Table of Contents

How to convert Tiff to Glimpse files:

1. Convert Tiff to Glimpse files in MatLab

- Create file with date of experiment in C:\Users\deciantis\MatLab\data\Tiff_Files\
  - (e.g. C:\Users\deciantis\MatLab\data\Tiff_Files\20160712\)
- Copy Tiff movie files from the server to the file you have just created
- Create a similarly named file in C:\Users\deciantis\MatLab\data\Glimpse_Files\
  - (e.g. C:\Users\deciantis\MatLab\data\Glimpse_Files\20160712\)
- Create subfolders in this new directory for each of the movies you want to convert
  - (e.g. C:\Users\deciantis\MatLab\data\Glimpse_Files\20160712\20160712_movie1\)
- Open Matlab
- Enter: user= 'deciantis'
- Enter: frames=[1:1000];
  - The user and frames commands only need to be entered once
- Enter:
  intiffpath= 'C:\Users\deciantis\MatLab\data\Tiff_Files\20160712\20160712_movie1.tif'
- Enter:
  out_path= 'C:\Users\deciantis\MatLab\data\Glimpse_Files\20160712\20160712_movie1\'
- Enter: glimpse_from_tiff(intiffpath,frames,out_path,user)
- You can open your newly created Glimpse movie folder and check that a '0.glimpse' file and a 'header.MAT' file have been created
- Repeat the last three commands for each movie you want to convert

Create a mapping file

1. ### Bead preparation for Mapping

   - Fluorescent labeled beads are used as markers for mapping of emission channel images.
   - TransFluoroSpheres streptavidin-labeled, 0.04 µm (488/645) beads from Molecular Probes (dark purple in color, found in "Chemicals A-M" bin in the 4ºC deli case)
   - Dilute beads 1:500 into 1xPBS. Do not vortex or spin down beads, gently mix by flicking or pipetting up and down, draw sample from center portion of suspension if possible.
   - Immobilize Beads on slide as per standard protocol

