**MATLAB FRET data analysis**

This readme aims to explain how to perform FRET analysis using Fiji and MATLAB 2012b scripts as performed in Kuijt et al., 2020.

**Note1**: Some MATLAB scripts may not work on more recent MATLAB versions due to the way tables and variables are handled. Unfortunately, I am not available to aid in updating these scripts.

**Note2**: When performing siRNA depletion, in some instances cellular stress results in formation of aggregates that completely obstruct analysis. These aggregates must be excluded from analysis

1. **Data organization and Fiji scripts**
2. **Organize your data:** Place all raw imaging data in a single folder (convenient for Fiji-script). Now generate the file structure where Fiji processed data will be saved: **1)** create copies of the ‘x\_Dummy\_Folder’ (found in 0\_FIJI-script\_KUIJTetal/) **2)** rename to specify experimental condition (e.g. control / inhibited/cell-line-X etc.). **3)** populate xy## folders with raw Z-stack data; named cfp.tif, yfp.tif (rfp.tif optionally). I highly recommend you write a script that performs these tasks. The script ‘Fiji-save\_script.jim’ is an example file that can handle ‘.nd2’ file containers and outputs data to xy## folders in the correct manner. See script for instructions on how to use it.

1. **Running Fiji\_FRET\_script.ijm**: To perform FRET calculations the background and a threshold to include pixels for analysis is to be calculated. In my datasets these values vary to such an extent that no single value can be used for all images and thus calculation for each image is needed. The **Fiji\_FRET\_script** generates these values in an unbiased, reproducible and automated manner. To start: Define the path where raw cfp and yfp images are placed. Draw ROI on pixels to be analysed (all kinetochores, but no some aggregate), a mask will be created to exclude other pixels The script does not overwrite raw data but creates \_msk.tif files and saves the masked image as image sequence (e.g. cfp001.tif, cfp00#.tif). Script defines BG ROI (excluding high intensity pixels) by series of thresholding and measures background in CFP & YFP. Then threshold is performed to define and measure kinetochore means. All these values are saved as a results.csv file. (if background or KT ROI is not defined properly, play with thresholding methods).
2. **Matlab FRET analysis instructions**
3. **Set Matlab path**: In Matlab, add the 1\_MATLAB-script\_KUIJTetal folder to the Matlab path list directory (*File>Set Path>Add Folder)*. This ensures that all the required FRET code can be found by the scripts for the entire Matlab session).
4. **Determine channel offset**: In matlab, navigate to xy##/ and run in the ImageAlign function “ImageAlign(1); ImageAlign(2); ImageAlign(3)” etc.”. The function returns a score of how good alignment is (1=perfect) and the corresponding x and y translation used to reach this improved alignment. Perform ImageAlign on several xy## images and use the best scoring x and y translations for the Master3D file (you input this into the Master3D file). Note: 1) Image rotation (θ) is incredibly hard to correct and is disabled by default. 2) Images are not corrected in Z-axis, so any misalignment must be resolved before acquiring images‼
5. **Set Master3D parameters**: *Use the files in folder: 3\_MATLAB-batch\_KUIJTetal.* In Master3D (\_redward).m set the correct number of slices (Nplanes) and timepoints (Ntime points). Also define the shrink parameters (if shrink\_objects = 1; enabled) to force large objects to be split and exclude small objects. At this point you can place the optimized Master3D.m file into each xy## folder and check if the parameters are good. Then proceed to step 4.
6. **Copying Master3D to each xy## folder:** To perform FRET calculations each xy## folder must contain either Master3D.m or Master3D\_redward.m (if rfp data is present). To quickly do this I provide a windows only script ‘PasteMaster3D.bat’. First place the modified Master3D.m (or *redward.m)* in each /Data/XXX folder (not yet inside the /xy##). Adjust the .bat script to now place Master3D(\_redward).m into each subfolder (these are all the xy## folder).
7. **Running Batchmode MaMaMaster.m**: Place the MaMaMaster.m file in /Data/XXX. Call the script (or drag it into MatLab) to execute. This script will search each xy## folder for Master3D.m or Master3D\_redward.m and execute it. Once done the script will scrape each folder for local\_mean.txt and obj\_mean.txt raw FRET ratio values and collect them into ‘All\_locmean.xlsx’ and ‘All\_objmean.xlsx’.

This pipeline can handle >1000 images with minimal user input in a few hours. I recommend to first prepare all data with the Fiji scripts. Next, run the Master3D copy .bat script for all Experimental Conditions. Then place MaMaMaster.m in each Experimental Condition folder. Lastly consecutively drag MaMaMaster.m files into Matlab and they will be queued them up so you are free to perform other activities.

The script has been modified to calculate CFP/FRET ratio’s. Furthermore, the script only shows on the first analysis a plot of the ratio (local and object if enabled). This plot is saved as a .jpg and is particularly handy to quickly see time lapse FRET calculations. Additionally, the script saves the aligned\_ images (cfp, yfp and rfp)

1. **Additional MATLAB script features.**

There are many additional features in the script I have never used. See publications from the Lampson lab to get an idea of what features are there.

Try the script on the provided test files.

Good luck‼

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