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, Part 1, is a semi-automatic tool for segmenting the cellular compartments of the neuron, the somas, nuclei, and dendrites. Each segmentation is stored as a binary image which we refer to as a print. Additionally, Part 1 generates skeletons for each dendrite, which are also stored as binary images. After running Part 1, FISHQuant is used to identify the coordinates of mRNAs (x, y, and z) in the smFISH images. Then the second part of the program, Part 2, uses both the spot coordinate (x and y) obtained from FISHQuant along with the skeletons and prints obtained from Part 1 to collect statistics about the localization of mRNA. In Part 2, we investigate the following: 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 (not used in this paper) and 4) The degree of colocalization of mRNA at synapses in the dendrites.

Program Layout

The program is composed of 6 modules, two of which are executable, part1.py and part2.py. Both Part 1 and Part 2 draw from ui.py and segmentation.py. The following graph describes the purpose of each module and the module dependencies.

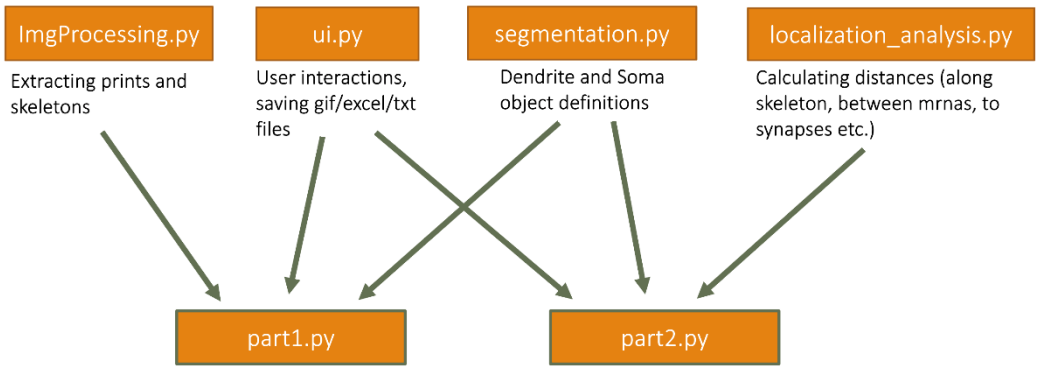


Figure 1. Schematic of the program layout, the function of each module and their dependencies. Only part1.py and part2.py are executable

Part 1

Terminology:

A **print** is a 2D binary image for a specified cellular compartment, where there is a white pixel where the cellular exists and a black pixel everywhere else

A **skeleton** is a 2D binary image of a single white line representing the midline of a print for a dendrite

MAP2 is an immunofluorescence using an antibody that detects the Microtubule associate protein 2 (Map2) which localizes in dendrites

DAPI (4’,6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain used to identify nuclei

Generating prints:

For dendrites, the program will look at each color used to annotate a dendrite in the annotation image and uses that color block as a mask for the 2D projection of the MAP2 image. In other words, the program extracts the map2 signal colored over by the annotation, and it does so separately for each dendrite. This MAP2 signal is subsequently binarized using the Otsu threshold generated from the original unmasked 2D MAP2 image. Thus, we obtain a binary image for each dendrite which is cleaned, smoothed, and then passed through an algorithm to account for inconsistent MAP2 signal.

Since synapses protrude out of dendrites, often the synapses are not contained by the Map2 stain, and thus also not contained by the dendrite prints. To account for this, when analyzing synapses, Part 1 of our program generates two prints for each dendrite, a regular one and another which is slightly dilated so that it contains all synaptic protrusions.

Because somas are easy to annotate accurately, when generating prints for somas, the program simply uses the masks generated from the annotations as a print. Thus, no binarizing is necessary, and only minimal cleaning is required to smooth the edges of the print.

