**Instructions for Matlab Analysis Program that determines spatial patterns of HIEL and identifies puncta that localize to HIEL**

This code is intended for users experienced in Matlab code since analysis does require manual input from the user to optimize thresholding for each image. This code works best for high magnification images. Threshold specificity for HIEL will decrease as you decrease the objective magnification.

1. Create a folder on your desktop that includes all of the Example Matlab codes included and all images that will be used in the program. Below are the current names of the images included in the code.
   1. M9V7Z1\_hydr.png
      1. Use to threshold holes in the internal elastic lamina (HIEL)
   2. M9V7Z1\_PS.png
      1. Use to threshold puncta in endothelium
   3. M9V7Z1\_PS\_hydr.png
      1. Use to threshold puncta that overlap in HIEL
   4. M9V7Z1\_cldn5\_1.png
      1. Secondary fluorescent signal used to determine spatial pattern of HIEL. In this case, it is the interendothelial junctions.
   5. M9V7Z1\_DAPI\_cldn5\_PS\_hydr.png
      1. Merged image with all fluorescent signals.
2. Open all code files and save under a new name that is specific for your image.
3. Using “Find” replace all directories to your current folder for all code files.
4. Using “Find” function replace all “M9V7Z1” to be your unique image identifier for all code files.
5. Add a breakpoint at 642 in ExampleCode.m and click Run. This will threshold the first image. Adjust variables within the first section until the threshold is satisfactory. Nonspecific detection will be addressed in the next section, so the goal here is to make sure that all HIEL are detected in this first step, even if it includes some nonspecific detection
   1. Note that some HIEL may be dark and difficult to detect since the IEL is wavy. In these instances, open up the image in ImageJ, increase the brightness around those HIEL, save the new image, and repeat the thresholding.
6. If there are remaining HIEL that were detected nonspecifically, these can be removed manually beginning at Line 402. You must manually remove the nonspecific HIEL from each of the following variables so that the final data matrix can be formed correctly: G, F2, distance\_, X, Y, AA, C\_f2,r,d, and label.
   1. Suggestion: add all nonspecific HIEL to the first variable list (G). Next, copy and paste that list into a blank Matlab file and use the “find” function to switch “G” to “distance\_”. Repeat this process for all variables in that section. This will allow you to avoid typing in the HIEL numbers multiple times.
7. Look at the secondary fluorescent signal thresholding (claudin-5). Adjust thresholding parameters until the threshold is satisfactory.
   1. If needed, use the ImageJ line tool to trace over low intensity areas of claudin-5 signal at the interendothelial junction to facilitate correct thresholding. Background noise can also be removed from the middle of the cell in ImageJ.
8. Remove the previous breakpoint and add one at 774 (right before the start of section 5).
9. Run the code. This should now show you the thresholded IEL and claudin5 and the output images of the ExampleMonteCarlo.m, ExamplePositiveControl.m, and ExampleNegativeControl.m.
10. After the simulation plot generates and you are satisfied with it, comment out the plot sections in each of the simulation files so that plots don’t generate for every iteration later on.
11. Next, run the puncta detection section. Suggested to put a breakpoint at Line 900.
12. Adjust the levels of thresholding in order to detect the puncta that you are interested in. This involves changing the levels in the “thresh” variable” and then removing the thresholding levels that do not correspond to your puncta of interest in the “I4\_p” variable.
13. Run the code for the next two figures to check if the puncta thresholding is satisfactory.
14. Add a breakpoint before section 6 and run the code.
15. Look at the puncta figures and see if there are any detected puncta that are either nonspecific or are not actually overlapping with an HIEL (this could be the case based on the Threshold\_p value, which is the maximum distance that a puncta can be away from an HIEL to be considered overlapping) We have found that a Threshold\_p value of 0.75m accurately detects puncta in HIEL.
16. Remove unwanted puncta by adding the puncta numbers individually in the “TF1, TF2, etc” variable sections.
17. Remove all breakpoints and run the entire code.
18. You should now see output images of the ExampleMonteCarloPuncta.m, ExamplePositiveControlPuncta.m, and ExampleNegativeControlPuncta.m.
19. After the simulation plot generates and you are satisfied with it, comment out the plot sections in each of the simulation files so that plots don’t generate for every iteration later on.
20. Once everything is thresholded, change the number of simulations to be at least 100 (variables sim1 and sim2). Upload the code to a supercomputing cluster to run high numbers of simulations.
21. Final output variables for analysis are listed in Table X.
22. After analyzing multiple images, you can use the ExampleCombiningData.m code to combine variables from multiple analyses into a single matrix. Then, these matrices can be copied and pasted into a data presentation software such as Prism to run statisticial tests.
23. To detect spatial patterns, run a Brown-Forsythe and Welch ANOVA statistical test on distance\_avgMIN\_hist\_M2V1Z1A1, distance\_mc\_avgMIN\_hist\_M2V1Z1A1, distance\_pc\_1um\_avgMIN\_hist\_M2V1Z1A1, and distance\_nc\_avgMIN\_hist\_M2V1Z1A1.

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| **Description** | **Variable Name** |
| Number of HIEL in an image | length(Param\_M2V1Z1A2) |
| Average radius of HIEL in an image | (mean(Param\_M2V1Z1A1(:,3))/scale1) |
| Minimum distance of all HIEL to cldn5 | distance\_avgMIN\_hist\_M2V1Z1A1 |
| Minimum distance of all **MC** HIEL to cldn5 | distance\_mc\_avgMIN\_hist\_M2V1Z1A1 |
| Minimum distance of all **PC** HIEL to cldn5 | distance\_pc\_1um\_avgMIN\_hist\_M2V1Z1A1 |
| Minimum distance of all **NC** HIEL to cldn5 | distance\_nc\_avgMIN\_hist\_M2V1Z1A1 |

**Table X. Main output variables for downstream analysis.**