Overview

The purpose of the program is to collect statistics about the localization of mRNAs in smFISH images of neurons. The program is broken into two parts. The first part one is a semi-automatic tool for segmenting the cellular compartments of the neuron, the somas, nuclei, and dendrites. Part 1 also generates skeleton for each dendrite. After running the first part, FISHQuant is used to identify the coordinates of mRNAs in the smFISH images. Then the second part of the program uses both the spot coordinate obtained from FISHQuant along with the skeletons and prints obtained from part 1 to collect statistics about the localization of mRNA. With part 2 we investigate the following...

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1. The density of mRNA in cellular compartments
2. The distribution of mRNA along the skeleton of dendrites
3. The degree of colocalization between two types of mRNA
4. The degree of colocalization of mRNA at synapses in the dendrites

Part 1

1. Setting up your folder of images

We recommend having a specific folder containing all the materials for a specific experiment. Within this experiment folder you should have a subfolder containing all your microscopy images in ".tif" file format.

2. Naming your images

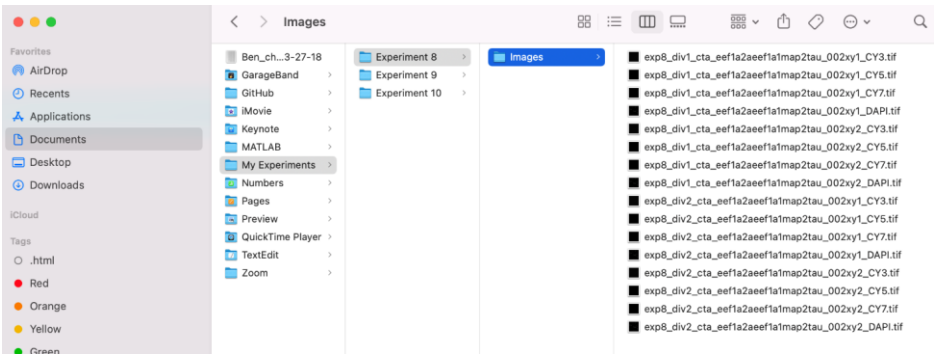
It is important that your images are named properly so that the program can read in all your images.

All images should be names as follows:

***ExperimentName*_(DIV or div)##*_treatment_listOfStains*_00#(xy or XY)##*_channel*.gif**

Where the DIV ## is the age of the neurons. The treatment is the experimental conditions. The numbers before and after xy/XY specify the Field of View (FOV), and channel is the code specifying the microscope channel used for acquiring that image. You should have one channel for Map2, one for DAPI, a few channels for various mRNAs, and an optional channel for a synaptic stain like PSD95.

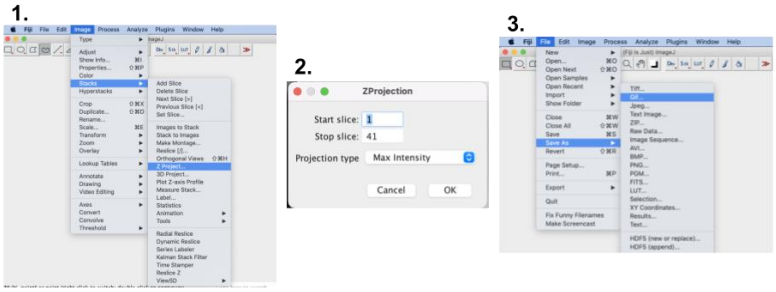
Below is an example of the folder setup and image names for a small experiment with div 1 and div 2, and a treatment called "cta".



Note 1: If you are doing other types of experiments such that you don't record the DIV number or treatment, you can either add a dummy word there or change the regex expression that the code uses to read images. To do this alter the regex expression in part1.py and part2.py and change the MaxImg object definition in ui.py.

