Downloading ImageJ (Fiji Package) and necessary plugins

Installing Fiji

1.) Go to https://fiji.sc/#download and select the installation for your OS
   a. The following uses Windows, so Mac or Linux may have slight variations
   b. Fiji is essentially an ImageJ package that comes with various plugins preloaded
2.) Extract the folder to your preferred destination
3.) ImageJ should now be installed
4.) Open Fiji by clicking the ImageJ icon
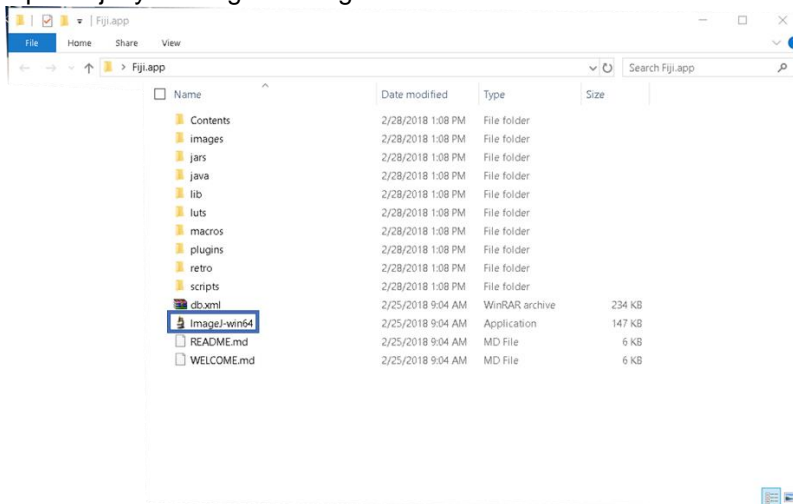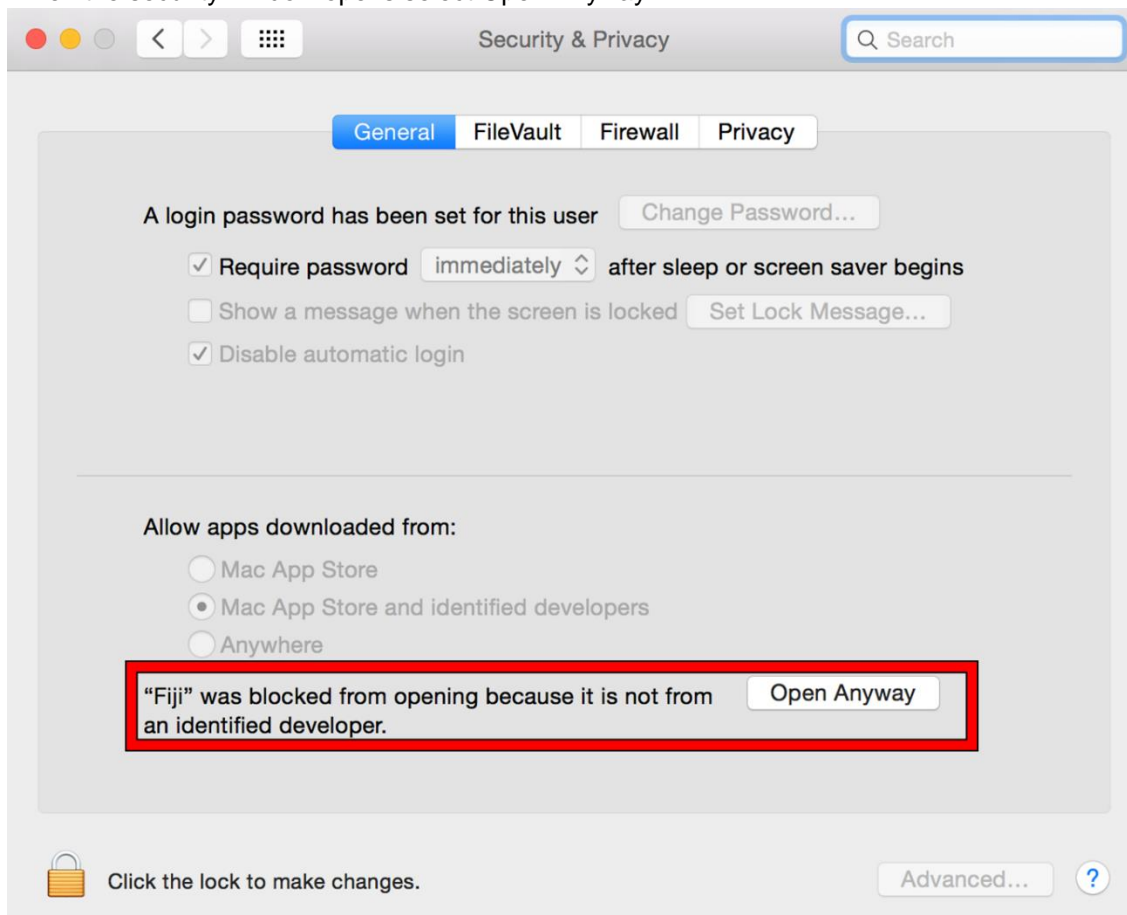

5.) The ImageJ program should open the following:
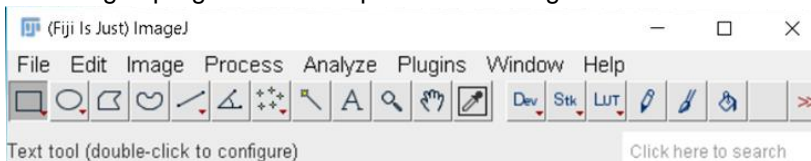
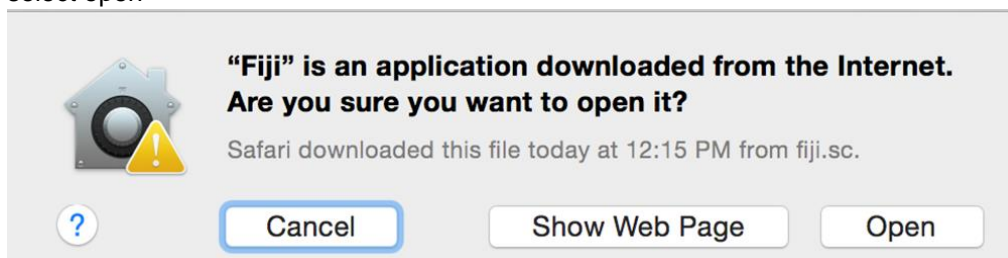6.) A common issue with Mac users is the following error when attempting to open Fiji

7.) To resolve this issue go to your system preferences and select Security and Privacy

