**Vascular zonation analysis of stitched retina images in Image J**

Purpose: Nuclear protein intensities such as Cdt1-mVenus and Gem-mCherry has already been extracted by masking with ERG from stitched images of each retina (background subtraction -> Z projection -> auto threshold -> ERG mask). Now we want to segment the entire retina into different vascular zones and quantify PIP-FUCCI levels in each EC nucleus from each zone. There are total 9 zones we defined:

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| Vascular zones | Abbreviation | Notes |
| Primary Artery | PA | Artery before branching into two arterioles |
| Arterioles | Art | Branches out directly from PA but before connecting to a capillary bed with uniform vessel diameter |
| Mature Capillary | MC | Capillaries in the mature region (behind angiogenic front) |
| Tip cell | Tip | A specific capillary EC at the very tip of a sprout and show filapodia |
| Angiogenic front Capillary | AFC | Capillaries within first 3-5 rows of endothelial cells of the angiogenic front AF |
| Venules | Ven | Branches out directly from PV but before connecting to a capillary bed with uniform vessel diameter |
| Primary Vein | PV | Vein before branching into two venules |

**Run image j script to get output jpeg images of each retina and csv files of IF intensities in each EC nucleus.**

1. Rename the stitched images with the format “litter-pup.oir” (no space in file name!!!) and move all stitched images into one folder named “stitched”. If you have multiple stitched files for a specific retina, rename all of them, e.g. “litter-pup\_00001.oir”, “litter-pup\_00002.oir”.
2. Make a new folder named “20x image j output”.
3. Start image j/Fiji, open the script file “Supplementary\_File\_1-Retina\_EC\_PIP-FUCCI\_int\_extraction\_by\_imageJ.ijm”. Click in the top bar “Run” -> “Run”, then in the new window popped up, select the folder “stitched” for “Choose image Directory”, then select the folder “20x image j output” for “Choose saving Directory”. The script will start to run, which takes a few to 15 mins and the results will be saved in the output folder. Make sure you have only oir files in the input folder and you don’t have space in any of the file names.
4. If the script stops in the middle, it’s usually due to out of memory of image j (your images are too big, e.g.,. when you have multiple stitched images for a retina). In this case, you will need to manually copy all the measurements in the “Results” window to a new csv file named with the retina numbers being measured. Basically, you “Ctrl + A” to select all measurements, and copy them into a new csv file. Now you need to go through image files in the output folder and rownames of the csv file/”Results” window, to find out which retinas have not been measured. Then make a new input folder, e.g., “stitched\_2”, copy the stitched images of the un-measured retinas into this new folder, and repeat step 3. You can still select the same output folder.
5. Make two new folders named “ERG” and “IB4” and move the corresponding output images ("xx\_ROI\_overlay\_ERG\_mask.jpg" and “xx\_IB4.jpg”) to those folders to help you do the polygons later.

**Vascular zonation:**

1. Go to the "ERG" folder, record tip cell ID for each retina in a new csv file “Tip\_cell\_ID.csv.” You can open the corresponding IB4 image of that retina to confirm sprouts in tip cells. Sometime you also need the ERG black and white images to confirm the intensity of ERG staining.

2. (Optional) Occasionally, vitreous vessels on the edge of retina and nonEC expressing weak ERG in the space between angiogenic front and edge of retina can be detected. If desired, we can exclude those nonEC and vitreous vessel EC from analysis. We can do that by manually draw one big polygon of the entire vascular region for each retina. First, make a new folder named “roi\_to\_exclude\_nonEC”. Then open image j and open the jpeg file of ERG mask one retina at a time, draw a polygon to exclude nonEC & vitreous vessels, rename the roi in the format “litter-pup” (one ROI per retina), then save rois for all retinas into one zip file under the new folder “roi\_to\_exclude\_nonEC”.

