1) Calibration procedure.

At the beginning or end of your FLIM recordings (each day) take 3 EB measurements. For this,

a) Make a solution of 1 mg/ml EB in pure water.
b) Take a large 0.17 mm thick coverglass.
c) Mount the coverglass and add a large drop of the EB solution.
d) Use the objective/laser that you used for your FLIM measurements
e) Change the filter to YFP (NB also for 442 and 488 excitation)
f) Turn the 488 or 514 laser to minimal power, 442 remains unchanged
g) Position the laser in the middle of the droplet (xy) and focus the laser INSIDE the EB droplet (preferably 1 mm inside (z-axis)).
h) Check by eye if you get a homogeneous field.
i) If you use the 488 or 514 laser then change the calibration exposure time: In the FLIM command window type: “edit calibrate.m” (without “”) and change the exposure to the indicated values for EB measurement. After changing it save the macro (from the file menu or ctrl+S) (see example 1).
j) Take a FLIM recording with 18 phase measurements, extra bleach image, and recorded bias. For 2x2 binned image approximately 20-40 ms exposure/frame.
k) Check the highest intensity in the EB image stack and in the calibration image stack (they are printed in the matlab command window). Both values need to be lower than 3900 and higher than 2000. For the EB you can adjust the exposure time in the FLIM acquistion window, for the calibration cube exposure time modify (if necessary) the exposure time in the calibrate.m macro. After changing save the macro.
l) Repeat step k until your maximum intensities for both the EB and the calibration image are between 2000-3900.
m) Move the EB sample to a slightly different xy position.
n) Take a FLIM stack and store it
o) Repeat step n three times.
p) After calibrating, calculate the phase and modulation in the EB-image. First load the image. Then take the calculate_lifetimes window, and check “adjust calibration”. Then press “bias from input image”. Note the phase bias (xxx.xx degrees=phase_ref) and the modulation bias (xx.xx percent=mod_ref). Subsequently uncheck “correct for bleaching” and calculate a phase image. Use the histogram command to get the mean phase from the centre half of the image. Note the mean value printed in the matlab command window (=phase_EB). Then calculate the modulation image. Again use the histogram command to get the mean modulation. Note this value (=mod_EB). Repeat step p for the three EB FLIM stacks.
q) Now calculate phase_correction=phase_EB - phase_ref - EB_corr and calculate mod_correction=mod_EB/mod_ref. Use for EB_corr 2.32 degrees if you measure at 75.1 MHz (This is the phase shift due to 86 ps EB lifetime).
r) Calculate phase_correction and mod_correction for all three EB measurements and check that they are consistent. If they phase_correction and mod_correction are larger than 1 degree or 1% then it is worth correcting for the phase/mod bias.
s) At the end of the calibration procedure change back the exposure times in the calibrate.m macro.

2) Export the data from Matlab to Image J.
a) Copy your FLIM data to the image processing PC
b) Start Matlab and the FLIM software
c) type "edit export_to_ij.m" in the command window of matlab (see example 2).
d) Here change the filename to the correct name (& path) of the FLIM stack
e) Optionally change the phase_correction or mod_correction to the values you determined with EB
f) S ave the changed macro (by in the file menu or by ctrl+S).
g) In the matlab command window type "export_to_ij" to run the maco
h) The macro is finished if your converted image appears in a window.
i) Repeat steps c-h for all your FLIM stacks

3) Display lifetimes of entire FLIM image in image J
a) Load the macro Lifetimes5.txt in Image J (copy it from c:\users\Flim_macros).
b) Run the macro and select "Open new file". The macro lets you open the converted FLIM stack (filename ends with "_ij.ics").

4) Display lifetimes of an ROI in aFLIM stack in image J
a) Load the converted FLIM image stack into image J (filename ends with "_ij.ics").
b) Adjust brightness/contrast in image J of the first slice.
c) Make an ROI (only rectangle) and crop the image
d) run the Lifetimes5.txt macro and select "work on current image".

5) Measure average lifetimes and intensities in individual cells (ROIs) in one FLIM stack.
a) a) Load the converted FLIM image stack into image J (filename ends with "_ij.ics").
b) Adjust brightness/contrast in image J of the first slice.
c) Load the ROI plugin "Multimeasure"
d) Set the measurements to include the mean grey value and the standard deviation
e) Then make your ROI (arbritary shape) in the image and press the spacebar
f) Repeat step E for all the ROIs
g) Inside the Multimeasure window press "select all" and press "measure"
h) Confirm that it needs to process the three slides.
i) You get a table with columns corresponding to your ROIs. The first row corresponds to the intensity image, the second row to the tau(phi) image (values x 1000) and the third row corresponds to the tau(mod) image (values x 1000).