2. ### How to create a mapping file

   - Follow the steps to convert the .tiff file to a .glimpse file above.
   - Type glimpseloadimscroll → select the header file just generated for the mapping experiment.
   - Click on Mapping at the top just right of center in the Glimpse window.
   1. Change the both field folder(s) to GLIMPSE
   2. Change both Autoscale(s) to manual
   3. Change MaxIntensity to 500 and press enter. Do so for both top and bottom
   4. Select sub regions with MouseInput1 (for top / red field) and MouseInput2 (for bottom/ green field).
   a. Right click on one corner and then the diagonal corner of the field to zoom in on.
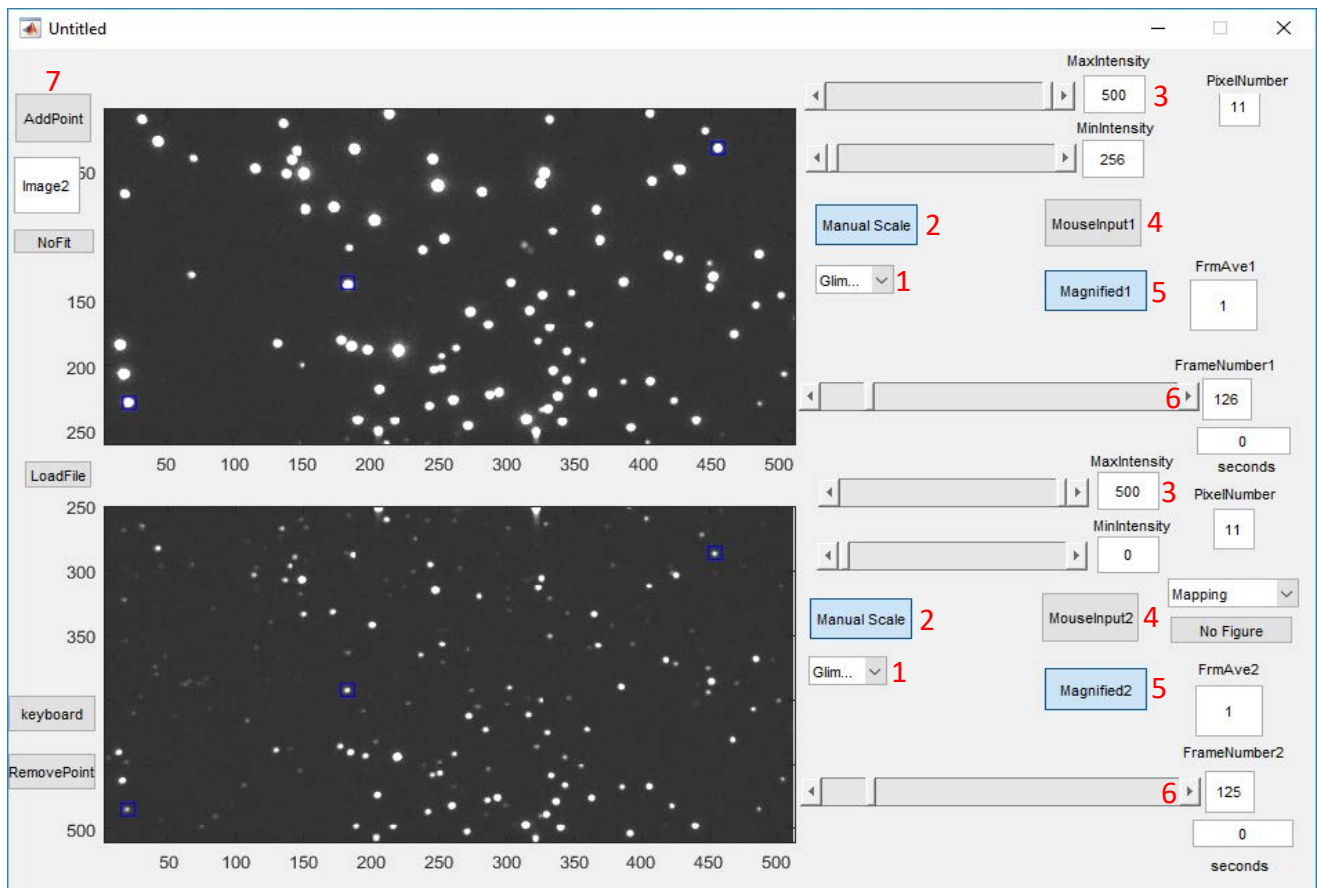
5. Change Full Screen1 to Magnified1. Do the same for Full Screen2.
6. Click forward one frame both displays to zoom in. Make sure that the values in "FrameNumber1" and "FrameNumber2" match
7. Click AddPoint and select the center of the dot in both the top and bottom image. Repeat.

