Guide to using MatLab to analyze smFRET data

**In MatLab**

Note 1: In MatLab if you press the up arrow button, this will bring up the most recently used commands for that beginning phrase. For all of these if the command isn’t complete, press the up arrow key to find the correct command.

Note 2: MatLab is case sensitive. Make sure you have the correct case when typing your commands.

Note 3: MatLab does not like spaces. When you name something use underscores if you want to space things apart.

**Creating a “glimpse” file. This converts a TIFF file that you obtain from the MetaMorph to a glimpse file that MatLab can understand.**

1. Type “Path” and press the up arrow on your keyboard. This should bring up the most recent “Path” chosen. This path is so MatLab knows where to look when entering later commands. Your path is probably a “MatLab” folder in your username.
   1. After typing in the path, make sure that in the drop down menu above the white window you select the same path. In the window on the left-hand side you should see the files from the folder that you designated in the path.
2. Intiff=’path’
   1. This is where you want to input the Tiff file that you made from the microscope. Copy the path as follows: ‘C:User/name/data/rawdata/1.tif’ Make sure that it ends with .tif
3. out\_path=’path’
   1. This is where the glimpse file will save. You probably want to have a different folder for every glimpse file you make or it will overwrite. Paste in the folder address followed by a “/”
4. user=’NAME’
   1. Enter your name between the apostrophes
5. frames=[#:#]
   1. Fill in the number signs with your range.
   2. Example: frames=[1:500]
6. glimpse\_from\_tiff(…)
   1. This will make your glimpse files
   2. It will make a header file that you will actually load into imscroll
7. glimpseloadimscrolll
   1. This loads imscroll
   2. It will prompt you to find the file. Select the correct file folder and choose the header.
   3. NOTE: imscroll does not have an “enter” command so if you make changes and want to press “enter” you have to advance the frame

**Mapping with Beads**

1. Turn your beads file into a glimpse file according to steps 2-7
2. Once you load imscroll, click the “mapping” button
3. Change the “folder” drop down menu to “glimpse” to show the beads glimpse file. Do this for both image screens.
4. Toggle “autoscale” to “manual scale” to change the contrast in the image. Change the number in the “Max scale” box to any number (e.g. 5000) and press enter/advance frame. A scroll bar should appear – adjust it until you can clearly see beads in both channels. Do this to both image screens.
5. In the mapping window you will get two identical images. Both images have the red and green channels (red = top half, green = bottom half).
   1. Choose beads by first selecting a bead in the red channel of the top image. Then selecting the corresponding bead in the green channel of the bottom image.
6. First choose three beads in different areas of the image (e.g. upper right, lower middle, middle left). Once you pick three beads, a new window will pop up. A straight line should be going through the three points indicating the quality of your mapping algorithm. Now the mapping program will identify the beads on the green channel from the points that you chose on the red path.
7. Continue to pick beads, and select or correct the beads that mapping has picked.
   1. If you have different fields of view in your beads file, you can advance the frame number and continue to select beads in order to cover the entire area.
8. After you have about 15-20 beads that covering the entire field, you can exit out of all the imscroll windows.
9. Navigate to the mapping folder and rename the “fitparms” file that was just created with the date (e.g. 20131022\_fitparms)
   1. This will be the name of the file you load into imscroll to load your mapping algorithm.