3. (Optional) Now we need to run an image j script to extract actual x y coordinates of all the ROI we drew, because later we need to feed these x y coordinates of each polygon into R to decide whether an EC is inside a specific polygon or not. First, we will make a new output folder “xy\_coordinates”. Then we need to open an IB4 jpeg image in image j for the script to work. Importantly, we need to open the image from the **largest** retina in this litter, otherwise if a polygon from a large retina goes beyond image boundaries of a small retina image, the script will report error. Now with the largest IB4 image open, open the image j script “Supplementary\_File\_2-Retina\_retrieve\_xy\_coordinates\_from\_polygon\_roi\_to\_exclude\_nonEC.ijm”. Click in the top bar “Run” -> “Run”, then in the new window popped up, select the folder “roi\_to\_exclude\_nonEC” for “Choose roi Directory”, then select the folder “xy\_coordinates” for “Choose saving Directory”. You should very soon find an output file called “Vacular\_Region\_polygon\_coordinates.txt” in the folder “xy\_coordinates.”

4. Now we need to draw polygons for vascular zonation. First make a new folder named “Vascular\_zonation”. Then open a IB4 jpeg image in image j, and use the pencil tools to drop red dots (size = 20, color = red) at branching points of arteries and veins to mark the separation between artery and arterioles and between veins and venules. Go to “Image”->”Overlay”->”Flatten”, then click “no”, then click on the new flattened image that popped out, go to “File”->”Save as”->”Jpeg” and save the image in the “Vascular\_zonation” folder.

5. Now we are ready to make polygons! First, make a folder called “ROI” under the “Vascular\_zonation” folder. Then on the IB4 image, select the polygon tool and make polygons for one PA, click t to add the ROI to ROI manager, then repeat this for all other PA, check “Show All” at the bottom of the ROI manager to see all the ROI. When done with PA for one retina **(!!! THIS IS CRITICAL FOR DOWNSTREAM R ANALYSIS!!!)**, we need to double check and make sure there is at least one EC in each polygon that was measured for nuclear intensity (cells can be excluded from measurement if there are multiplets). To do that, open in image j the “ROI\_overlay\_ERG\_mask.jpg" image in the "ERG" folder (the same one you used for finding tip cells but make sure to open it in image j not windows photo viewer). Then click “Show All” to see all PA ROIs overlaid on the image and make sure you see **at least one cyan (NOT WHITE) AND numbered EC nucleus in each polygon**. If you see polygons without any cyan nucleus, delete that ROI from the ROI manager. Now select all ROI left in the ROI manager, click “more” ->”Save”, and save all PA ROI in the “Vascular\_zonation/ROI” folder. Name it following this format “ROIsets\_litter-pup\_PA”. To save an image for record, go to “Image”->”Overlay”->”Flatten”, then click “no”, then click on the new flattened image that popped out, go to “File”->”Save as”->”Jpeg” and save the image in the “Vascular\_zonation” folder. Close the flattened image window. Now delete all ROI in the ROI manager then repeat this process for Art, Ven and PV, and AFC. **No need to do for MC** as all the leftover cells will be MC.

6. Now we need to run another image j script to extract x y coordinates of the ROIs we drew above, similar to what we did in step 3. Again, you need to open an IB4 jpeg image of the largest retina (this is important) in the batch of retinas you’re analyzing. Then you need to open in image j the script “Supplementary\_File\_3-Retina\_retrieve\_xy\_coordinates\_from\_polygon\_roi\_for\_vascular\_zonation.ijm” by “File->Open”. Click in the top bar “Run” -> “Run”, then in the new window popped up, select the folder “ROI” under “Vascular\_Zonation” for “Choose roi Directory”, then select the folder “xy\_coordinates” for “Choose saving Directory”. You should very soon find in the output folder “xy\_coordinates” a file called “Vacular\_Zonation\_Original\_polygon\_coordinates.txt”.

7. Finally we are done with image j! Now copy all **csv** files (hopefully your image j has enough memory and only generates one csv file) in the folder “20x image j output” **AND** the two **txt** files under “xy\_coordinates” into the folder where R analysis will be done.