Ridwaan Joghee – University College London – Macro Publishing – Macros Written by Ridwaan M. Joghee

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TREM2 Expression and Microglial Density Analysis

Significance

In Alzheimer's disease, abnormal levels of the Aβ protein aggregate together to form Aβ plaques. The response to plaques by microglial cells is characterised by changes in gene expression and an increase in cell density surrounding plaques. This set of semi-automated macros calculates both microglial density and changes in protein expression intensity within concentric circles drawn every 10µm from plaques. The macros used in this analysis were scaled for images taken on a 20X objective. The macros can be edited appropriately.

Plaque Thresholding Macro

This macro reduces the threshold value of the plaque image stepwise whilst calculating the corresponding percent coverage value. Then, plotting the threshold values against percent coverage, one can calculate the correct value for to threshold your plaque image, this value is necessary as an input for the Plaque ZIP File Macro.

Instructions

This macro requires the 'Read and Write Excel' plugin for ImageJ. Make sure the code is edited to suit the scale of your image and will save the excel sheet in the right file path.

1. Load the macro as an .ijm file & load the plaque image as a .tiff image into ImageJ.
2. Run the macro.
3. Using the saved excel sheet for each threshold value, calculate the percent coverage of the thresholded area to the total image area.
4. Using GraphPad prism (or another analysis software), plot threshold value against percent coverage. Draw a line of best fit between threshold values 30-45 (plateau).
5. The smallest value that remains on this line of best fit is the chosen threshold value for the plaque image. Save this number for use in the next macro.

Code

```
run("Restore Selection");

run("Set Scale...", "distance=1 known=0.378 unit=um global");

run("8-bit");

run("Set Measurements...", "area display redirect=None decimal=3");

run("Measure");

Title=getTitle();

run("Read and Write Excel", "file=[enter file path/ enter chosen file name.xlsx] sheet="+Title);

run("Clear Results");

for(i=6; i<45; i++)

{

thresholdValue=i+1;

run("Threshold...");

setThreshold(thresholdValue, 255);

setOption("BlackBackground", true);

run("Analyze Particles...", "size=10-Infinity display exclude");

n=nResults;
```

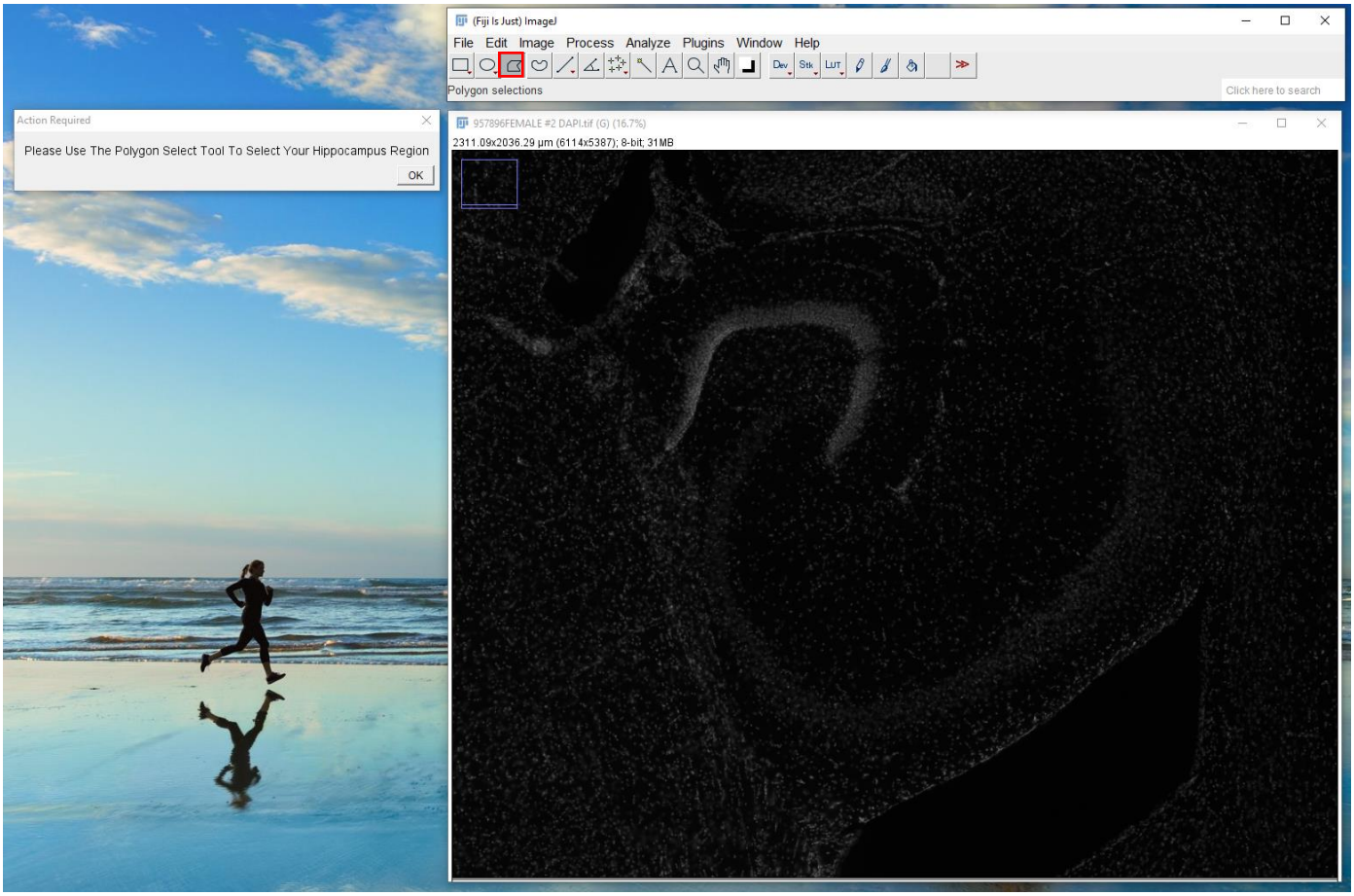
```
for(l=0; l<n; l++)
{
setResult("Label", l, thresholdValue);
}
Title=getTitle();
run("Read and Write Excel", "file= [enter file path/ enter chosen file name .xlsx] sheet="+Title);
run("Clear Results");
}
```