8.) When the security window opens select Open Anyway

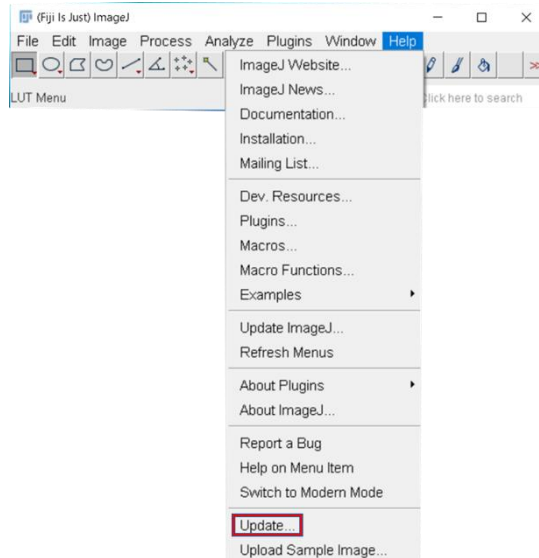9.) Open the Fiji application. A new window may appear asking if you are sure want to open Fiji, select open
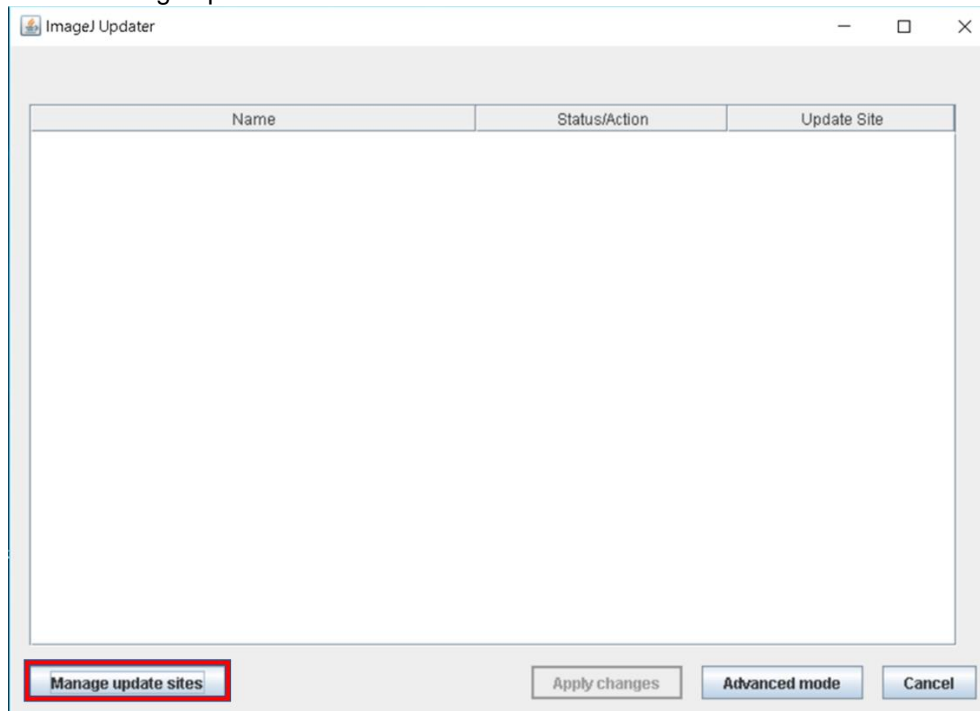
10.) ImageJ is now ready to be used

Installing ImageScience plugins (NeuronJ)

Fiji allows users to select some packages to install and update without having to manually place them in the plugins folder
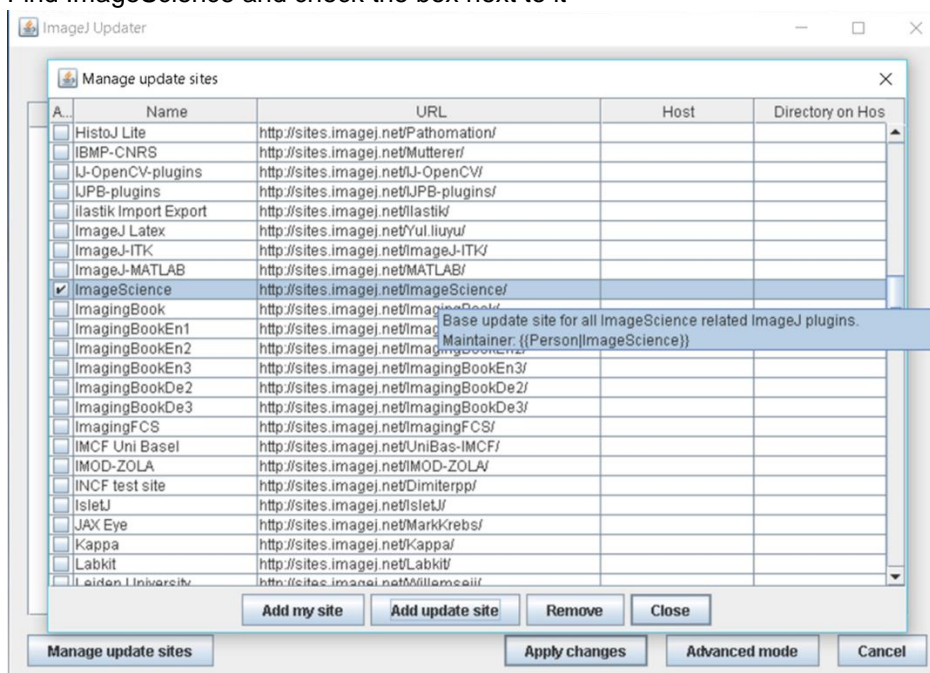
1.) Open Fiji
2.) Go to help, update

3.) Select manage update sites

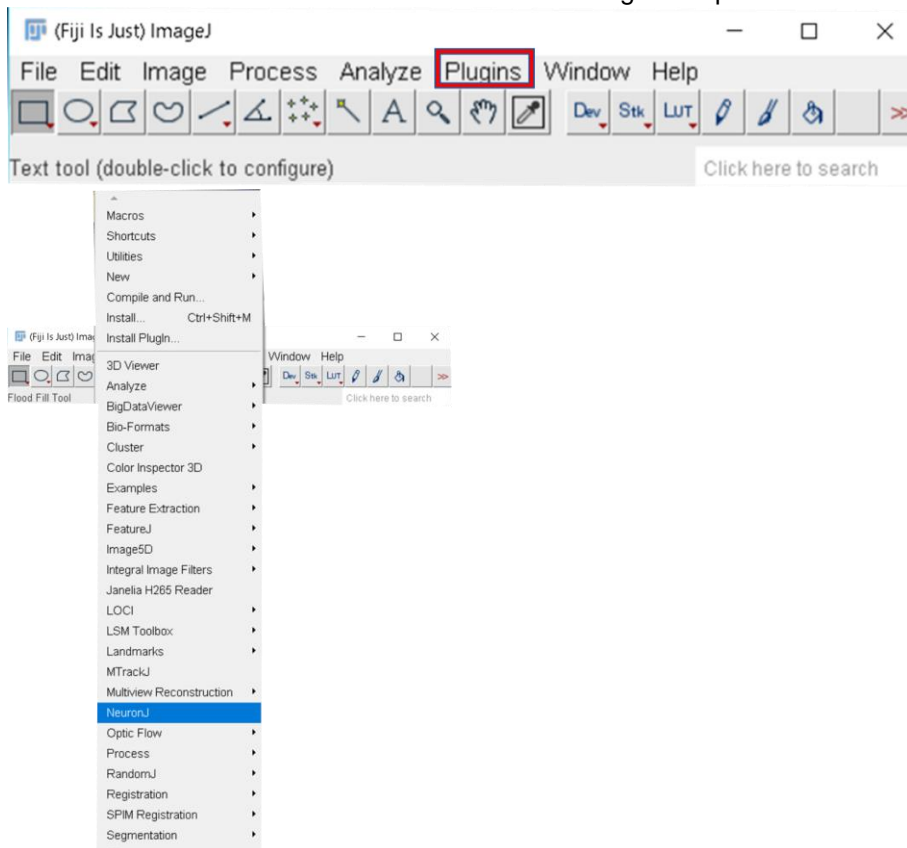4.) Find ImageScience and check the box next to it


5.) Select close
6.) Select Apply Changes
   After the changes have been installed, restart Fiji
7.) Ensure NeuronJ is now available to select in the Plugins drop-down menu
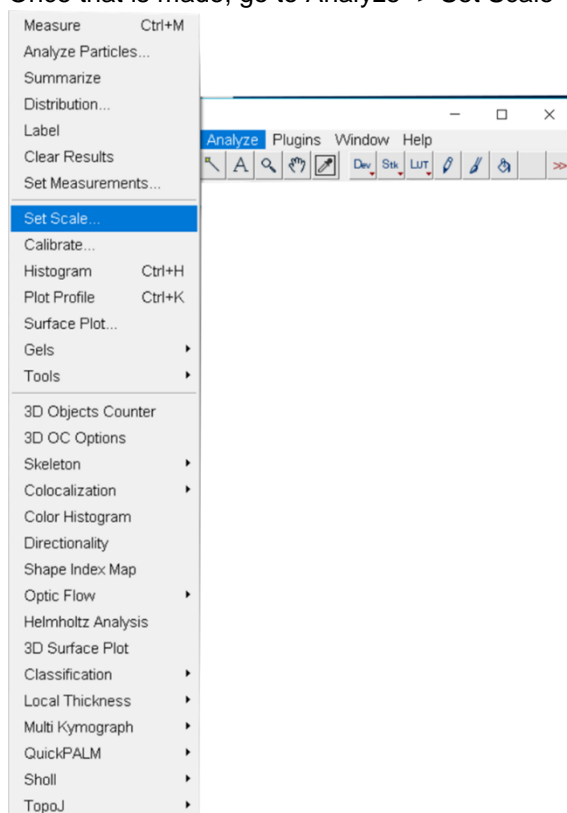
Calibrating distances in ImageJ