- Be sure to consistently select a point first in the top image and second in the bottom image. Switching the order will throw off the entire process and you will have to start over.
- After 3 points have been selected, a linear fitting graph for each axis will be generated (see below.) The goal is to get an even distribution of points along the fit in both axis. 20-30 points are usually sufficient.
- Subsequently, whenever a point is picked on the top figure a predicted placement will be shown on the bottom figure with a small green box based on the current mapping file up to that point. You still need to select the optimal placement of the matched point in the lower figure.
- The mapping conversion parameters are saved automatically in C:\Users\deciantis\Matlab\mapping\FitParms.dat. Go into this folder and change the filename to include the date collected for reference later. (e.g. 03282017FitParms.dat)
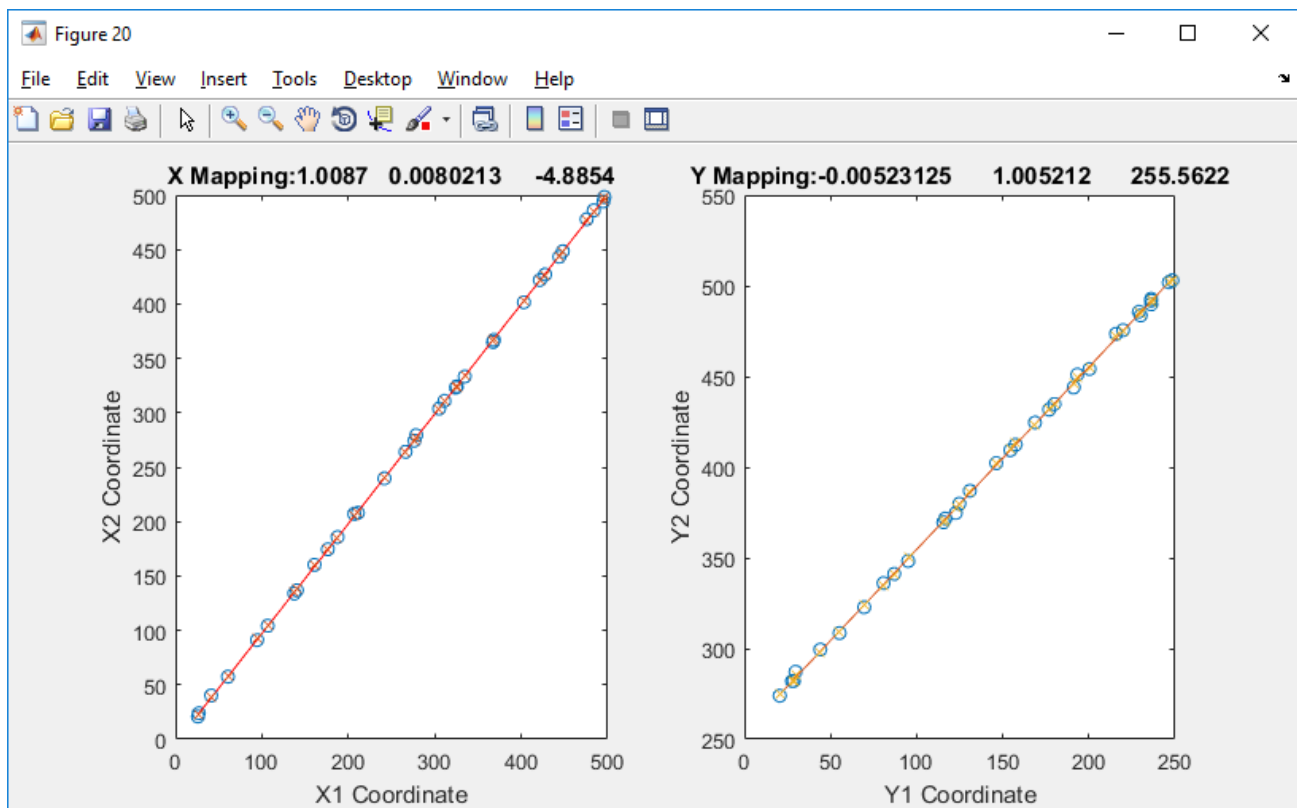
How to analyze glimpsedata:

1. Analyze data in MatLab

- Open MatLab
- Enter glimpseloadimscroll → window to open files will pop up
- Search your header file and "open"
- NOTE: Should be in …MatLab\data\Glimpse_Files*file* you made, name your files with easy names and without spaces, otherwise MatLab will show an error and doesn't open it