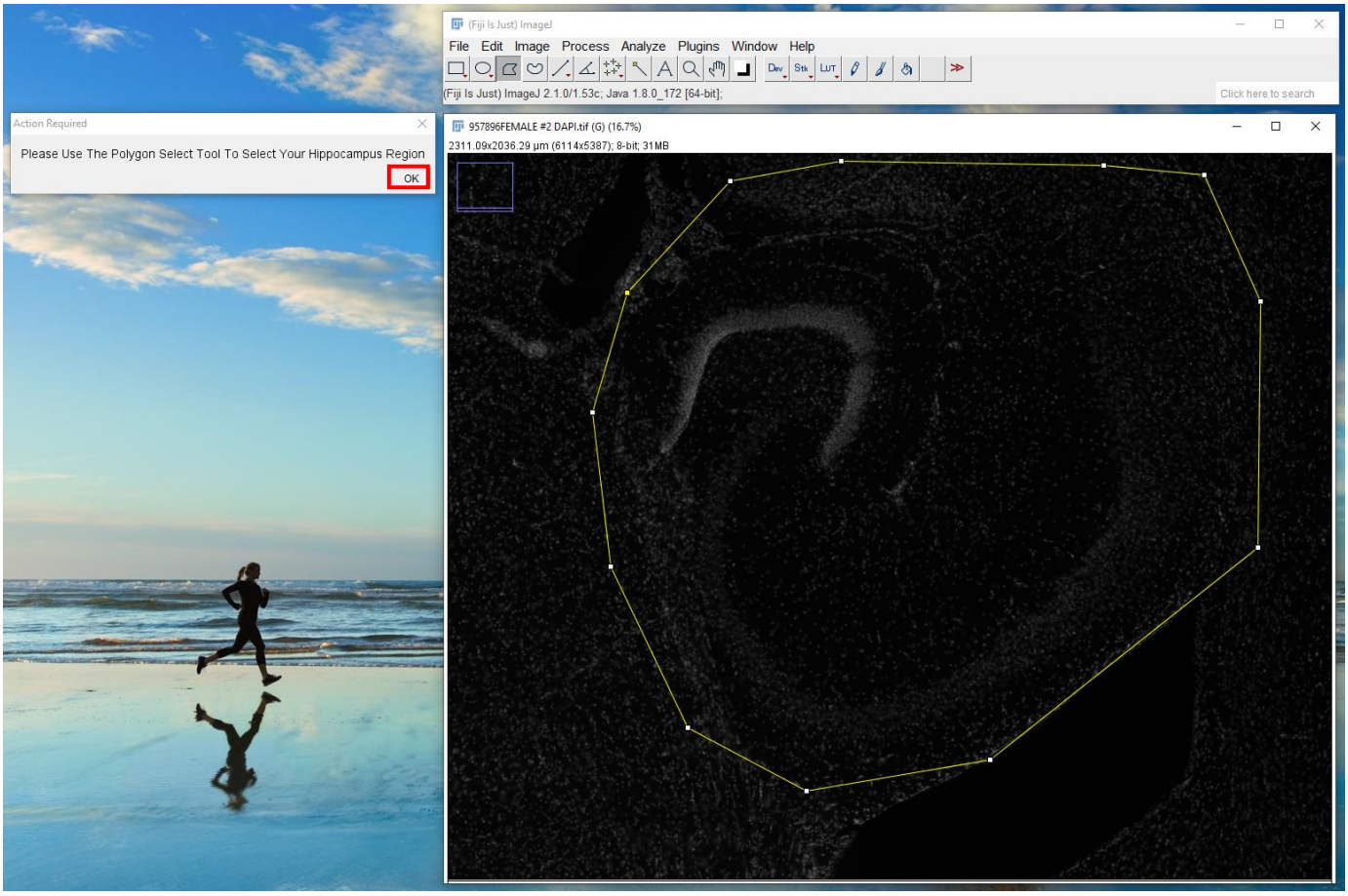
Plaque ZIP File Macro

The plaque/pseudoplaque ZIP file macros calculate plaque area and for each plaque in the image will precisely draws an initial ROI around the plaque circumference. Then, every 10µm, concentric circles are drawn outwards equidistantly until it reaches another plaque. Here, the concentric circles are stopped at half the distance between the two plaques. This enables quantification within each plaque territory to be calculated individually with minimal overlap with other plaques. The values outputted by this macro are in µm2 with the assumption that 1 pixel is equivalent to 0.378 µm2.

Instructions

1. Open FIJI
2. Load macro on FIJI – *Plugins > Macros > Run*
3. Open hippocampus (DAPI) image
4. Use polygon tool to select hippocampus region – press ‘OK’





5. Open plaque (AMYTRACKER) image and enter threshold value *(check how to threshold plaque image above)*
6. Click 'OK' and wait whilst plaque image is processed, and concentric circles drawn
7. Save RoiSet on ROI Manager to create a ZIP file - Ctrl+A > More > Save *(check how to do this above)*

Code

```
print('Please Open Hippocampus (DAPI) Image');

run("Open...");

run("8-bit");

setOption("BlackBackground", false);

run("Set Scale...", "distance=1 known=0.378 unit=um global");

setTool("wand");

waitForUser("Please Use The Polygon Select Tool To Select Your Hippocampus Region")

roiManager("add");

print('Please Open Plaque (AMYTRACKER) Image');

run("Open...");

run("8-bit");

setAutoThreshold("RenyiEntropy dark no-reset");

temp1=getNumber("Enter Threshold Value",46)

setThreshold(temp1, 255);

setOption("BlackBackground", false);

roiManager("Select", 0);
```

```
run("Clear Outside");