1. Open ImageJ
2. Open an image with known distance markers that was taken on the same setup and the same magnification as the image you will be analyzing
    a. We use a hemocytometer
3. Select the straight line from the toolbar
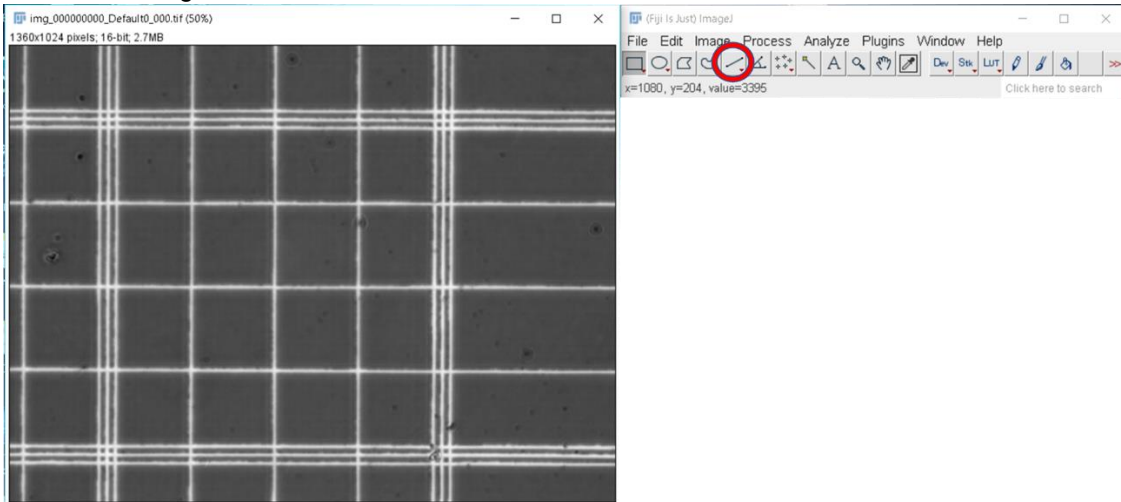
4. Make a straight line from one point to another of known distances (yellow line)
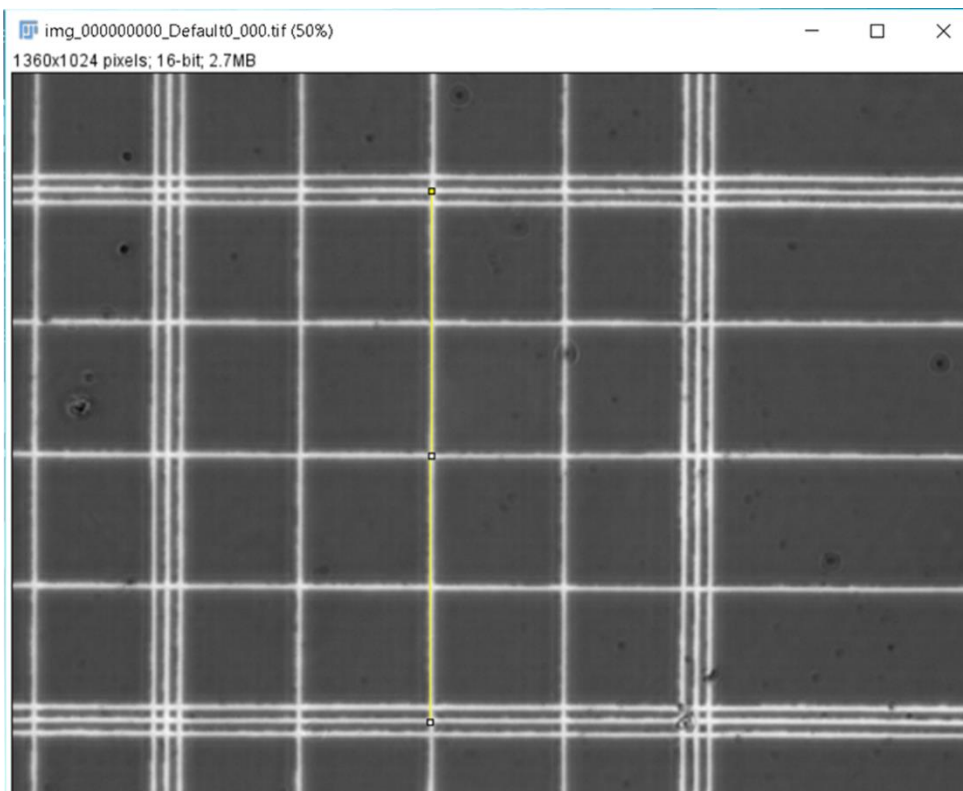
5. Once that is made, go to Analyze -> Set Scale

6. Enter your known distance and unit of length (um for micrometers) and check Global
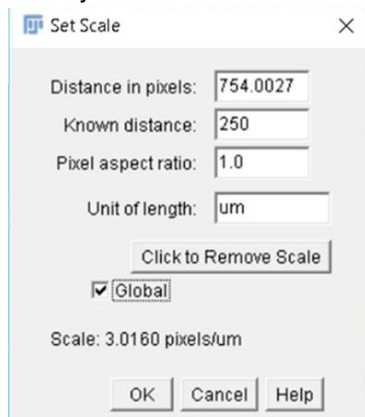
7. Select OK
8. When you open another image, if a dialogue box opens asking to disable global calibration, unselect that box


Counting in ImageJ

1. Open ImageJ software
2. Open an image that will be traced

3. Adjust the image as needed to clearly visualize the what you are counting (e.g. the contrast needs to be changed because you cannot distinguish if something is one cell or more)
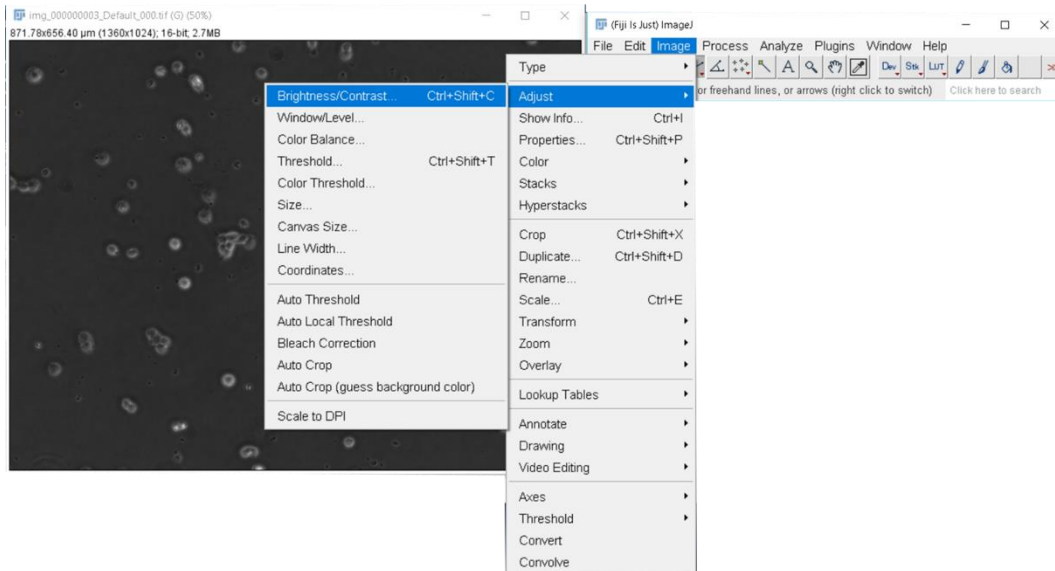
a. Go to Image -> Adjust -> Brightness/Contrast (or press Ctrl+Shift+C in window)
b. This will open another window with a graph, 4 sliding bars (minimum, maximum, brightness, and contrast), and four buttons (Auto, reset, set, apply)
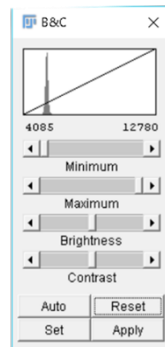
c. Change the minimum and maximum values to a point where the neurite stands out, but the cell body is not overly bright as to hide neurites or blend cells bodies together
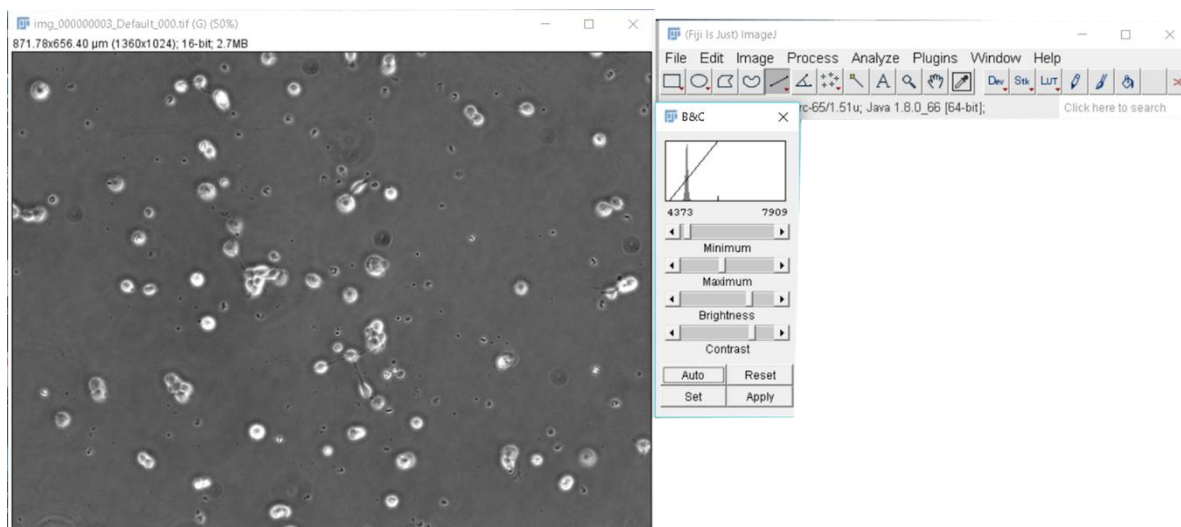
      d.   Once the desired contrast is obtained, press Apply

