**Step 1**: Get MIPs if you don’t already have them

**Step 2**: Extract just the IBA1 channel using the batch splitting macro in Fiji

Note: (CANNOT be done in OneDrive folder bc fiji doesn’t like filepath spaces)

**Step 3**: Load a few representative sample images into Ilastik to start the training process

**Step 4**: I always like to train on nuclei first, to get raw counts for the microglia

* Make an ilastik project named something like “Iba1 Nuclei Output”
  + This should be a “Pixel + Object Classification” project
  + Try to train the program to recognize processes in one color, background in another, and the bright nuclei/somas of the microglia as a third color.
  + You will then want it to count the somas as object in the object classification.
  + You can then batch this on all your images, and it should output the object identities of each image. Ask someone for help, or write your own python script that will combine all the outputs for analysis.

Once you have the output from this step, move them into a folder labelled “Nuclei” and move on to the next step, which is making for TMEM or other homeostatic proteins!!

**Step 5**: Binary mask segmentation

* For this one, make a separate project and name it somewhere in the realm of “Mgla Mask IBA1”
  + This project will be just a “Pixel Classification”
* You can use the same Iba1 MIP images from the nuclei thresholding
* Make sure you output as a simple segmentation, and a tif. Batch process all files and move the output tiffs into a folder labelled “simple\_segmentations”

**Step 6**. Run the “MASKING SIMPLE SEGMENTATIONS.ijm”Fiji Macro

* + \*NOTE\* there may be issues for importing the files into fiji based on your filenaming conventions. To remedy this, adjust the numbers in the parenthesis in lines 31, 35, and 64. You want those index locations to include the full filename of the original MIP files minus the .tif file extension. Ask Ben if you have questions about this
    - Also, the script is written such that it will print out each filename as it goes. Double check that it is predicting the names correctly based on the names of the images that you have in your simple\_segmentations folder.
  + Also, you may need to change which channels you want to measure from. For example, if you have DAPI in channel 1, TMEM119 in channel 2, and Iba1 in channel 3, you will want to only readout from channel 2 since that is what you are trying to measure. This can be adjusted by changing the “ch\_nbr” variable in the script. It is provided with 4 blocks of code that tell it to measure all 4 channels. Only include the chunks that you need.
* It should run through all the images in the folder, and you will now have all the csv output files in the main directory.
* Once again, ask someone for help or write your own python script that will combine all the output for analysis. I have provided an example script here to work off of.