roiManager("Delete");
run("Convert to Mask");
run("Set Scale...", "distance=1 known=0.378 unit=um global");
setTool("wand");
run("Analyze Particles...", "size=10-Infinity show=Masks display exclude clear in_situ");
setThreshold(1, 255);
setOption("BlackBackground", false);
run("Convert to Mask");
run("Set Scale...", "distance=1 known=0.378 unit=um global");
setTool("wand");
run("Analyze Particles...", "size=10-Infinity show=[Overlay Masks] display exclude clear add");
numROIs = roiManager("count");
arrayX = newArray(numROIs);
arrayA = newArray(numROIs);
arrayY = newArray(numROIs);
arraylength = newArray(numROIs);
for(i=0; i<numROIs;i++) {roiManager("Select", i);
run("Set Measurements...", "area centroid stack limit redirect=None decimal=3");
run("Measure");
A= getResult("Area");
rad=A/3.14;
rad=Math.sqrt(rad);
rad=rad/0.378;
X=getResult("X");
Y=getResult("Y");
X=X/0.378;
Y=Y/0.378;
arrayX[i] =X;
arrayY[i] =Y;
arrayA[i] = A;}
nmaxroi = roiManager("count");
for(i=0; i<numROIs;i++) {
A = arrayA[i];
rad=A/3.14;
```

```
rad=Math.sqrt(rad);
rad=rad/0.378;
X=arrayX[i];
Y=arrayY[i];
setTool("wand");
doWand(X, Y);
um10_step =54;
um10=um10_step;
run("Measure");
num = i+1;
string1 = "Plaque"+num+" "+0+"um" ;
Roi.setName(string1);
roiManager("Add");
lengthMeasurements = arraylength[i];
jj=lengthMeasurements;
j=lengthMeasurements;
print(j);
xx=0;
setTool("oval");
z=1;
while (j==jj)
{makeOval(X-(rad+um10/2), Y-(rad+um10/2), (rad*2)+um10,(rad*2)+um10);
run("Measure");
string1 = "Plaque"+num+" "+um10/54*10+"um" ;
Roi.setName(string1);
roiManager("Add");
Overlay.show;
lengthMeasurements = getResult("Area");
print(lengthMeasurements);
xx=xx+1;
if (xx < 2)
{j= lengthMeasurements ;
jj=lengthMeasurements ;}
else
{j= lengthMeasurements ;}
```

