**Example MATLAB scripts for seFRET microscopy**

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<http://laser.cheng.cam.ac.uk/wiki/index.php/Resources>

This “readme” document contains brief notes and instructions on the example MATLAB scripts which are provided for seFRET image analysis.

These MATLAB scripts are provided as a guideline for seFRET image processing. They can be used to perform seFRET analysis on the sample data provided in the “Example\_Data” folder. They may also be useful for performing seFRET analysis on your own experimental data, or as a starting point for developing a similar method. Please note that these scripts are provided for guidance only, and have no warranty (as specified in the GPL version 3+ license).

These scripts were written to accompany a Springer Protocols book chapter:

**A quantitative protocol for live cell FRET imaging.**

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**Instructions for using these seFRET scripts.**

1. Start MATLAB.

You will need access to an installation of MATLAB, together with the MATLAB image processing toolkit. These scripts were verified to work with MATLAB version 2011b. As an alternative to running these scripts in MATLAB, you may use a compiled version of these routines, which can be run using the MATLAB compiler runtime (<http://www.mathworks.co.uk/products/compiler/>).

2. Run seFRET\_Calibrate

(a) A Dialog box will prompt you to confirm some image processing parameters.

For processing the Example\_Data the default values are suitable, so click OK.

Dialog boxes will prompt you to identify images containing data for calibration from the “Acceptor Only,” “Donor Only,” and “Linker Construct” samples.

(b) The image names in the “Example\_Data” folder should be self explanatory; however refer to the abbreviations list below for more information.

The file “AcceptorOnly\_AmAx\_YFP\_ch04.tif” refers to the Acceptor-Only sample, measured with the “Acceptor Emission” spectral channel (Am) and with “Acceptor Excitation” illumination (Ax). The “YFP” in the file name is the fluorophore in this sample, and the “ch04” refers to the automatic file naming convention on our Leica SP5 setup. This image is, of course, a “tif.” The MATLAB scripts provided are programmed to look for “tif” files to open – this aspect of the scripts will need editing if it is necessary to open experimental data in other formats.

(c) The seFRET\_Calibrate script will determine values of AER, DER, α and β. These will be echoed to the MATLAB console, and stored in the base workspace.

(d) It is recommended to repeat this procedure on different areas of the calibration sample. This will provide better accuracy (by averaging the results for AER etc.) and will allow you to check for consistency and repeatability in the calibrated results.

3. Run seFRET\_Analyse

(a) You will need the calibration parameters AER, DER, α and β. They should be available in the base workspace after running the seFRET\_Calibrate script.

(b) A Dialog box will prompt you to confirm some image processing parameters.

For processing the Example\_Data the default values are suitable, so click OK.

The script prompts you for the “Specimen\_AmAx…” “Specimen\_AmDx…” and “Specimen\_DmDx” tif images from the “Example\_Data” folder.

(c) The script will then produce some images of normalized dFRET and aFRET, as well as histograms of dFRET and aFRET.

(d) Editing the visualizations so that they look perfect for presentation is recommended.

**Notes on seFRET image processing.**

1. Thresholding (Identifying Background Regions and Good Signal Regions)

Previous versions of seFRET required the user to identify regions of (a) background and (b) FRET signal. This was slow and could be error-prone. These MATLAB scripts use a heuristic (“Rule of Thumb”) filter to do the job automatically.

Pixels which are darker than a “Low Threshold” are selected. This area is subjected to a “mask erosion” to exclude the edges. The reduced area is used to identify the d.c. background level of each image. It is essential to remove d.c. background, as it would otherwise contaminate the intensity-ratio measurements that are essential to doing correct seFRET. So identifying background regions accurately is very important.

Pixels which are brighter that the “Low Threshold” but darker than the “High Threshold” are identified as areas of good FRET signal. These are used to determined AER, DER, α and β on the relevant calibration samples, and for identifying reliable dFRET and aFRET values. Similar to the background mask above, this region is made conservative via an “mask erosion.”

2. Mask Erosion (Avoiding Edge Effects)

To avoid edge effects which may contaminate the signals obtained in seFRET, the masked areas that are selected by thresholding are “eroded.” This shrinks the regions, so that the final regions selected for measuring (a) background, and (b) AER, DER, α and β are selected conservatively.

3. Abbreviations used in the example code

**AOS** = Acceptor only sample

**DOS** = Donor only sample

**Linker** = CFP-YFP linker sample, with known dFRET and aFRET

**AmAx** = Acceptor Emission, Acceptor Excitation spectral channel

**AmDx** = Acceptor Emission, Donor Excitation spectral channel

**DmDx** = Donor Emission, Donor Excitation spectral channel

**Im** = image (assumed 16 bit)

Users are prompted to confirm (or edit) some important imaging processing values via a dialog box. (The scripts could easily be edited to set these manually, too.)

**ThresholdLow**: pixels within a region darker than ThresholdLow are

assumed to be background. Used for d.c. background

estimation and subtraction

**ThresholdHigh**: pixels within a region that is brighter than ThresholdLow

AND darker than ThresholdHigh are identified as signals.

The reason for ThresholdHigh is that some detectors may

become nonlinear at high brightness, and such nonlinear

regions are unsuitable for this linear seFRET analysis.

ThresholdLow and ThresholdHigh are set at the start of this script. They may (and should) be optimised by the user for their own system.

**KnownDFRET** = the dFRET value of the Linker construct sample,

which must be known (e.g. from TCSPC / lifetime measurement)

in order for alpha and beta to be fitted in this script.

In the book chapter, this is the *E* value of the Linker-construct sample.

**MaskErodeRadius**: Radius for eroding the masks so that the selected regions exclude edge effects.