      e.   If adjustments are made, save the new image with the original name-CellCount

4.   Go to Plugins -> Analyze -> Cell Counter -> Cell Counter
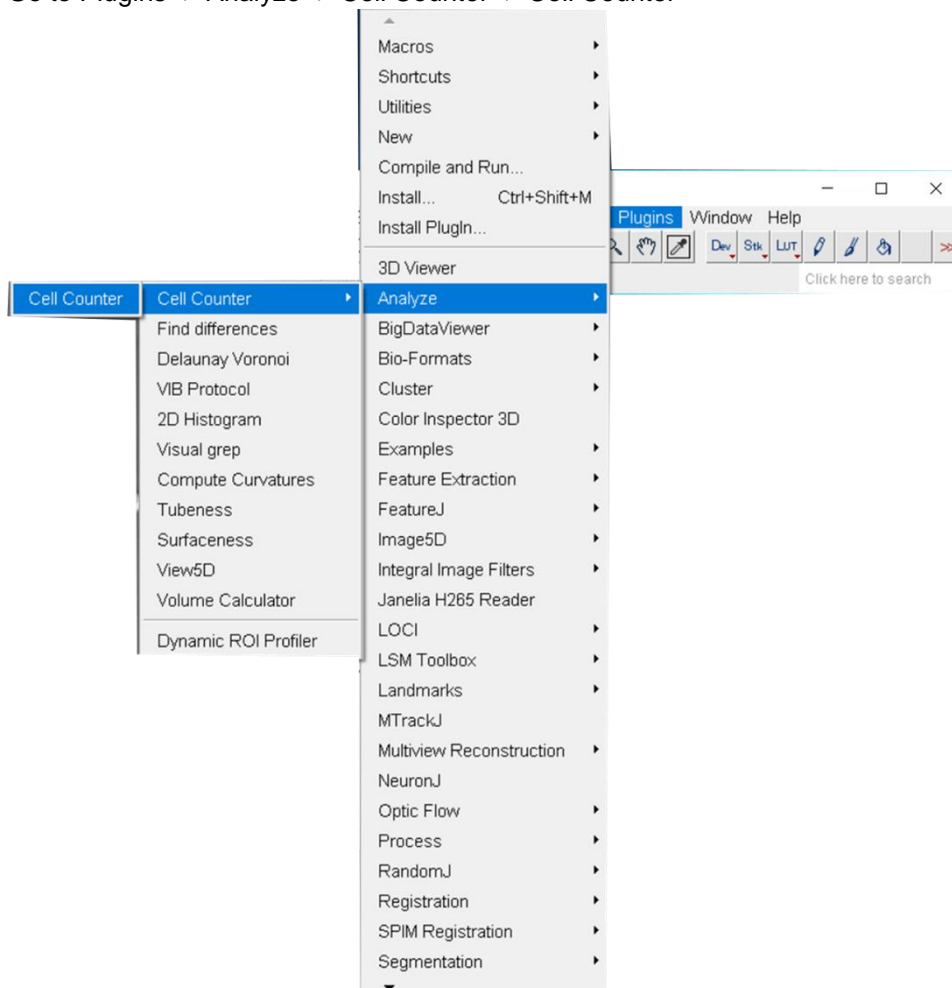
5. Click on the Initialize button
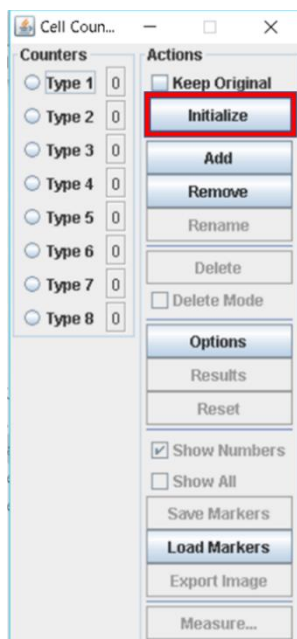
6. If observations other than cell number are going to be counted, you can select different "types" for different counts
   a. These can be labeled differently than 'type #' by selecting the type and pressing the rename button
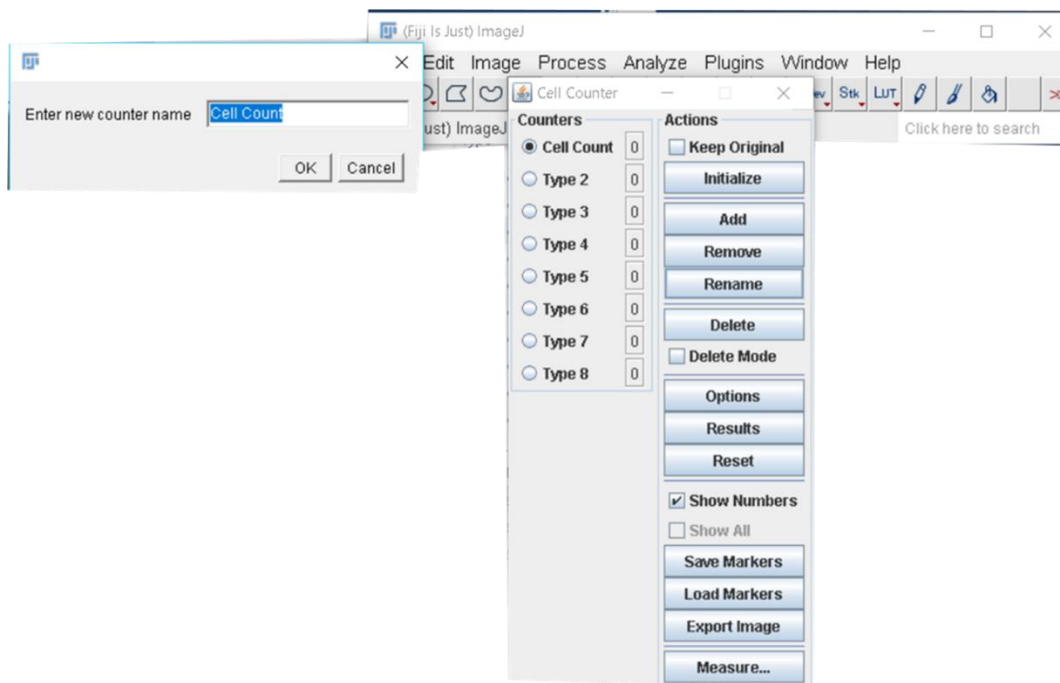
7. After selecting the type, simply click on the spot that is being counted. This should place a colored number (blue here) on the selected spot, increase the number next to type by one, and put a number where you clicked
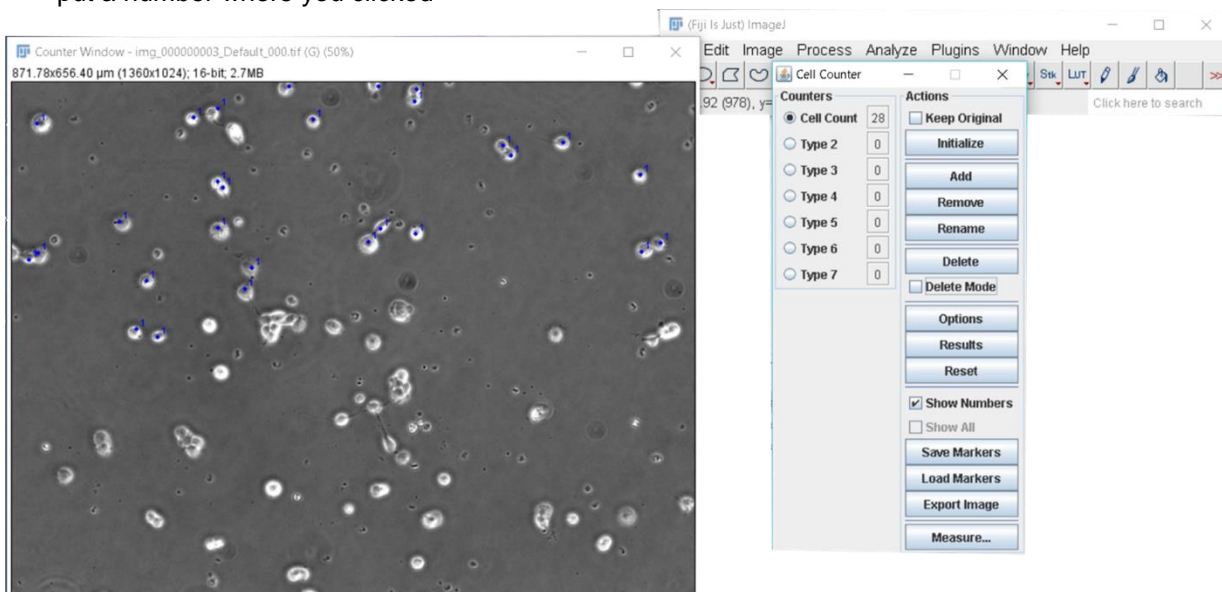
8. If mistakes are made, you can check the Delete Mode box and select the spot to be deleted.
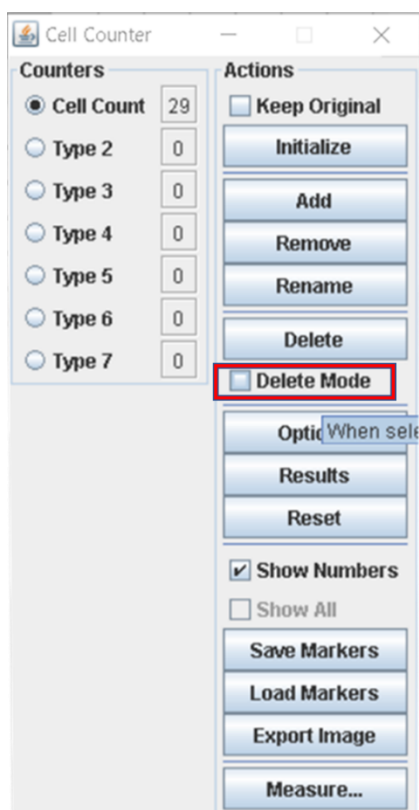
9. You can then Save by selecting Save Markers