1. Change the field "folder" to "GLIMPSE" on left side of the MatLab window and go to the next frame
2. NOTE: always go back and forth between the frames if you changed a setting in the software, otherwise MatLab will not show you the change (click on slider arrows at bottom of window)
3. Now you should see the picture of the red and green channel in frame 2, go back to frame 1
4. "Autoscale" → "Manual Scale" set "Max" Intensity set to 3000
5. Adjust intensity to get a good picture (413 upper, 78 lower in my case)
6. Zoom in: Click "Mouse input" and mark the edge of your new field of view
7. Change "full screen" to "Magnified" by clicking on it and change to frame 2 again
8. Zoomed picture should show up
9. Check how many frames are in the file (drag slider fully to the right, read "frame number")
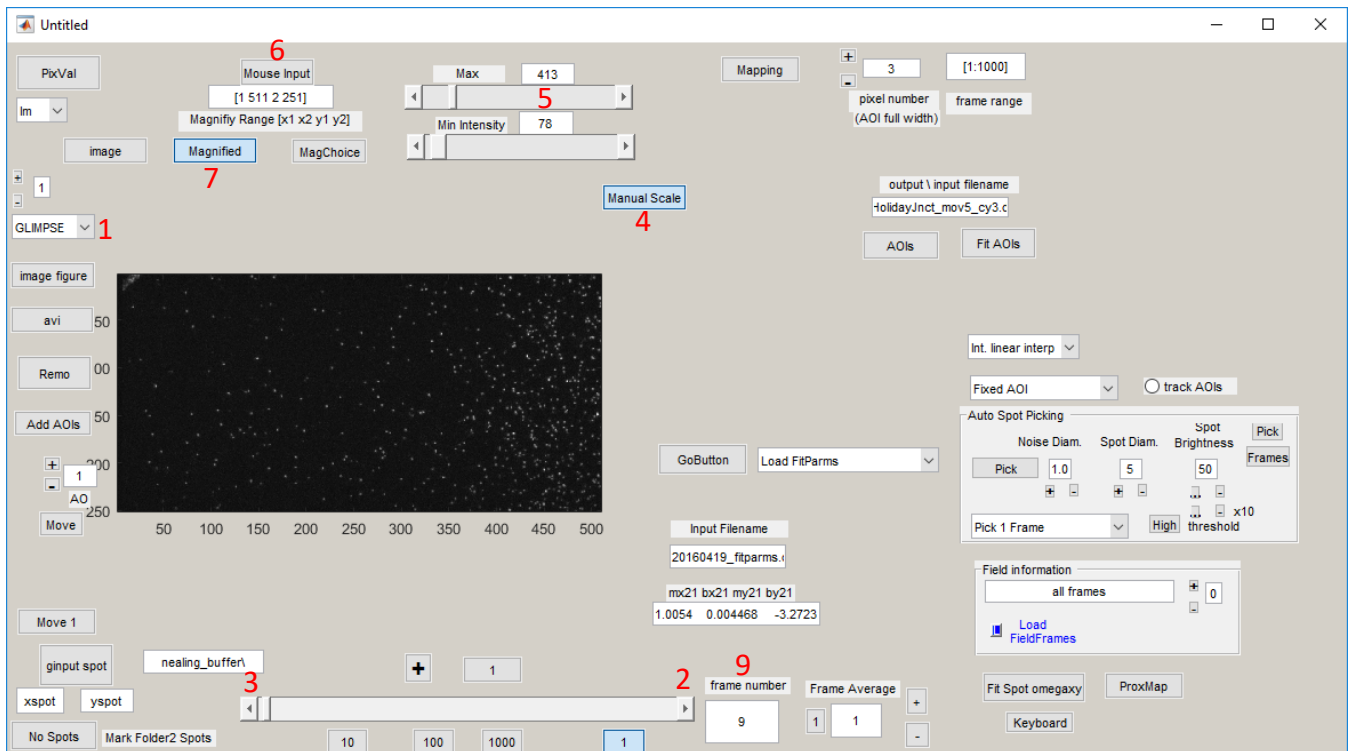
1. Now load mapping file into "Input filename" (copy name from file, which is in matlab → mapping → ST20131104_fitparms.dat)
2. Select "Load Fitparms" and hit "GoButton" (parameters should appear below window "mx21 bx21 my21 by21")