**Imscroll Analysis**

1. Make a glimpse file of the data you want to eventually analyze (steps 2-7)
2. Open imscroll
3. In the dropdown menu next to the black window, change “folder” to “glimpse” and then advance the frame.
4. Click “autoscale” to adjust contrast and advance the frame. Now you can manually change the intensity. Change the intensity so you can see the spots.
5. Zoom in to just the red channel.
   1. Press “mouse input” and crosshairs will appear
   2. Select the upper left and lower right corners
   3. Right click to get rid of crosshairs
   4. Toggle “full screen” to change to “magnified”
   5. Advance frame
   6. Now your field of view should just be the red channel spots
6. Now, in the box on the right hand side of the program window, click “Pick”
   1. You can adjust the parameters in the Pick section. First change the “brightness” threshold but do not go below 10. The program is defaulted at 50.
   2. You should also change the pixel size (at the Top Right of the screen) from 10 to something smaller (probably around 4). You want the boxes to be small enough to just be around individual spots and similar in size to the spot itself.
7. Once you are happy with the number of spots chosen, it is time to remove all boxes that contain more than one spot
   1. This is best done by zooming into individual areas on the screen and focusing on 1/4 or 1/5 of the screen until you have gone through the entire channel
   2. You click “Remove AOIs” and individually click the spots. If you mess up you can either just forget about the spots or you can start all over. Selecting another AOI individually gets rid of all the other AOIs.
8. Once you are satisfied that all your boxes (AOIs) contain only one spot, zoom out.
9. Now in the white box titled “Output/Filename” type in the full name of the data that you want to save
10. Then change the dropdown menu that is below that box from “Gaussian” to “Int. linear interp”
11. Change the frame range from [1:10] to the range you wish to fit over.
12. Then click “Fit AOIs”
13. This will pop up a new window (Fit AOIs window). There will be three large white boxes. The top one is for looking at the AOIs, the second and third are for looking at the data of the AOIs that you chose.
    1. We want to check and see if your data plots so first in the middle box next to the “Load AOIs” button, type in your file name (make sure it ends in .dat)
    2. Then change the drop down on the right side of the white box from “Amplitude” to “Intensity”
    3. Press “Load AOIs”
    4. Then hit the small “P” button next to the arrows and advance the AOI number
    5. If you can see the Intensity vs. frame trace, close out of the “Fit AOIs” window and go back to the main imscroll window
14. Now, back on imscroll, you want to load your mapping file
    1. Enter in the filename of your mapping file (created in steps 8-16) below the Go Button on the imscroll screen (e.g. 20131021\_fitparms.dat)
    2. Select “Load fitparms” from the dropdown menu
    3. Press the Go Button and advance the frame
    4. The parameters for the mapping file should appear below the filename box (mx,my,mz…)
15. To map the AOIs from the red channel onto the green channel, select “Map AOIs” from the drop down menu.
    1. AOIs should be visible in the red channel before you can map.
    2. If there are no AOIs, you can load the AOIs by entering in the filename of your Fit file in the Output/filename box (above the Fit AOI button), adjust the dropdown menu to “Load AOIs”, press the Go Button and advance the frame.
16. Once you have the AOIs mapped onto the green channel, change the filename under a new name in the “Output/FileName” box.
17. Click “Fit AOIs”
18. In the “Fit AOIs” window, load both your red and green channel data into the middle and bottom windows (as you did in step 29)
19. Now go through both data sets and look for FRET
    1. An analyzable trace has the following attributes:
       1. Cy5 was present at the beginning of the movie (when only the red laser was on)
       2. Cy3 has photobleached (so you can detrend the trace)
       3. There are anti-correlated peaks signifying FRET is occurring (e.g. Cy5 signal goes away at the same time that Cy3 signal photobleaches)
       4. There is only one molecule in each AOI
       5. Both spots have their brightest pixel within the AOI
    2. Write down all the AOI numbers that you want to analyze
    3. Note the range of frames for which the molecules are undergoing FRET
20. Once you have determined all the AOIs you will analyze, begin analysis. You will have to do all these steps for each point unless specified:
    1. Load Cy3 or Cy5 data in both windows
    2. On the drop down menu on the right hand side of the “Fit AOIs” menu, select “Load vid time” and hit the “DataOperation” button. This will bring up your files and choose the correct “header” file for your data. YOU ONLY HAVE TO DO THIS ONCE BEFORE THE FIRST POINT.
       1. For the prism microscope, this time base is WRONG. You can change it later if it is necessary (i.e. for dwell times of different FRET states)
    3. Now change the drop down menu to “Set detrend frame range” and hit “DataOperation”
       1. This will bring up crosshairs
       2. Left click to select to baseline range (left click on the left most edge and then on the right edge)
       3. Double right click off the image to get rid of the crosshairs
    4. After the double right click, the drop down menu should just automatically have changed to “Detrend trace.” Click “Data Operation”
    5. Now the menu should have changed to “Set mean/std frame range”
       1. Hit data operation. Once again you have the crosshairs so repeat choosing the baseline data with the crosshairs
    6. After double right clicking, the drop down menu will show “Find Intervals”
       1. THE FIRST TIME: change the dropdown to “Set Interval Frame Range” and click “DataOperation”
       2. In “Set Interval Frame Range” select the entire range that the green laser is turned on with the crosshairs.
       3. Double right click after you are done
    7. After setting the mean/std frame range, hit “DataOperation” when the dropdown menu is on “Find Intervals”
    8. Select “add trace to interval data structure” from the dropdown and press “DataOperation”
    9. Now only THE FIRST TIME change the dropdown to “UI Save Interval Data Structure”
       1. Hit “DataOperation”
       2. Navigate to the “Fits” folder and save the data under a specific name, include the channel you are analyzing (i.e. Cy3 or Cy5)
       3. Your data will now save in this specific file from now on
    10. After adding the trace to interval data structure, go to the next AOI and repeat 36 c-h for all data points
    11. After finishing with all your AOIs, on the dropdown select “Save Interval Data Structure” and press “DataOperation”
    12. After finishing, repeat all of 36 with the other data set. Save the fitting files accordingly.