3. Obtaining 2D projections

The first step is to use ImageJ (aka Fiji) to obtain 2D max projections of your ".tif" images. To do this: 1. Open each image in Fijij and go to Image > Stacks > Z Project. 2. In the pop-up window, make sure the projection type is "Max Intensity". 3. Save your image as a gif into your folder of images.



Note 2: If you are only analyzing somas and dendrites, you will only need max projections of your Map2 (dendrite stain) images. If you are also analyzing nuclei, you will also need to get max projections of your DAPI (nuclei stain) images.

4. Making annotations

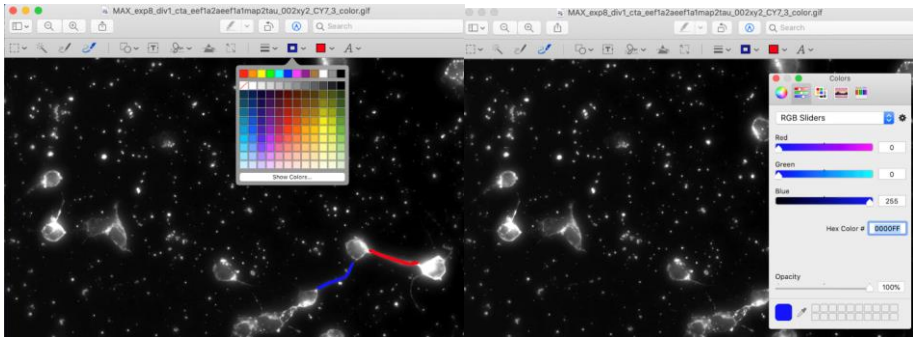
To annotate dendrite or somas open the Map2 max projection .gif in a simple image editing software, for example Preview on a Mac or Paint on a PC.

Color over up to 9 dendrites or somas of interest **using the following colors in the following order**

1. Red [RBG: (255, 0, 0), hex: #ff0000]

2. Green [RBG: (0, 255, 0), hex: #00ff00]
3. Blue [RBG: (0, 0, 255), hex: #0000ff]
4. Orange [RBG: (240, 134, 51), hex: #f08633]
5. Yellow [RBG: (255, 255, 0), hex: #ffff00]
6. Purple [RBG: (143, 57, 182), hex: #8f39b6]
7. Teal [RBG: (130, 210, 208), hex: #82d2d0]
8. Mint [RBG: (214, 253, 208), hex: #d6fdd0]
9. Salmon [RBG: (255, 128, 102), hex: #ff8066]

You must be sure to use exactly these colors with these RBG values. If you are using Preview, you can change the precise color of your pen by clicking on the color, clicking on "Show colors..." and specifying the Hex code, or sliding the RBG values.



Note 3: We have found that sometimes when saving gif images from Paint on PC's, the colors of the annotations can change. This affects the ability of the program to detect the annotations. If you annotate image on a PC, we recommend that you open images of the annotations you have made and record the RBG values of the color that the annotations were saved as. Then open part1.py and on lines 11-20 edit the RBG values of each color accordingly.

```
 9  def main():
10      #####Set colors######
11      colors = {"red": [255,0,0],
12              "green": [0,255,0],
13              "blue" : [0,0,255],
14              "orange" : [240, 134, 51],
15              "yellow" : [255, 255, 0],
16              "purple" : [143, 57, 182],
17              "teal" : [130, 210, 204],
18              "mint" : [214, 253, 208],
19              "salmon": [255, 128, 102]
20              }
```

4.a If analyzing dendrites:

- Make a copy of each 2D Map2 gif and open it in your image annotating software
- Color over up to 9 dendrites, using the colors in order as listed above
- Prints for dendrites are generated by extracting the signal behind your annotations, so it best to cover the dendrites of interest completely
- Save the gif
- Rename the file by adding the tag “dendAnnot_N” before “gif”, where N is the number of dendrites you colored in that image

4.b If analyzing somas/nuclei:

- Make a copy of each 2D Map2 gif and open it in your image annotating software
- Color over up to 9 somas, using the colors in order as listed above
- Soma prints are generated by taking the area of the annotations, so it is more important that soma annotations are accurate and do not extend past the edge of the soma
- Save the gif
- Rename the file by adding the tag “somAnnot_N” before “gif” where N is the number of somas you colored in that image

4.c Tips for Annotating

- Try to avoid having your dendritic annotations touch the border of the image as it can distort the skeleton of dendrites
- Before annotating a soma, open the DAPI max projection to make sure the nucleus for that soma is clearly visible
- Avoid annotating cellular compartments that overlap significantly with other dendrites or somas because FISHQuant will overcount the number of mRNAs in that cellular compartment
- Careful annotations lead to better prints and skeletons

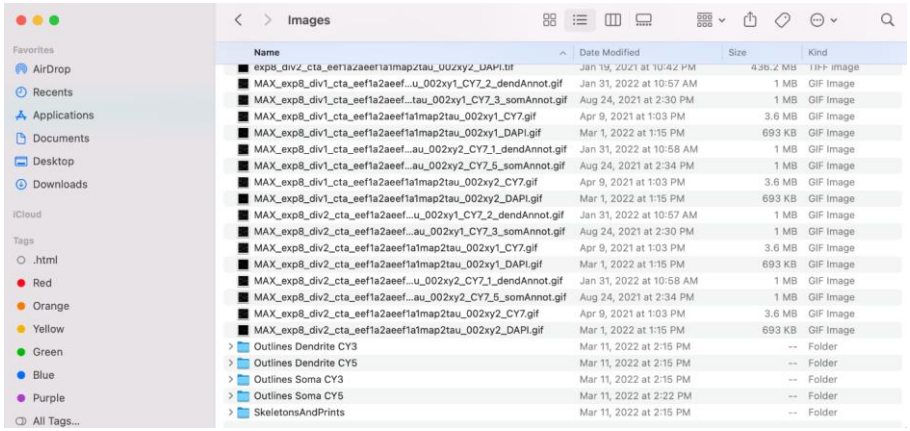
Below is an example of how your 2D max projections and annotations should be named.

MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy1_CY7_2_dendAnnot.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy1_CY7_3_somAnnot.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy1_CY7.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy1_DAPI.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy2_CY7_1_dendAnnot.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy2_CY7_5_somAnnot.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy2_CY7.gif
MAX_exp8_div1_cta_eef1a2aeef1a1map2tau_002xy2_DAPI.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy1_CY7_3_dendAnnot.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy1_CY7_3_somAnnot.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy1_CY7.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy1_DAPI.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy2_CY7_1_dendAnnot.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy2_CY7_6_somAnnot.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy2_CY7.gif
MAX_exp8_div2_cta_eef1a2aeef1a1map2tau_002xy2_DAPI.gif

5. Now you are ready to run part1.py!

After running part1.py, your folder of images should have a new folder containing the text file outlines for dendrite and somas specific to each mRNA channel. You should also have a folder called **"SkeletonsAndPrints,"** which will contain all the gif images of the skeletons and prints for every annotated compartment.

At this point, your folder of images should look like the following:



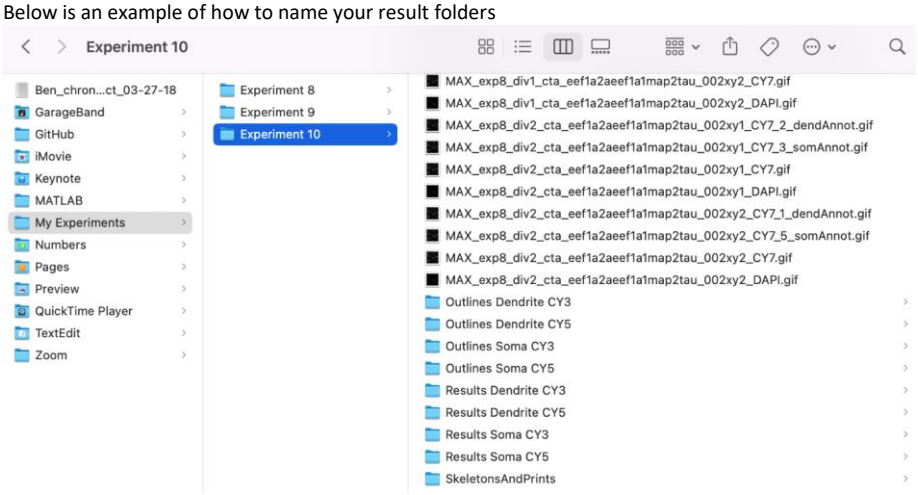
Within the **SkeletonsAndPrints** folder, each print/skeleton for a compartment is labeled with a unique **"segmentation number."** This segmentation number is recorded as the cell number in the text file outlines. The segmentation number for a soma is the same segmentation number as its corresponding nucleus.

Note 5: If an annotation was done with a wrong color, if a soma had no clear nucleus in the DAPI image, or if there was any other technical issue with generating a print for a compartment, the print will not be saved as a gif. In this case, we skip this compartment in the outline text files and we also skip a segmentation number.

The matching segmentation numbers for a soma and its corresponding nuclei are circled below

Now you should make new folders in your folder of images so that you have a place to save your result files from FISHQuant. You should name your folders **"Results Dendrite/Soma *channel*"** for each mRNA channel (and synaptic channel) you are using.

Below is an example of how to name your result folders



FISHQuant

You are now ready to run FISHQuant!

When you run FISHQuant, with batch processing you can set the root folders. Below is an example of how you would set the root folder for analyzing Dendrites for the CY3 channels...

- Root folder for images: "Images"
- Root folder for outlines: "Outlines Dendrites CY3"
- Root folder for results: "Results Dendrite CY3"

If you would like to do synaptic localization analysis in Part 2, you will need to also use FISHQuant to find the coordinates of every synapse. You can do this by treating your synapse stain the same as you would an mRNA stain.

For more information about using FISHQuant, reference this article (https://www.nature.com/articles/nmeth.2406)

Part 2

When you run part2.py the command prompt will ask you which of the following functionalities you would like to use:

1. **Density Analysis**
   - Input: None
   - Output: Excel file called SomaDensities.xlsx/DendriteDensisties.xlsx
   - Statistics: The density of mRNA per square nanometer in each annotated cellular compartment
   - Example:

Number	Channel	Number of mRNA	Area (sq. nanometer)	Density
1	Cy3	254	14087068.75	0.000018030720550008
1	Cy5	96	14087068.75	0.00000681476052283766
2	Cy3	132	6309712.5	0.00002092012908670 56
2	Cy5	73	6309712.5	0.00001156946532825 39
3	Cy3	183	8956093.75	0.00002043301523055 18
3	Cy5	124	8956093.75	0.00001384532179556 52
4	Cy3	110	5639450	0.00001950544822633 41
4	Cy5	68	5639450	0.00001205791344900 66
5	Cy3	348	11845156.25	0.00002937909746863 83
5	Cy5	184	11845156.25	0.00001553377567307 31
6	Cy3	242	13347468.75	0.00001813077854181 15
6	Cy5	92	13347468.75	0.00000689269266878786

2. **Distribution Analysis**
   - Input: None
   - Output: Excel file called DistrAnalysis.xlsx
   - Statistics: The number of mRNA at 0-25, 25-50, 50-75, 75-100, 100-125, 125-150, >150 nm away from the soma for each annotated dendrite. Distances are measured along the skeleton for each dendrite
   - Example:

Dendrite Num	Channel	0-25 (um)	25-50 (um)	50-75 (um)	75-100 (um)	100-125 (um)	125-150 (um)	>= 150 (um)
1	Cy3	89	35	33	32	35	22	0
1	Cy5	47	9	8	13	10	6	0
2	Cy3	52	36	42	2	0	0	0
2	Cy5	45	16	12	0	0	0	0
3	Cy3	67	40	26	48	2	0	0
3	Cy5	61	25	13	25	0	0	0
4	Cy3	110	0	0	0	0	0	0
4	Cy5	68	0	0	0	0	0	0
5	Cy3	90	64	68	71	55	0	0
5	Cy5	51	40	26	35	32	0	0
6	Cy3	74	43	34	30	31	16	0
6	Cy5	38	14	13	4	9	3	0
7	Cy3	59	36	35	0	0	0	0
7	Cy5	52	20	10	0	0	0	0
8	Cy3	181	0	0	0	0	0	0
8	Cy5	96	0	0	0	0	0	0
9	Cy3	101	69	49	30	4	0	0
9	Cy5	73	26	12	5	1	0	0
10	Cy3	67	59	57	6	0	0	0
10	Cy5	56	31	25	4	0	0	0

3. **Colocalization Analysis**
   - Input: 2 channels of mRNA that are to be analyzed, max recorded distance, desired increment to record distances in the excel table

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Commented [MVUD4]: @Zoe Of what?

- Output: Excel file called ColocAnalysis.xlsx
- Statistics: In each dendrite, for each mRNA of type A we record the minimum distance to another mRNA of type B, and for each mRNA of type B we record the minimum distance to an mRNA of type A. This is also done for distance from type A to mRNA themselves and distance from type B mRNA to themselves. These distances are binned based on the desired increment and reported in the excel file. To provide a computational control, we randomly simulate the coordinates of both type A and type B mRNA 100 times and report the average statistics using these simulated coordinates in the last two columns of the excel file.
- Example:

Distance (nm)	Cy3 to closest Cy5	Cy5 to closest Cy3	Cy3 to closest Cy3	Cy5 to closest Cy5	Sim-Cy3 to closest Sim-Cy5	Sim-Cy5 to closest Sim-Cy3
0-75	487	489	94	109	0	0
75-150	1172	1190	387	359	0	0
150-225	1216	1198	710	550	0	0
225-300	1203	1113	988	671	0	0
300-375	792	655	1183	628	0	0
375-450	516	308	1191	643	0	0
450-525	448	187	1108	587	0	0
525-600	385	107	1087	518	0	0
600-inf	2863	277	2910	2168	9082	5524

4. Synapse Localization Analysis

- Input: Name of the synapse channel, threshold distance to consider mRNA localized at a synapse.
- Output: Excel file called SynapseAnalysis.xlsx
- Statistics: For every annotated dendrite and every synapse, we calculate the number of mRNA with the threshold distance to the synapse. We sum over all dendrites and record the number of synapses with X mRNAs localized at them, where X is 1, 2, 3, etc. To provide a computational control, we randomly simulate the coordinates of each type of mRNA 100 times and report the average statistics using these simulated coordinates in the columns titled "Sim *mRNA Channel.*"
- Example:

Num of mRNA	Real Cy3	Sim Cy3	Num of mRNA	Real Cy5	Sim Cy5
0	1724	2164.61	0	2148	2394
1	525	109.73	1	358	140.48
2	150	78.5	2	100	60.24
3	32	46.14	3	16	23.12
4	5	23.15	4	6	7.87
5	1	10.21	5	0	2.4
6	1	3.84	6	1	0.64
7	0	1.39	7	0	0.2
8	0	0.32	8	0	0.04
9	0	0.1	9	0	0.01
10	0	0			
11	0	0			
12	0	0.01			

Note 6: Part 2 will calculate the localization statistics separately for dendrites/somas of different DIV #s and treatments. Each excel file will have a sheet for each DIV treatment combination used in the experiment.