For nuclei, the program will look at each color used to annotate a soma as a mask on the 2D projection of the DAPI channel. So, the program isolates the nuclei associated with each soma. The masked image is then binarized using a scaled Otsu threshold of the 2D DAPI image. Qualitatively, we found that scaling the threshold down by 0.65 worked best. DAPI stains tend to be stronger around the perimeter of the nucleus and spotty in the center, so once a binary image of each nucleus is obtained, it is cleaned, by filling in holes and smoothing edges.

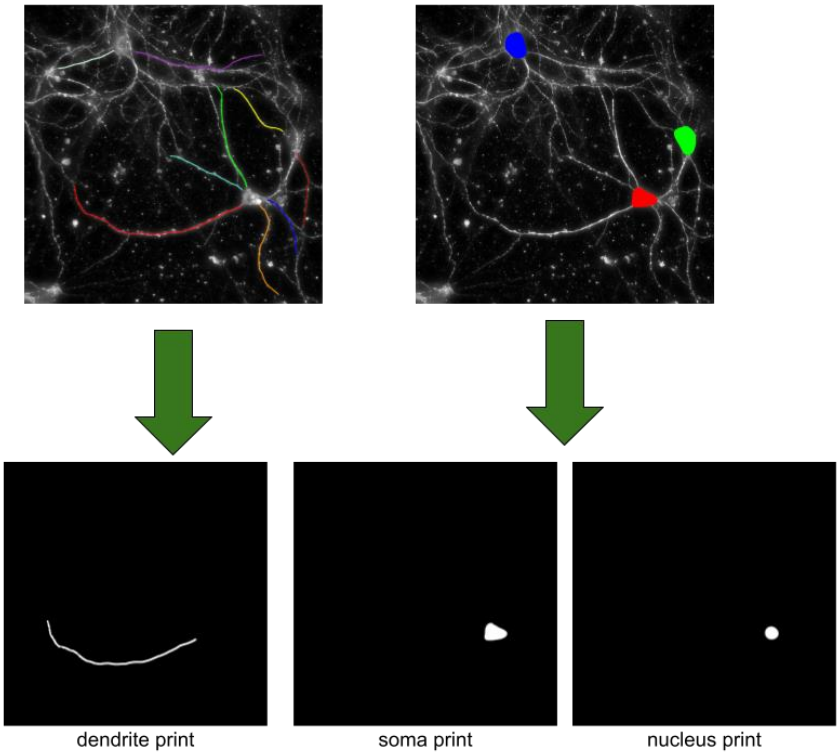


Figure 2. Schematic of input and output of part1.py. Images of annotated dendrites and somas are used to generate prints and skeletons.

Algorithm for inconsistent signal

The MAP2 stain for dendrites varies in intensity even along the same dendrite. To obtain connected prints for each dendrite we merge fragments of the dendrite print with the following algorithm:

Input: 2D projection of a dendrite binarized by Otsu threshold

Algorithm:

- Save a copy of the input
- Obtain an over-dilated dendrite by dilating every pixel by a radius of 4 and then erode every pixel by a radius of 3 until all the white pixels in the image are part of 1 connected component
- Skeletonize the over-dilated dendrite
- Dilate the skeleton by a 5-pixel radius
- Add the dilated skeleton to the copy of the original input and obtain the final print