3. Select "Int. linear interp." from drop down menu
4. Reduce pixel number to 3
5. Set frame range (here [1:1000]) (start is almost always 1, 2nd # if from max frame number)
6. Now select spots with "auto spot picking" OR by hand → just play around with Noise Diameter, Spot Diameter and Brightness
7. Zoom in more (if a lot of spots are there, zoom into quadrants) "Mouse Input" & "Magnified"
8. Get rid of spots that are not real spots or not in the center of a rectangle or overlapping with "Remo" button
9. Add spots with "Add AOIs" button underneath Remo
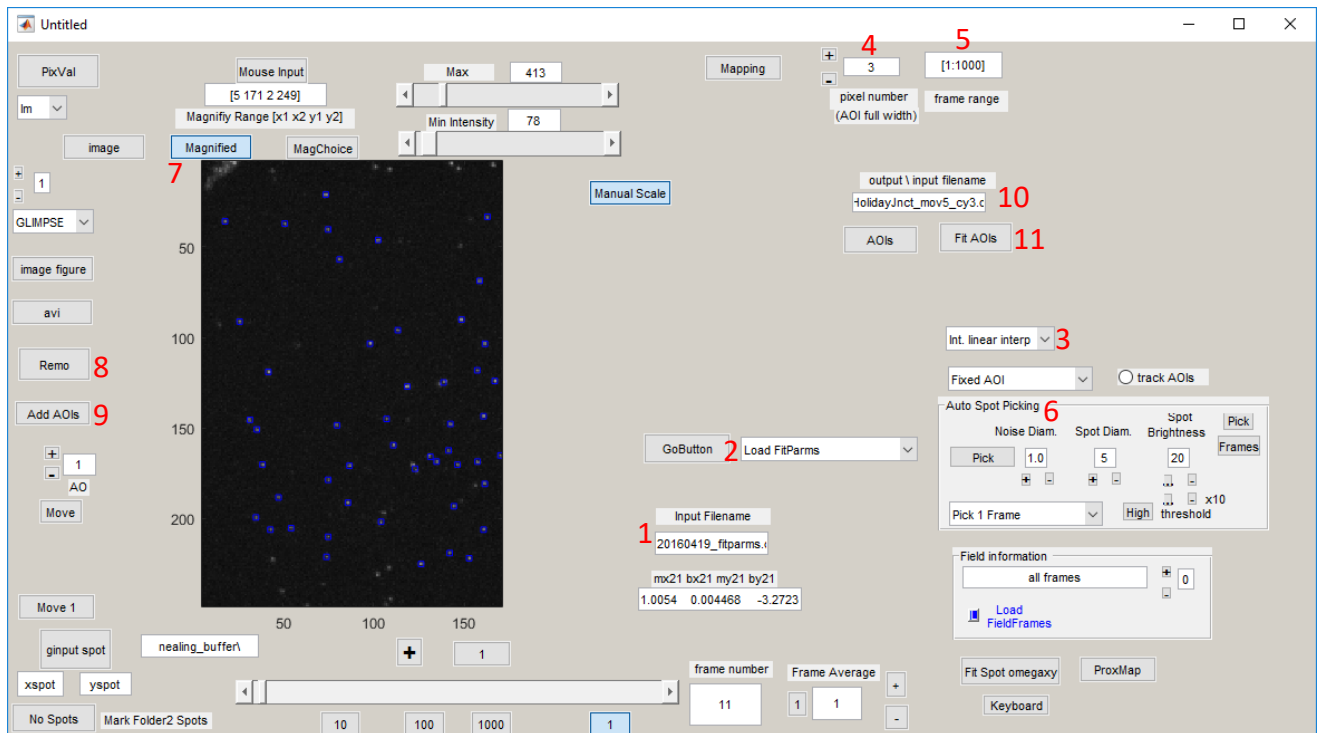
10. Once you're happy with the number of spots, Rename the file from default.dat to something like 20150130_cus2mutant_1_FRET.dat
11. Click "Fit AOIs" -- Matlab will process, when done a fit window will pop up, close this new window
2. Map onto the bottom channel: click "Map AOIs (x2y2)" and "GoButton"
10. Rename the file from default.dat to 20150130_cus2mutant_1_Cy3.dat
11. Click "Fit AOIs"
- Wait while MatLab is processing and creating a new window
- A New Fit window pops up