Preparing phase contrast images for neurite tracing (Neuron J)

1. Open ImageJ software
2. Open an image that will be traced
3. Adjust the contrast if necessary to ensure the neurites stand out against the background as described previously
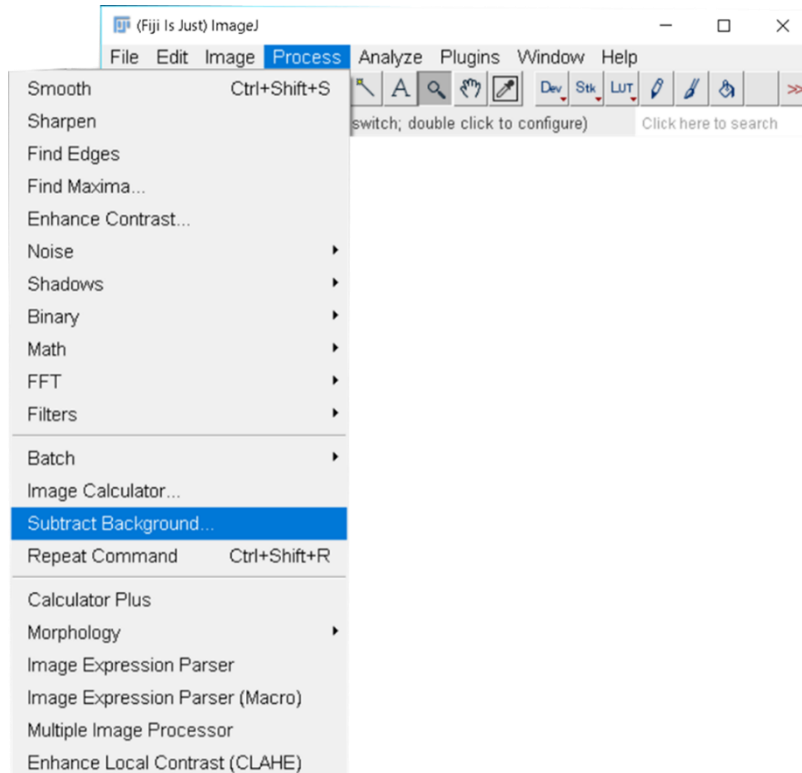4. For the program to trace the neurites most efficiently, the background should be as different as possible from the neurites.
5. Go to Process -> Subtract Background

6. A window will appear that has a manual rolling ball radius, and check boxes (Light background, Create background, Sliding paraboloid, Disable smoothing, Preview)
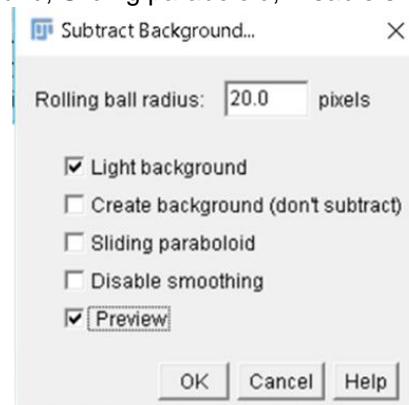
7. Check the boxes Light background and Preview (the others should not be checked)
8. After the preview is checked, you should see what your image looks like if the current rolling ball radius is applied