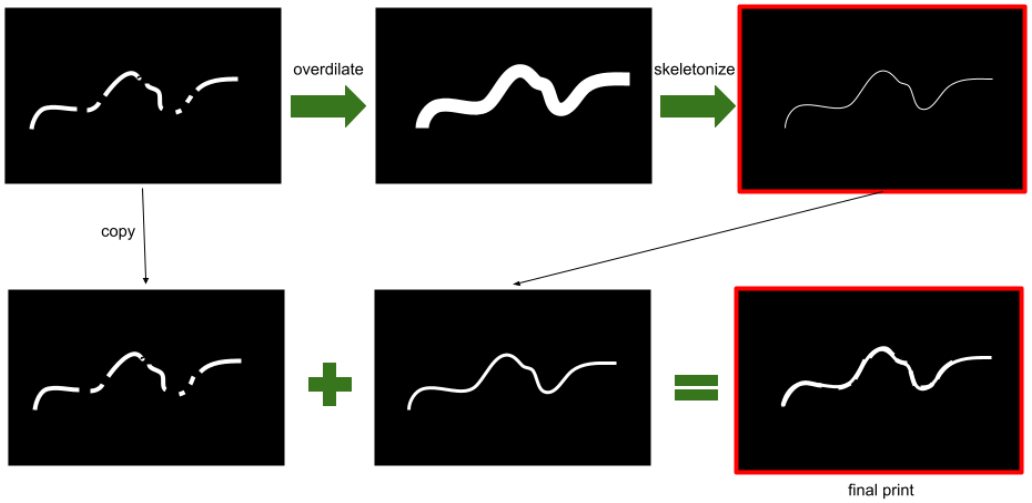


Figure 3. Schematic of the algorithm for inconsistent MAP2 signal

We choose to dilate by a radius of 4 pixels and erode by a radius of 3 pixel because it both smooths the edge of the image while also having the net effect of dilating each pixel in the dendrite by a radius of 1. Also note that because the dendrite is over-dilated by expanding each pixel equally in all directions, the midline (ie skeleton) of the original input should not be greatly affected by the algorithm.

Trimming Skeletons

Initial skeletons are obtained using the skimage skeletonize function. However, sometimes these original skeletons have some small branches. To trim these branches, use the FilFinder library to extract the part of the skeleton corresponding to the longest path. Additionally, the initial skeletons on occasion contain small circle or line segments disjoint from the main skeleton. In these cases, our CleanBinaryImage function is applied, which keeps only the largest component of the skeleton image.

Cleaning Binary Images

Even though every dendrite print is passed through the algorithm for inconsistent signal the final prints may still have white blobs which are disjoint from the main print. This can happen when the saved copy of the original dendrite print captures a signal that is noise and not from the dendrite. So even though the algorithm for inconsistent signal dilates the print into 1 connected component, when the skeleton is added back to the saved copy, the extraneous white blobs are still present.

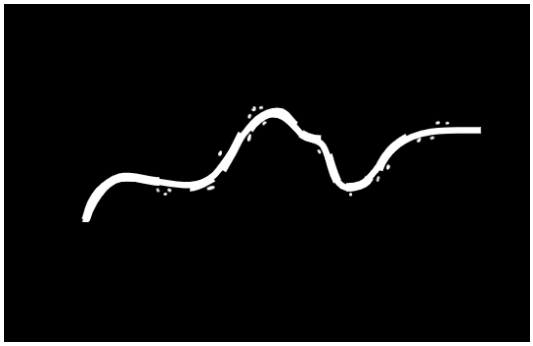


Figure 4. Example noisy signal spots surrounding print after applying the algorithm for inconsistent signal

This issue is accounted for with a function for cleaning binary images, named CleanBinaryImage. This function looks at every connected component in the print and deletes all components except for the one with the largest area. However, it first checks that the second-largest component has an area smaller than 20% of the area of the largest component. If the print does not pass this requirement, it is evidence that there is an abnormally large extraneous signal or that something else has gone wrong in the generation of the print. In this case, the program will not delete any small blobs from the print, will report the problem to the user, and will not add this dendrite to any of the outline files.

Note the program also contains a similar function for refining soma and nucleus prints, named refineSomaOrNucPrint which calls the Clean Binary Image function. Because DAPI stains tend to be spotty at center of the nucleus, in addition to deleting small white blobs the program also fills in holes

Saving Skeletons and Prints

All prints and skeletons are saved as black and white gif images in a folder called SkeletonsAndPrints, so that they can be accessed by the second part of the program.

FISHQuant

FISHQuant is a program that searches smFISH images for Gaussian distributions of signals to detect mRNAs (Mueller et al., 2013). Furthermore, the program can distinguish between multiple mRNA spots if their signals overlap, making it particularly useful for counting the number of mRNAs in transcription sites. We use version one of FISHQuant, which is written in Matlab, to find the coordinates of all the mRNA in each dendrite and soma.