2. Analyzing the spots that show up on each AOI

1. Change "folder" to "glimpse"
2. Click "display AOIs" → AOIs show up in upper trace
3. [1 10 13 3] means it shows frame 1 – 10, makes 13 frames in row (each row has a top and a bottom box, which in this case the same, will be interesting in three color experiments) and 3 rows in total (this example is changed to show frames 1-39 by changing the 2nd # to 39)
4. load the files by pasting the names into the left hand side bar
5. press "Load aoifits"
6. Select int.aoi in middle and lower trace
7. Click "P" and + in both the middle and the lower trace
8. Graphs will appear

- Go through molecules and make a list in excel of the ones you think should be analyzed.
- NB: Things to keep in mind for FRET:

- o You want to have a photobleaching event to support that it is a single molecule
- o Anti-correlation between the signal in the two channels is supportive of FRET
- o The AOI should only contain a single "spot" and it should be centered:

  9. Big Red Circle in figure above: This area allows for a close inspection of the AOI and surrounding pixels:

    - "Xpand" allows zooming in or out on the AOI
    - Clicking on the box below "AOI" toggles the view between channel 1 (top/cy5) & 2(bottom/cy3)
    - Frame number is shown in the lower box (you can type a number, but it only updates the view when you push "+" or "-")

- Now onto analysis:

For the first molecule (access pull down menu in right-middle of window – see figure below):

- Click "load vid time base" (click Data Operation) and load exact same header file that you loaded to analyze the data with the glimpse file (only required once in the beginning)
- Click "Set Detrend Frame Range" (click Data Operation)(select start & end of photobleached region)
- Click "Set mean/std frame range" (click Data Operation))(select start & end of photobleached region)
- Click "Set interval Frame Range" (click Data Operation) (only once, sets start and end)(select region where Green laser is on only)
- ➔ Should show something like figure on right:
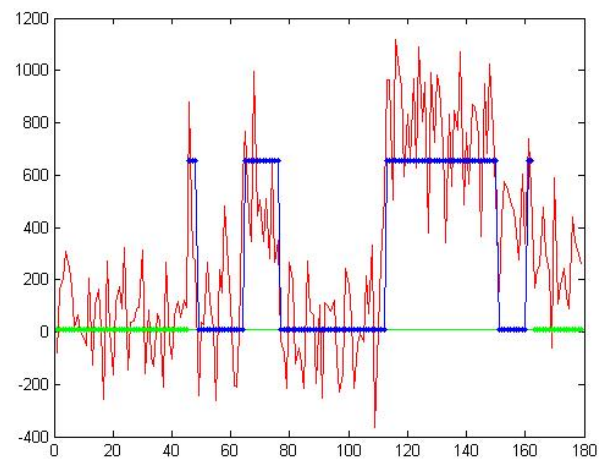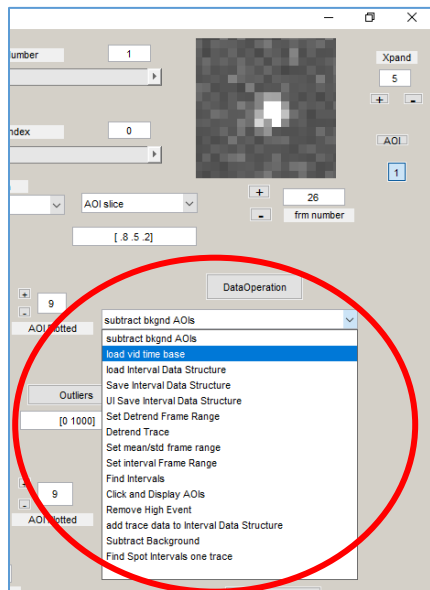
- If you want to add this trace to the data analysis, click "add trace data to Interval Data Structure" (click Data Operation),
- Save with "U1 Save Interval Data Structure" (click Data Operation) ➔ save into "fitting" folder (create new folder for new data date) (only once in the beginning required, to save data to the file you only need to click "Save Interval Data Structure" ➔ usually every 10 analyzed traces)
- Save the file as 20150130_mutantcus2_mov1_intervals_FRET.mat
- Go back to "Set Detrend Frame Range" and advance to next molecule.
- Click "Set mean/std frame range" (click Data Operation)
- If you want to add this trace to the data analysis, click "add trace data to interval" (click Data Operation),
- Advance to next molecule and detrend. Continue with all molecules to analyze.
- After all molecules have been analyzed, click "Save Interval Data Structure" (click Data Operation) before quitting

- Check to see if the intervals file was saved, by locating it in the fitting folder and dragging and dropping the file into matlab. The number of molecules in the intervals file should match the number of molecules you analyzed.