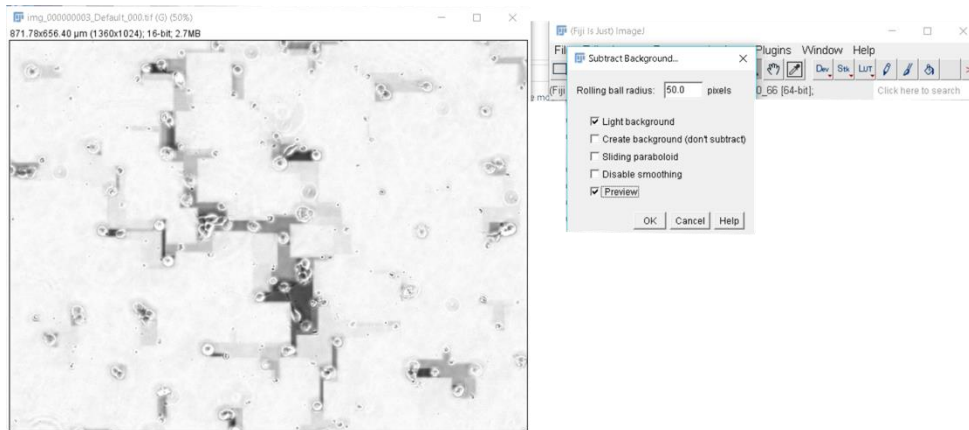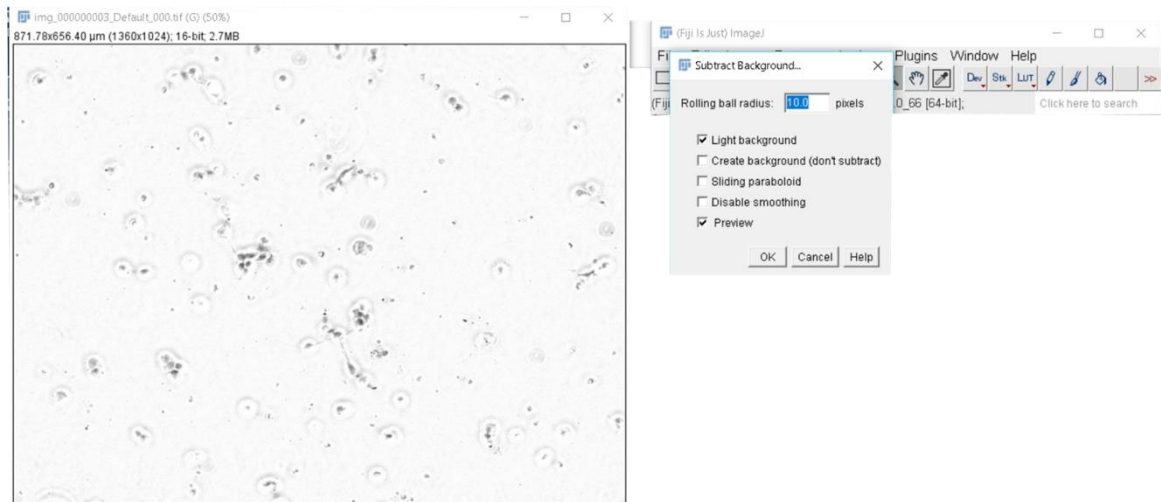a. If there is a high density of neurites and/or cells or the rolling ball radius is too large, there may be grey shades in areas that should be background

b. If there is a low density or the rolling ball radius is too small, some of the smaller neurites may have disappeared

9. To fix either of these issues, change the rolling ball radius
    a. Lower to get rid of the grey shades and higher to bring small neurites back
    b. Unless everything is perfectly spaced, you will likely have to leave some patches of grey background so neurites do not start disappearing. That is fine, just make sure you can clearly see where the neurites and cells are. You can overlay images to double check everything at a later time.
    c. A good range for the radius is usually 10 – 30 pixels depending on cell and neurite density
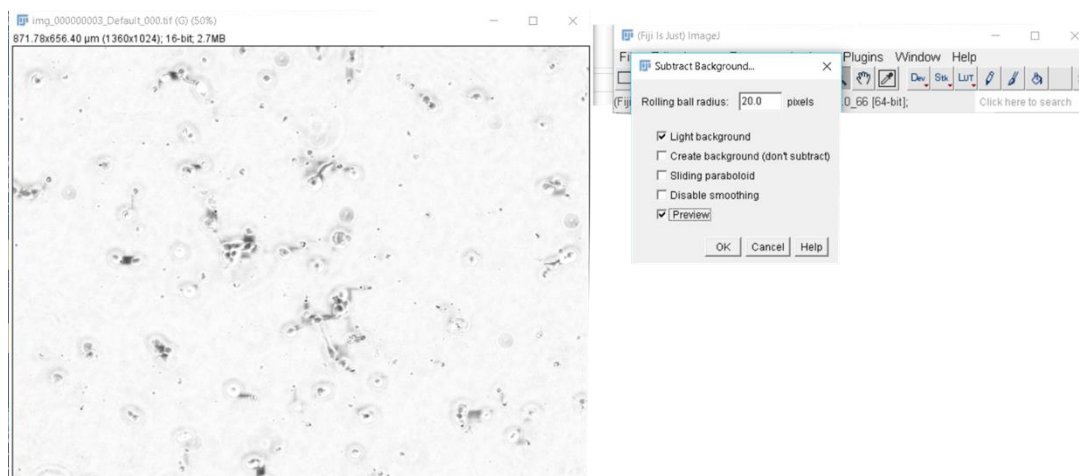
10. Select OK
11. Go to Image -> type and ensure that 8 bit is selected
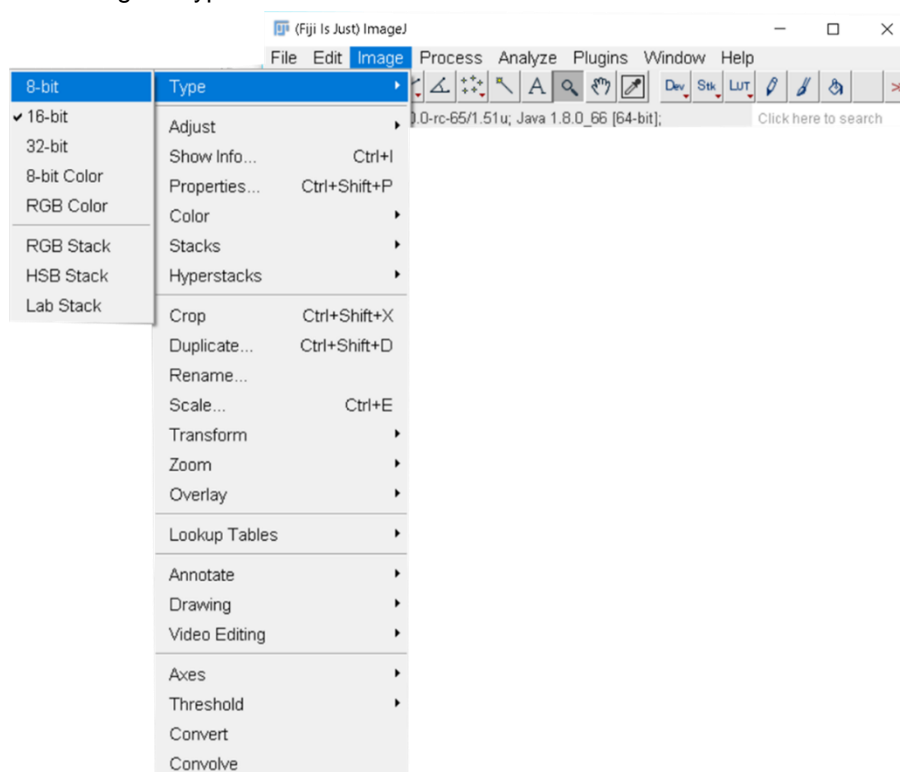
12. Save this image with the original file name + SubBack
    a. Do not overwrite the original image; you always want to have the original + any that you have made changes to.
    b. SubBack just means subtract background
13. Do this for every image
14. Now they are ready for neurite tracing
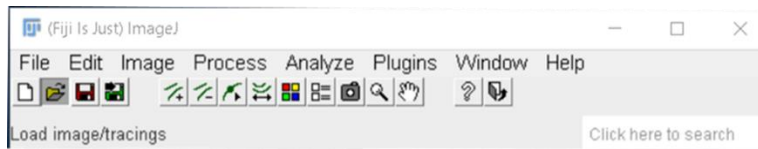

Neurite Tracing in NeuronJ

At any point to save your tracings, select the save tracings icon on the toolbar (red floppy disk)