**Back in MatLab and Creating a Histogram**

1. To load the intervals file in MatLab type [fn fp]= uigetfile;
2. Then type in “eval([‘load’[fp fn]” and press the up arrow key to get the appropriate command
3. You will now see an intervals file in the Workspace
4. Open the intervals variable in workspace by double clicking it.
5. This will bring up your intervals table with the following information:
   1. Column 1: Name of file
   2. Column 2: AOI you fit
   3. Column 9: Time range
   4. Column 12: Detrended Trace intensities
6. Change the interval file name to either Interval\_FRET (if acceptor) or Interval\_Cy3 (if donor) so you do not write over your interval file when you load the next interval file
7. Now load the next interval file by repeating 37-41
8. Extracting FRET efficiency information using the following steps. First take out the time and intensity arrays and assign them a variable. Type:
   1. x=Intervals\_FRET.AllTracesCellArray(:,9);
   2. y=Intervals\_FRET.AllTracesCellArray(:,12);
9. Type: FRET=[x,y];
   1. This will make a two column data table. The first column will be your time, the second will be your intensity from your individual AOIs
10. Repeat 41 and 42, but this time with variables “a” and “b” with your Intervals\_Cy3 file
11. Make sure both FRET and Cy3 variables have the same dimensions in workspace
12. Now you will make another table, but first you have to assign another set of variables
    1. z=FRET(Row#,2):c=z(:,2);FRET\_Intesity=c(range of FRET)
       1. example z=FRET(1,2):c=(:,2);FRET\_Intensity=c(5:200)
       2. The 1,2 refers to the row and column of the data. So row 1 would be your first AOI and 2 would be the intensity from that row
       3. Make sure that the AOI number matches between FRET and Cy3 data.
    2. For Cy3: h=Cy3(1,2):i=z(:,2);Cy3\_Intesity=i(range of FRET)
13. To calculate the FRET efficiencies, type in E\_values and press the up arrow key
    1. This will bring you to the command to determine FRET efficiency values
    2. Press enter
    3. This will bring up a chart with the FRET efficiency values for all the frames for the specific AOI
14. Type in “list=[];
    1. This will make a new open table called “list”
15. Copy all the values from the E\_values table onto the list table
    1. This is so you can save your values and not accidentally overwrite them
16. Repeat 48 and 49 for every AOI (you have to change the “1” to the specific AOI and the “range of FRET” to the individual range for every AOI). Make sure that you copy and paste in your efficiency values to the “list” table.
17. Now you have to filter your data to eliminate all the extreme values (you want the range of FRET values to fall between -0.1 and 1.2). Type in this:

data1=list

data2=data1<1.2;

data3=data1(data2,1);

data4=data3>-0.1;

data5=data3(data4,1);

Data5 corresponds to your filtered data.

1. Type in “difittool”
   1. This tool will make your histogram
2. This will open Distribution Fitting Tool GUI
3. Change display to “density” to plot a histogram
4. Click “Data” button and select data5
   1. In this interface, you can select the bin width as well as any exclusion rules.
5. Select “Create Data Set”
6. Now you can do things like add a fit and save the figure.
   1. To add a fit, select “Fit” and adjust the parameters to your liking and press “apply”
   2. The equation and mean/std of your histogram will be displayed in the Fit window
7. Click “Print to Figure” from the menu and this will make a MatLab figure
   1. In the matlab figure, you can save it as a TIF or Bitmap image.
8. In MatLab figure window, you can change things like the color and the axes by selecting “Plot Tools” on the toolbar.
9. Save everything!
   1. Save your workspace in Matlab so you have all your raw and analyzed data as well as FRET efficiency list in case you need to change it later
   2. Save your histogram as a .dfit file as well as a matlab figure or image file

DONE!