Part 2

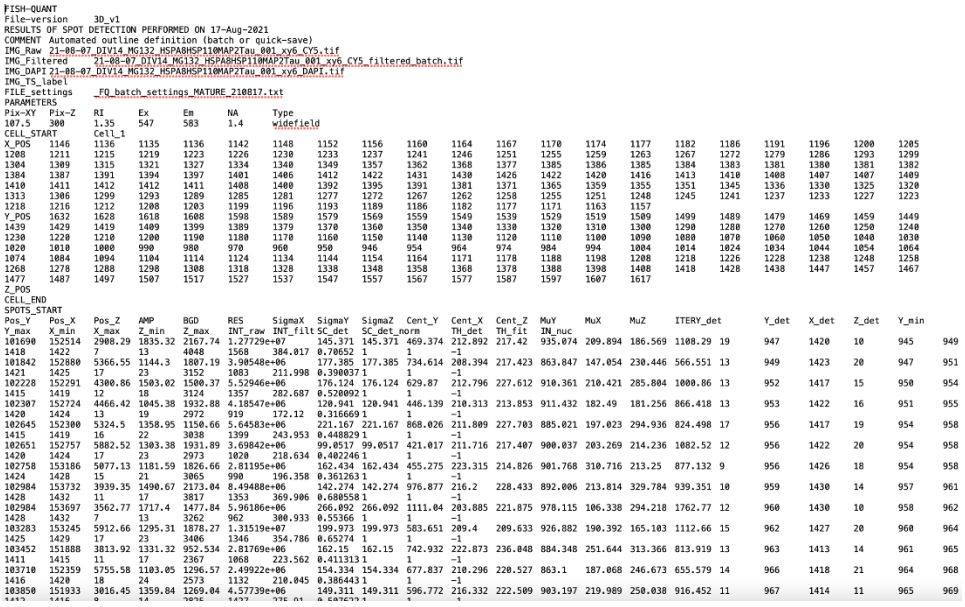
This part of the pipeline can be used to extract different information and statistics from the mRNA/synapse coordinates and dendrite morphology to provide insights for different biological questions.

Currently functionalities include finding:

1. Density of mRNAs within each dendrite and soma under different experimental conditions
2. Distribution of mRNAs along the dendrites based on how far they travel from the soma
3. Colocalization of different mRNA species based on how far each mRNA is from its closest mRNA from the other species
4. Colocalization of mRNAs with synapses based on how many mRNAs are within a threshold distance from each synapse