1. Open ImageJ software that has NeuronJ plugin installed.
2. Go to plugins -> NeuronJ
   a. This will change the toolbars on the ImageJ window

   b. For an explanation of the toolbar icons, go to the NeuronJ manual (https://imagescience.org/meijering/software/neuronj/manual/)
3. Open the image you wish to trace from the NeuronJ toolbar 'Load image/tracings'
   a. If you open the image in ImageJ and not from the NeuronJ toolbar, it will not work
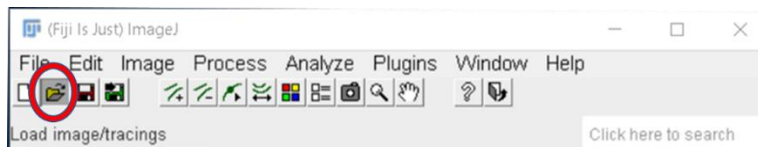
4. Select the Parameters button from the toolbar
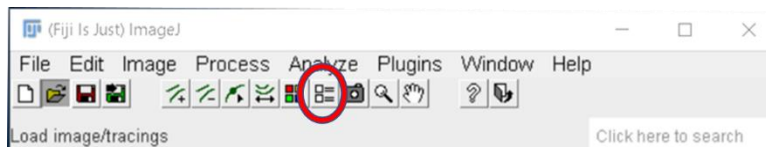
   a. Ensure the neurite appearance is dark if using phase contrast, light if fluorescent from the drop down, and select automatically save tracings
   b. Select save
5. If you want to zoom in for easier tracing, select Zoom in/out
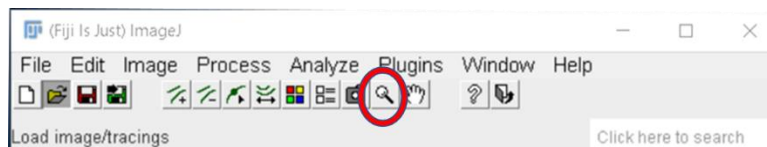
   a. Left click to zoom in and right click to zoom out in Windows
   b. After zooming in, you can select the hand from the toolbar to move around in the image. There should be a square with a smaller square inside on the top left corner of the image that shows you what you are seeing relative to the whole image
6. Select the add tracings button on the toolbar (has green lines and a +)
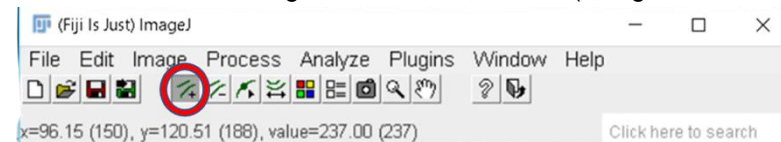
7. To begin tracing a neurite, left click on the area the neurite begins

871.78x656.40 µm (1360x1024); 8-bit; 1.3MB
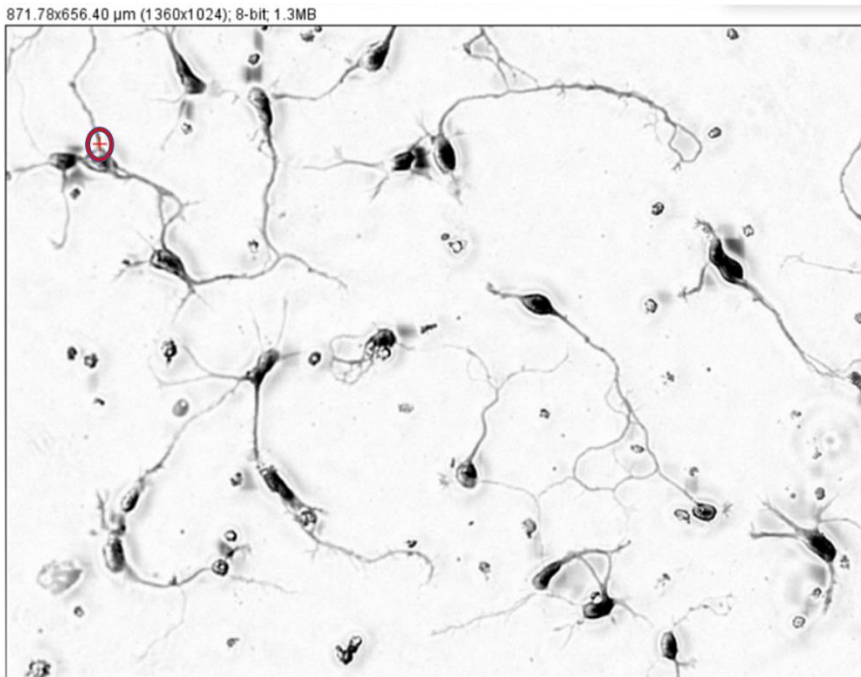
8. Follow the path of the neurite, and then double click the area where the neurite terminates

871.78x656.40 µm (1360x1024); 8-bit; 1.3MB

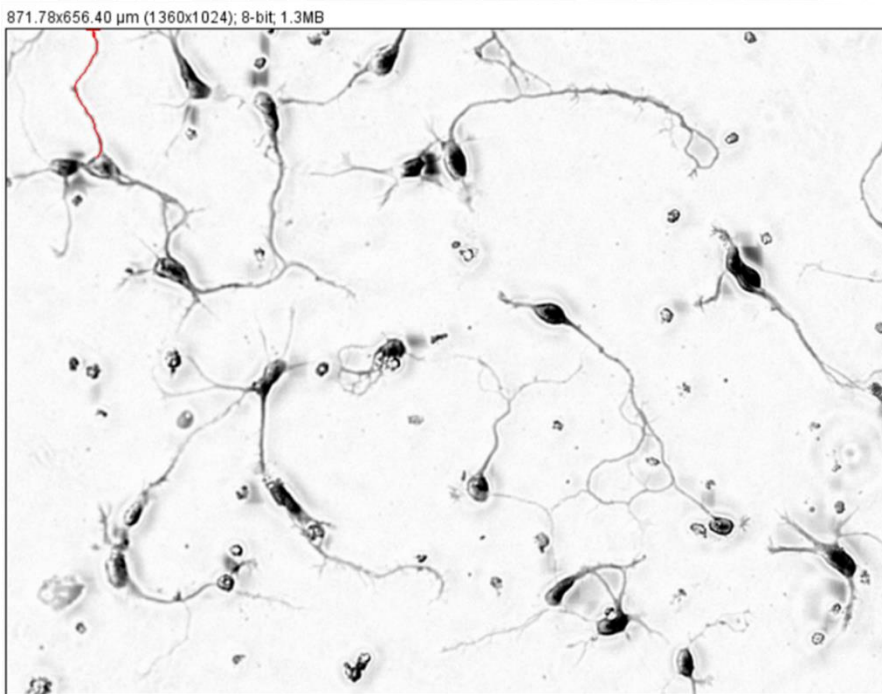   a. If the trace is not following the correct path at a certain point, you can make anchor points. A single click will create an anchor point for the neurite, so the program will not try to take a different path than what you wish to trace

9. Since most neurites are not just a single primary neurite, meaning they have branches, the branches must be measured from their originating neurite accurately.

10. If a mistake is made, you can delete incorrect traces by selecting the delete traces button and clicking the trace to be deleted
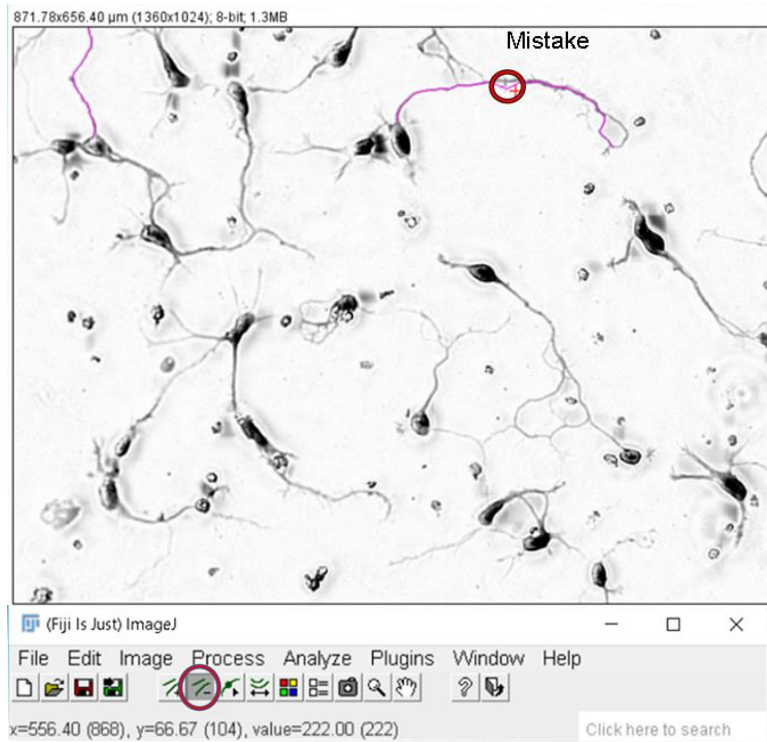
a. The program will ask you if you wish to delete that trace, select yes

