposure time and repeat from step 8. To see the lifetime image select the right tab.
- 12 Save sample as file (using file menu or shortcut with disk containing "S"). Use the .fli standard output. *If you save the selected tab you may only save the lifetime image which prevents any post-processing using the original raw phase image data.*
- 13 In case of highly accurate data press averaging $\geq 2x$ (top left acquisition) repeat from 10.

- 14 Press “**EYE**” on Nikon microscope to direct light to the oculars.
- 15 To display lifetime statistics, select the lifetime image (right tab) and draw an ROI in the right monitor (dotted square symbol) and then on left monitor right window, press “statistics”: button and check information on lifetime. Histograms will appear as well as average lifetime. Also multiple ROIs can be drawn. These can be saved if required.
- 16 (Optional) for easy output to ImageJ, select lifetime image tab & “**save selected tab**”.
- 17 For next cell or other sample repeat steps 2-14.

Regular Lifetime Image processing on FLIM2

- 1) (optional) (if no lifetime tab was saved):
 Startup Lambert Instruments LI-FLIM 1.2.23 software.
 Open reference file.
 Open sample file.
 Recalculate reference and recalculate sample.
 Select on right monitor the lifetime image & Save “selected tab” (4 images).
 Repeat above for every sample-ref combination you want to display.
- 2) Start Image J**.
- 3) Import newFLIM_to_ij_v2.ijm macro (on desktop).
- 4) Run macro. This converts the FLIM output in a ‘12-bit’ int-tau(phi)-tau(mod) stack.
- 5) Import Lifetimes14.txt macro (on desktop) & display lifetime images as usual.

*** Requires Image J \geq v1.49e, LOCI Bioformats & Image_correlator plugins installed.*

Image processing Complex & perhaps more accurate (needs phase stacks of reference & sample, requires Matlab & Image J conversion)

Complex Lifetime image processing on FLIM2

- 1) Start Image J
- 2) Import Macro convert_fli_to_ics2.ijm
- 3) Run above Macro to convert reference files and sample files
- 4) Open the ics reference files in Matlab old FLIM software
- 5) Type in command window: Store_bleachflag(A,0);
- 6) Type in command window: Store_nstack(A,1);
- 7) Do 3x3 blurring and calculate reference phase (not tau-phase) (in calculate lifetimes menu)
- 8) Take histogram (0-360) and note average value in middle ROI of image
- 9) Subtract 1.24 degrees from value noted in 8 (for EB 0.086 ns lifetime @ 40 MHz).
- 10) Calculate reference modulation (not tau-modulation) (in calculate lifetimes menu)
- 11) Take histogram (0-360) and note average value in middle ROI of image
- 12) Open the ics sample files in Matlab old FLIM software
- 13) Type in command window:
 Store_bleachflag(A,0);
 Store_hfreq(A,40000000);
 Store_nstack(A,1);
 Store_phase(A,phase); *** use for phase the number in step 9***
 Store_modulation(A,mod); ***use for mod the number in step 11***
- 14) Save image A in matlab FLIM software. This effectively completes conversion.
- 15) Use export_to_imageJ_newFLIM.m macro to export files for image J processing. This can involve blurring of phase stack for lifetime analysis (does not affect intensity image. Usually 3x3 blurring is fine. Do not use > 5x5 blurring. Note that iris correction can be done using the Iris_newFLIM2.ics file in this macro (assumes a 2x2 binned file).
- 16) Start Image J*
- 17) Import Lifetimes14.txt macro
- 18) Run Lifetimes14.txt macro (same as before) using your exported file.