Input Extraction

We first parse the information in the text files generated by performing spot detect in FISHQuant which records the coordinates of every mRNA and synapse. Thus, we first extract all the coordinates and which dendrite or soma they came from. It should be noted that the output of FISHQuant's spot detection function flips the x and y coordinates. We account for this in our own program.



```
FISH-QUANT
File-version    3D_v1
RESULTS OF SPOT DETECTION PERFORMED ON 17-Aug-2021
COMMENT Automated outline definition (batch or quick-save)
IMG_Raw 21-08-07_DIV14_MG132_HSPA8HSP110MAP2Tau_001_xy6_CY5.tif
IMG_Filtered    21-08-07_DIV14_MG132_HSPA8HSP110MAP2Tau_001_xy6_CY5_filtered_batch.tif
IMG_DAPI 21-08-07_DIV14_MG132_HSPA8HSP110MAP2Tau_001_xy6_DAPI.tif
IMG_TS_label
FILE_settings    _FQ_batch_settings_MATURE_210817.txt
PARAMETERS
Pix-XY  Pix-Z   RI      Ex      Em      NA      Type
107.5   300     1.35    547     583     1.4     widefield
CELL_START      Cell_1
X_POS   1146    1136    1135    1136    1142    1148    1152    1156    1160    1164    1167    1170    1174    1177    1182    1186    1191    1196    1200    1205
1208    1211    1215    1219    1223    1226    1230    1233    1237    1241    1246    1251    1255    1259    1263    1267    1272    1279    1286    1293    1299
1304    1309    1315    1321    1327    1334    1340    1349    1357    1362    1368    1377    1385    1386    1385    1384    1383    1381    1380    1381    1382
1384    1387    1391    1394    1397    1401    1406    1412    1422    1431    1430    1426    1422    1420    1416    1413    1410    1408    1407    1407    1409
1410    1411    1412    1412    1411    1408    1400    1392    1395    1391    1381    1371    1365    1359    1355    1351    1345    1336    1330    1325    1320
1313    1306    1299    1293    1289    1285    1281    1277    1272    1267    1262    1258    1255    1251    1248    1245    1241    1237    1233    1227    1223
1218    1216    1212    1208    1203    1199    1196    1193    1189    1186    1182    1177    1171    1163    1157
Y_POS   1632    1628    1618    1608    1598    1589    1579    1569    1559    1549    1539    1529    1519    1509    1499    1489    1479    1469    1459    1449
1439    1429    1419    1409    1399    1389    1379    1370    1360    1350    1340    1330    1320    1310    1300    1290    1280    1270    1260    1250    1240
1230    1220    1210    1200    1190    1180    1170    1160    1150    1140    1130    1120    1110    1100    1090    1080    1070    1060    1050    1040    1030
1020    1010    1000    990     980     970     960     950     946     954     964     974     984     994     1004    1014    1024    1034    1044    1054    1064
1074    1084    1094    1104    1114    1124    1134    1144    1154    1164    1171    1178    1188    1198    1208    1218    1226    1228    1238    1248    1258
1268    1278    1288    1298    1308    1318    1328    1338    1348    1358    1368    1378    1388    1398    1408    1418    1428    1438    1447    1457    1467
1477    1487    1497    1507    1517    1527    1537    1547    1557    1567    1577    1587    1597    1607    1617
Z_POS
CELL_END
SPOTS_START
Pos_Y   Pos_X   Pos_Z   AMP     BGD     RES     SigmaX  SigmaY  SigmaZ  Cent_Y  Cent_X  Cent_Z  MuY     MuX     MuZ     ITERY_det       Y_det   X_det   Z_det   Y_min
Y_max   X_min   X_max   Z_min   Z_max   INT_raw INT_filt SC_det  SC_det_norm     TH_det  TH_fit  IN_nuc
101690  152514  2908.29 1835.32 2167.74 1.27729e+07     145.371 145.371 469.374 212.892 217.42  935.074 209.894 186.569 1108.29 19      947     1420    10      945     949
1418    1422    7       13      4048    1568    384.017 0.70652 1       1       -1
101842  152880  5366.55 1144.3  1807.19 3.90548e+06     177.385 177.385 734.614 208.394 217.423 863.847 147.054 230.446 566.551 13      949     1423    20      947     951
1421    1425    17      23      3152    1083    211.998 0.390037 1      1       -1
102228  152291  4300.86 1503.02 1500.37 5.52946e+06     176.124 176.124 629.87  212.796 227.612 910.361 210.421 285.804 1000.86 13      952     1417    15      950     954
1415    1419    12      18      3124    1357    282.687 0.520092 1      1       -1
102307  152724  4466.42 1045.38 1932.88 4.18547e+06     120.941 120.941 446.139 210.313 213.853 911.432 182.49  181.256 866.418 13      953     1422    16      951     955
1420    1424    13      19      2972    919     172.12  0.316669 1      1       -1
102645  152300  5324.5  1358.95 1150.66 5.64583e+06     221.167 221.167 868.026 211.809 227.703 885.021 197.023 294.936 824.498 17      956     1417    19      954     958
1415    1419    16      22      3038    1399    243.953 0.448829 1      1       -1
102651  152757  5882.52 1303.38 1931.89 3.69842e+06     99.0517 99.0517 421.017 211.716 217.407 900.037 203.269 214.236 1082.52 12      956     1422    20      954     958
1420    1424    17      23      2973    1020    218.634 0.402246 1      1       -1
102758  153186  5077.13 1181.59 1826.66 2.81195e+06     162.434 162.434 455.275 223.315 214.826 901.768 310.716 213.25  877.132 9       956     1426    18      954     958
1424    1428    15      21      3065    990     196.358 0.361263 1      1       -1
102984  153732  3939.35 1490.67 2173.04 8.49488e+06     142.274 142.274 976.877 216.2   228.433 892.006 213.814 329.784 939.351 10      959     1430    14      957     961
1428    1432    11      17      3817    1353    369.906 0.680558 1      1       -1
102984  153697  3562.77 1717.4  1477.84 5.96186e+06     266.092 266.092 1111.04 203.885 221.875 978.115 106.338 294.218 1762.77 12      960     1430    10      958     962
1428    1432    7       13      3262    962     300.933 0.55366 1       1       -1
103283  153245  5912.66 1295.31 1878.27 1.31519e+07     199.973 199.973 583.651 209.4   209.633 926.882 190.392 165.103 1112.66 15      962     1427    20      960     964
1425    1429    17      23      3406    1346    354.786 0.65274 1       1       -1
103452  151888  3813.92 1331.32 952.534 2.81769e+06     162.15  162.15  742.932 222.873 236.048 884.348 251.644 313.366 813.919 13      963     1413    14      961     965
1411    1415    11      17      2367    1068    223.562 0.411313 1      1       -1
103710  152359  5755.58 1103.05 1296.57 2.49922e+06     154.334 154.334 677.837 210.296 220.527 863.1   187.068 246.673 655.579 14      966     1418    21      964     968
1416    1420    18      24      2573    1132    210.045 0.386443 1      1       -1
103850  151933  3016.45 1359.84 1269.04 4.57739e+06     149.311 149.311 596.772 216.332 222.509 903.197 219.989 250.038 916.452 11      967     1414    11      965     969
```