11. To correct this mistake, begin tracing the neurite then create an anchor point at the furthest point the trace is correct, then continue tracing
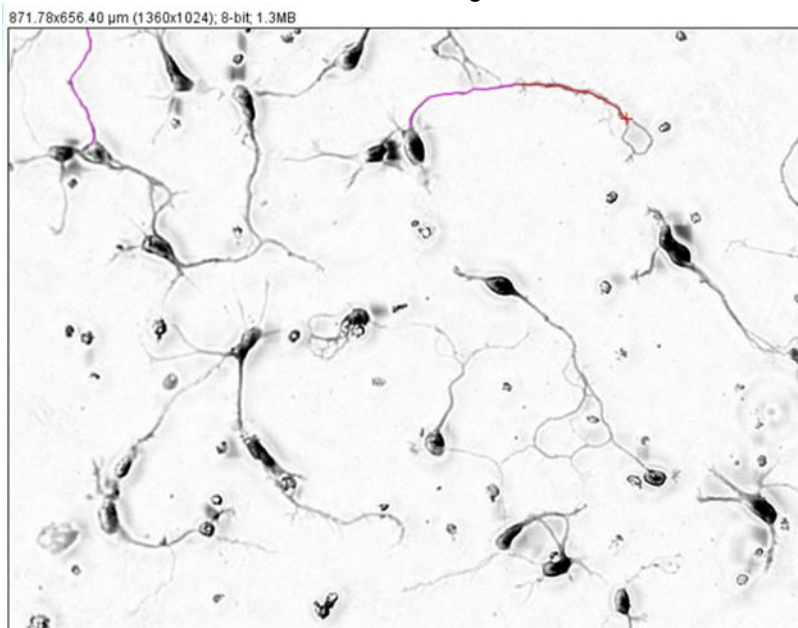
12. At this point, neurites can begin to be labeled. Select the label tracing button

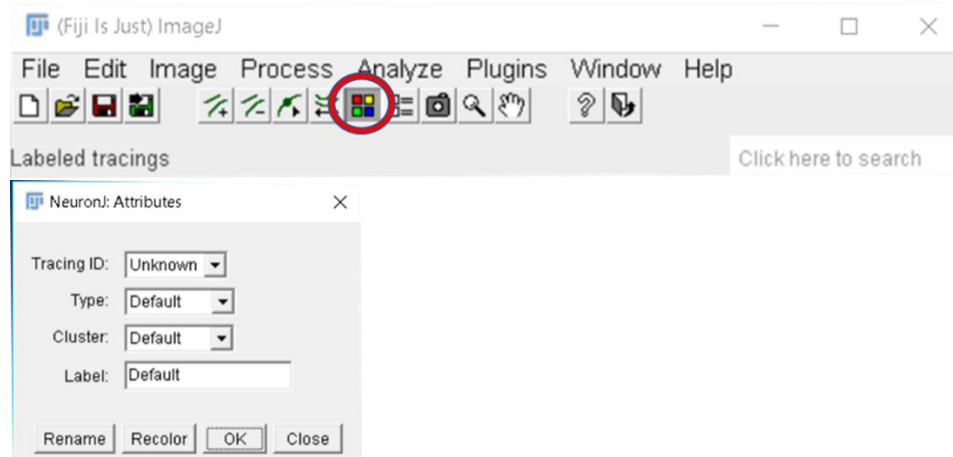13. Tracings can be selected in two ways. The Tracing ID drop down menu allows you to select a single tracing. You may also select the tracing by clicking on the tracing, which allows selecting multiple tracings at once
    a. Note that if you are selecting multiple tracings, this will cause all selected to have the same properties once you hit OK. So you if you already have primary and secondary type neurites labeled and want to put them into cluster 1, you have to do that separately for each type.
14. Label the selected tracings as needed for your experiment, and select OK after each to apply the label. In this example, primary neurite type is given, and the neurites are labeled by cell number
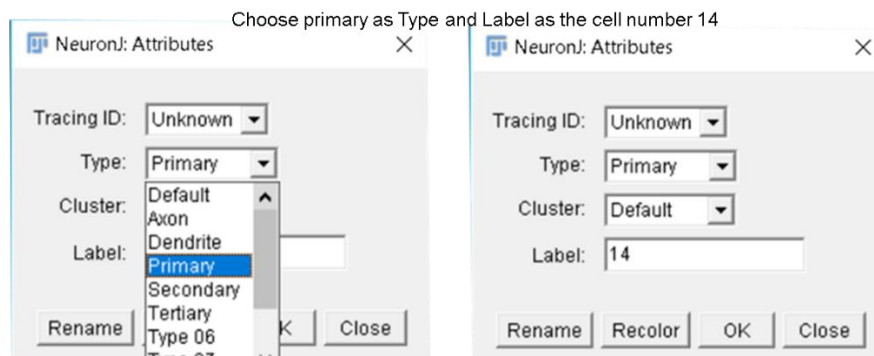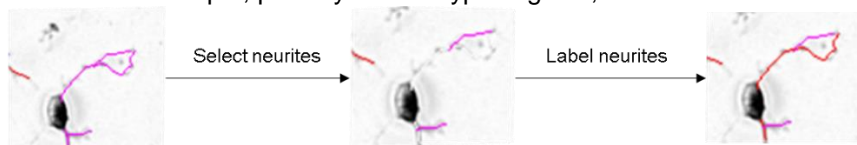
15. Once all neurites are traced and traces are labelled, you can measure tracings by selecting the Measure tracings icon
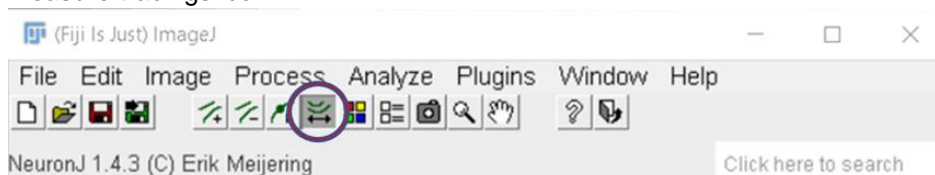
   a. This brings up a window that allows you to select the tracing type and cluster (if they have been labeled)

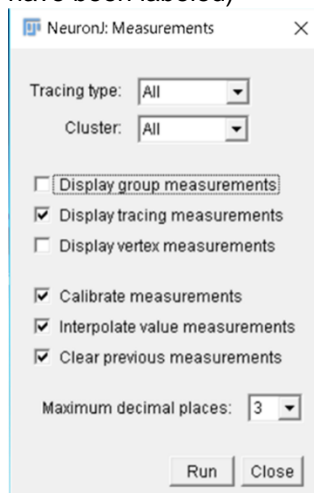16. Depending on what exactly you are measuring in your experiment, what you have selected will differ. Tracing measurements and group measurements are typically the most useful

17. Make sure that the numbers that are in the measurements window are in μm and not pix
   a. If the measurements are in pixels, you must do a global calibration in ImageJ as described previously

File Edit Font

Image	Tracing	Cluster	Type	Label	Length [μm]	Mean Val [a.u.]	SD Val [a.u.]	Min Val [a.u.]
	N1	Default	Primary	1	156.75	225.607	25.644	146
	N2	Default	Secondary	1	12.511	214.19	12.844	188
	N3	Default	Primary	1	48.964	188.368	21.009	132
	N4	Default	Secondary	1	17.013	224.923	20.702	179
	N5	Default	Primary	1	85.248	190.61	29.58	96
	N6	Default	Primary	1	20.909	85.742	37.714	0
	N7	Default	Primary	2	134.546	172.67	30.008	89
	N8	Default	Primary	2	106.069	152.02	26.428	84
	N9	Default	Secondary	2	24.069	208.694	18.257	169
	N10	Default	Primary	2	20.841	146.29	35.919	55
	N11	Default	Primary	4	66.692	147.287	33.367	42
	N12	Default	Primary	3	29.954	139.739	34.237	72
	N13	Default	Secondary	3	40.086	198.82	22.725	150
	N14	Default	Secondary	3	11.849	179.524	18.79	144
	N15	Default	Primary	3	34.854	215.863	18.282	153
	N16	Default	Secondary	3	58.111	207.686	34.181	123

18. Save and export all necessary data into excel or other programs for further analysis