3. Analyze second data set

- If the intervals file saved correctly, quit imscroll windows.
- Re-open imscroll with glimpseloadimscroll and pick the same header file you were analyzing.
- Pick AOIs with the auto pick and press Fit AOIs (leave as default – this is just to re-open the fit window).
- Now load in the other (cy3 or FRET .dat file) into the lower window in the fit window.
- Start analyzing the dataset as before to make an intervals file for the other dataset.

4. Generating vbFRET input

- With both intervals files, load them into Matlab by dragging and dropping one at a time. Drag the FRET intervals file into Matlab and rename it in the variable editor to "Acceptor"
- Drag the Cy3 intervals file into Matlab and rename it as "Donor"
- Look at the traces in column 12 of the intervals file and find the frame range for when the green laser is on. Something like 23-988. And calculate total frames (i.e. 988-23+1)
- Edit the following code in red with the correct frame range:

```
frames = 955;

 num_acceptors = size(Acceptor.AllTracesCellArray,1);

 num_donors = size(Donor.AllTracesCellArray,1);

 pairs = ones(min(num_acceptors, num_donors),2,frames);


%match donors and acceptors

matches = 0;

for i = 1:1:num_donors;

   curr_ID = Donor.AllTracesCellArray{i,2};

   for j = 1:1:num_acceptors;

     if curr_ID == Acceptor.AllTracesCellArray{j,2};

       matches = matches + 1;

       pairs(matches,1,:) = Donor.AllTracesCellArray{i,12}(30:984,2);

       pairs(matches,2,:) = Acceptor.AllTracesCellArray{j,12}(30:984,2);

     end

   end
```

end

for i=1:size(pairs,1)

   for j=1:size(pairs,3)

data{1,i}(j,1)=pairs(i,1,j);

data{1,i}(j,2)=pairs(i,2,j);

   end

end

- Copy the code and paste it into matlab.
- If there is no error, continue. If there is an error, the frame range is incorrect.
- Save "data" variable as input into vbFRET in the fitting folder for these experiments (20150130_cus2mutant_mov1_vbFRETinput)
- Use vbFRET (type in vbFRET into matlab and press enter, then file -> load) to identify molecules that need to be corrected for blinking. Correct for blinking in matlab by selecting both columns where the numbers fluctuate around zero and pressing delete.
- Re-save the "data" variable as vbFRET input
- Reload vbFRET and reload vbFRET input file.
- Go to Edit -> remove photobleaching and press remove.
- Now the traces should have been truncated.
- Save the rawdata by going to File -> Save data -> toggle menu bar to save rawdata and browse to the fitting folder (you can rename it here or just rename it later).
- Load the rawdata file into matlab by dragging and dropping.
- The variable "FRET" should appear in the variable window
- Copy and paste this command into matlab and press enter: list= vertcat(FRET{:,:});
- This creates a variable called "list" with all the FRET values from all the molecules you analyzed.

5. Make a histogram with all FRET values

- First define the bin centers in the variable 'X'. Copy and paste this into matlab: X=[0.01:0.02:1.21];
- Now use the histc command to bin the FRET efficiency values in data5 in a new variable 'Y': Y=histc(list,[0:0.02:1.2]);
- Enter cftool in matlab
- Change the variables in the upper left hand corner to X and Y variables. Histogram should show up. Change the fitting equation to Gaussian and modify the number of gaussians you want to fit it to (4 or 5 for Cus2 data).
- Go to Edit -> Generate Code
- Copy and paste all the code into matlab (except first line "function…")
- A new figure with the histogram pops up
- Copy and paste the equation for the fit from the left lower hand corner of cftool into a powerpoint.
- Close the code generated, cftool and other windows open in matlab except for the figure window.
- Press the right-most button on the toolbar in the figure (plot tools)
- Select a data point and change it to bar (lines can be added using "other properties -> line style '-')
- Once you are finished, save the file as a ".emf" so it can be edited later in powerpoint/illustrator.