Figure 5. Example of output file from FISHQuant. The x and y coordinates of the mRNA or synapse signals are extracted for analysis.

Calculating Density

To calculate the area of a dendrite or soma, the program counts the number of pixels in the corresponding print generated from part 1. The area in pixels can then be converted to squared nanometers (nm^2) based on the conversion factor specific to each microscope. We then can take the ratio of the number of mRNA in and the area of each dendrite/soma to obtain compartment-specific mRNA density.

Number	Channel	Number of mRNA	Area (sq. nanometer)	Density
3	CY3	36	2808168.75	0.0000128197424033011
3	CY5	36	2808168.75	0.0000128197424033011
4	CY3	36	2657937.5	0.0000135443365391398
4	CY5	36	2657937.5	0.0000135443365391398
6	CY3	36	2877506.25	0.0000125108329477999
6	CY5	36	2877506.25	0.0000125108329477999

Figure 6. Example of compartment-specific mRNA count, area (in pixels) and mRNA density.

Finding the Endpoints of Dendritic Skeleton

To find the two endpoints of the skeleton, the program looks at the neighbors of each white pixel in the skeleton. If a skeleton point is an endpoint, only one of its neighboring pixels will be white, while all other points in the skeleton will have two neighboring white pixels. Since the skeleton can have a curved structure, the neighboring points of a skeleton point can be anywhere inside a 3 by 3 grid centered at the skeleton point of interest. As a result, the program counts the number of white pixels in a 3 by 3 grid centered at each point in the skeleton. If there are 3 white pixels in the grid, the point of interest is considered to not be an endpoint. If there are only 2 white pixels, the skeleton point centered is an endpoint.

If the program finds less than 2 or more than 2 endpoints for a particular skeleton it is an indicator that the outline for this dendrite was not generated properly. In this case, the program will not include the dendrite associated with the skeleton in the statistical analysis.

Determine the Soma Endpoint

Once two endpoints of the skeleton are found, the program aims to determine which of the two endpoints belongs to the soma end. Since dendritic annotation does not keep track of the soma end, the program needs to determine it using available information. With thousands of images from previous smFISH experiments, it has been observed that the soma end of each dendrite almost always has more mRNAs than the distal end. Biologically, this observation can be explained by the fact that the

transportation of mRNAs out of the soma is limited. Thus, we assume that the end of the dendrite with more mRNA is the end closer to the soma.

Distance of mRNA to Soma

To find how far the mRNAs are from the soma, the program first projects each mRNA to the closest pixel on the skeleton. Then it counts the number of pixels along the skeleton from the soma endpoint to the projected mRNA. As we count pixels along the skeleton, we keep track if an adjacent or diagonal pixel step was taken, so that we can calculate the precise distance of each step (sqrt(2) for a diagonal step and 1, for an adjacent step). Note that the distance along the skeleton from an mRNA to an endpoint is more accurate than the Euclidean distance because it considers the curved morphology of the dendrite. Since the skeleton is the center axis of the dendrite, the skeleton and dendrite share the same curvature. The calculated distances are then binned into groups of 25 micrometers before being written to an excel file.

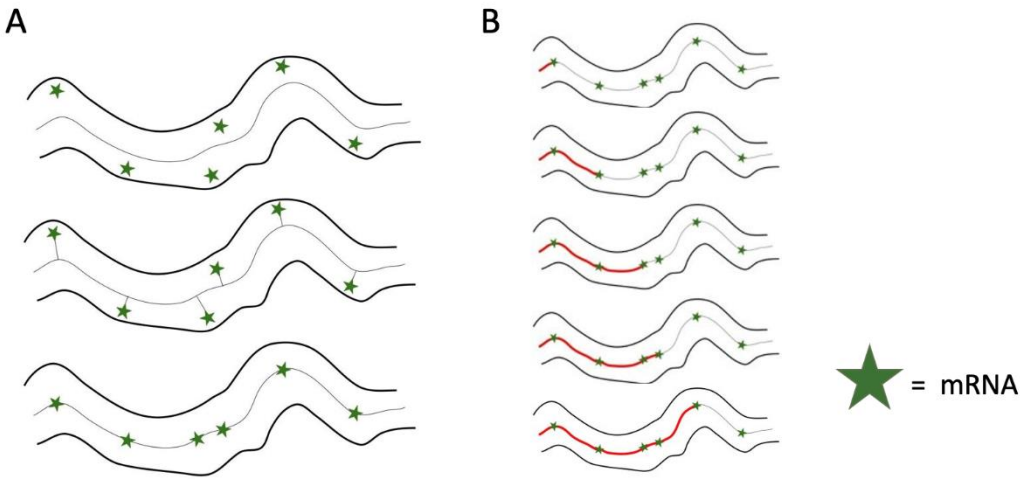


Figure 7. A) Diagram of mapping mRNA spot coordinates to the skeleton B) Measuring distance of mRNA to soma by counting pixels along the skeleton

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

Figure 8. Example of mRNA distribution based on distance to soma along the skeleton, each dendrite is assigned a unique number

mRNA Colocalization Statistics

Our motivation for calculating colocalization statistics for two kinds of mRNA is to see if the mRNAs are moving together down the dendrites in granules. The program will loop through every mRNA of species A and find the distance to the closest mRNA of species B. The same calculation can be done the other way around, finding the distance between each mRNA of species B to the closest mRNA of species A. Additionally, the program calculates self-colocalization, the distance between an mRNA of species A to another mRNA of species A (not including itself). To have a computational control, the same colocalization statistics were calculated with simulated mRNA coordinates. The simulation process is described below in the section “Simulation as Control.” Nearest distances are then binned into user-defined groups before being written to an excel file.

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

Figure 9. Example of mRNA colocalization statistics: mRNAs are binned based on Euclidean distance to the closest mRNA of the other species of interest

Synapse Statistics

We seek to understand how often synapses are being served by mRNA. We assume that mRNA localizing near a synapse are servicing that synapse. We take advantage of the fact that PSD95 staining for a single synapse looks like a single mRNA spot and detect the position of synapses using FISHquant. When part2.py is run, the program will ask the user to define a colocalization threshold which will be used to determine whether a certain mRNA is localized at a synapse. Then the program loops through every synapse coordinate of each dendrite and counts the number of mRNA which are within a radius of the threshold distance. We record the number of synapses with X localized mRNA, for X equal to 0, 1, 2, 3, etc. As with the mRNA colocalization functionality, a computation control is required to interpret the statistical significance of the degree of synaptic localization. We generate fake mRNA coordinates, and again calculate the number of synapses being served by X fake mRNA.

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			

Figure 10. Example of synapse colocalization statistics: synapses are binned based on how many real or simulated mRNAs of interest are within its threshold radius

Simulation as Control

It is necessary to verify that the colocalization patterns (between mRNA and to synapses) observed are due to meaningful biological mechanisms (e.g., active transport of mRNA to synapse, co-transportation of different mRNA species) rather than restricted space and randomness. To do this, the program picks N fake mRNA sub-pixel coordinates uniformly random from each print, where N is the number of real mRNA in that dendrite. The program then uses these fake mRNA coordinates to calculate statistics in the same way it would with real mRNA coordinates. This simulation is repeated 100 times (50 times in the older version of the program), and averages of each statistic are taken. The simulation serves as computation control because if the simulated mRNAs show the same degree of colocalization as the real mRNAs, then colocalization patterns are likely due to having many mRNAs crammed into a restricted space. However, if the real mRNAs show strong colocalization, while simulated mRNAs do not, it suggests the existence of an underlying biological mechanism supporting mRNA localization.

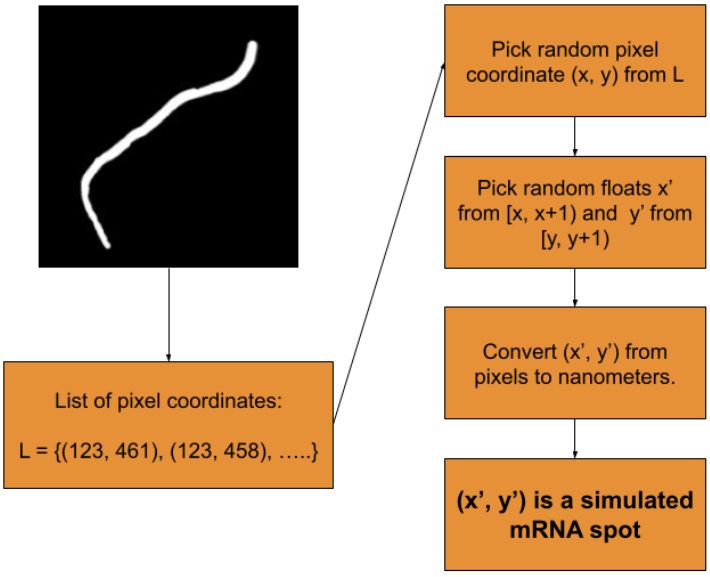


Figure 11. Steps used to uniformly randomly sample sub-pixel coordinates from dendrite print to use as simulated mRNA spots

Writing to Excel Files

The results of all distribution and colocalization analysis are organized into tables and then saved as excel files. The tabular results can then be used to generate plots for visualization and examination.

Disclaimer: The program layout below is for the newer modularized version of our code. An older version of the code was used to collect the data presented in the paper. Both versions used the same fundamental algorithms, but the older version was organized so that each functionality of part2 was in its own executable script.

References

Mueller, F., Senecal, A., Tantale, K. *et al.* FISH-quant: automatic counting of transcripts in 3D FISH images. *Nat Methods* 10, 277–278 (2013). https://doi.org/10.1038/nmeth.2406