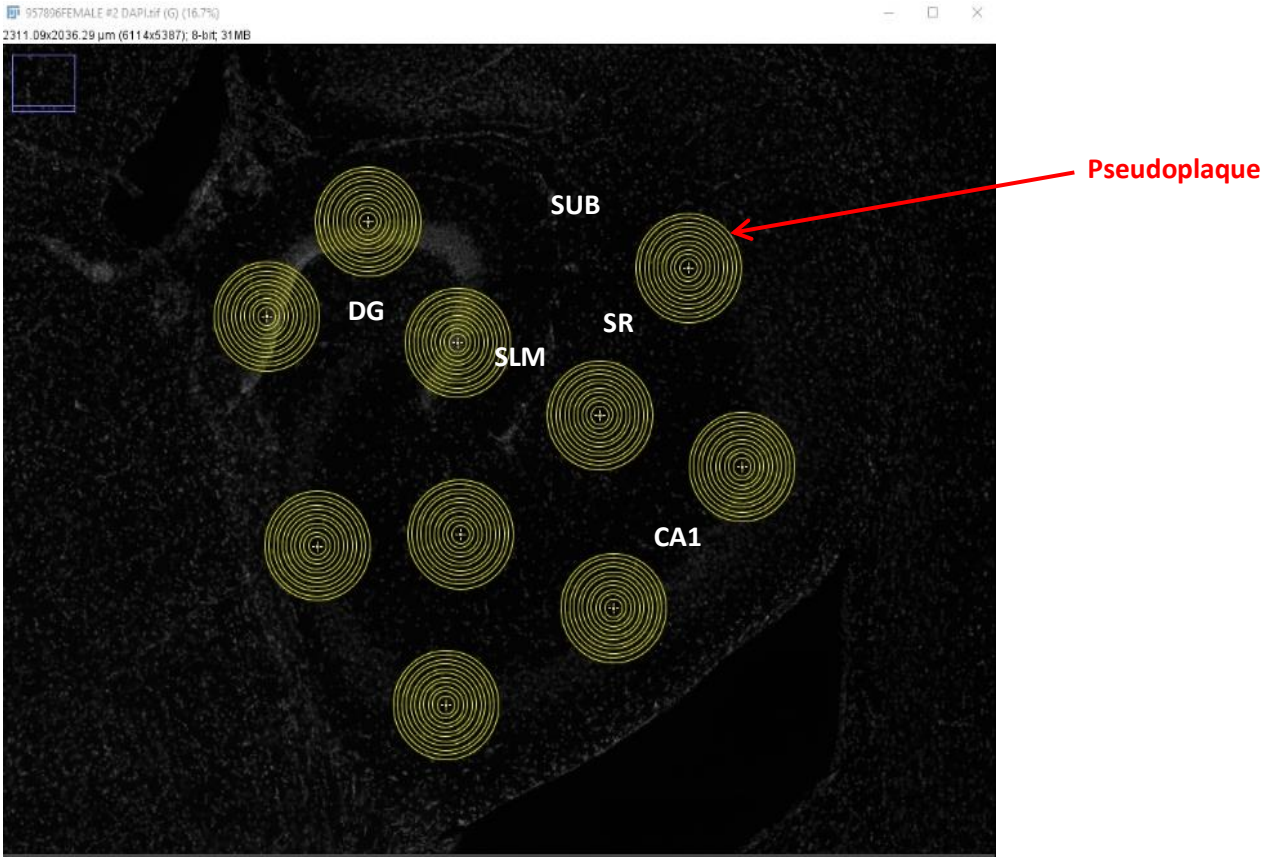
```
um10=um10+um10_step;
z=z+1;}
half=z/2;
while (z>half)
{roiManager("Select", (z-1+nmaxroi));
roiManager("Delete");
z=z-1;}
nmaxroi = roiManager("count");}
roiManager("Deselect");
roiManager("save","");
;
```

Pseudoplaque ZIP File Macro

Due to the lack of plaques in the control groups, it is necessary to create fake plaques (pseudoplaques). This macro is designed to create both the pseudoplaques and radiating concentric circle ROIs. This RoiSet ZIP file can be used in the same manner as the experimental group RoiSet ZIP files above.

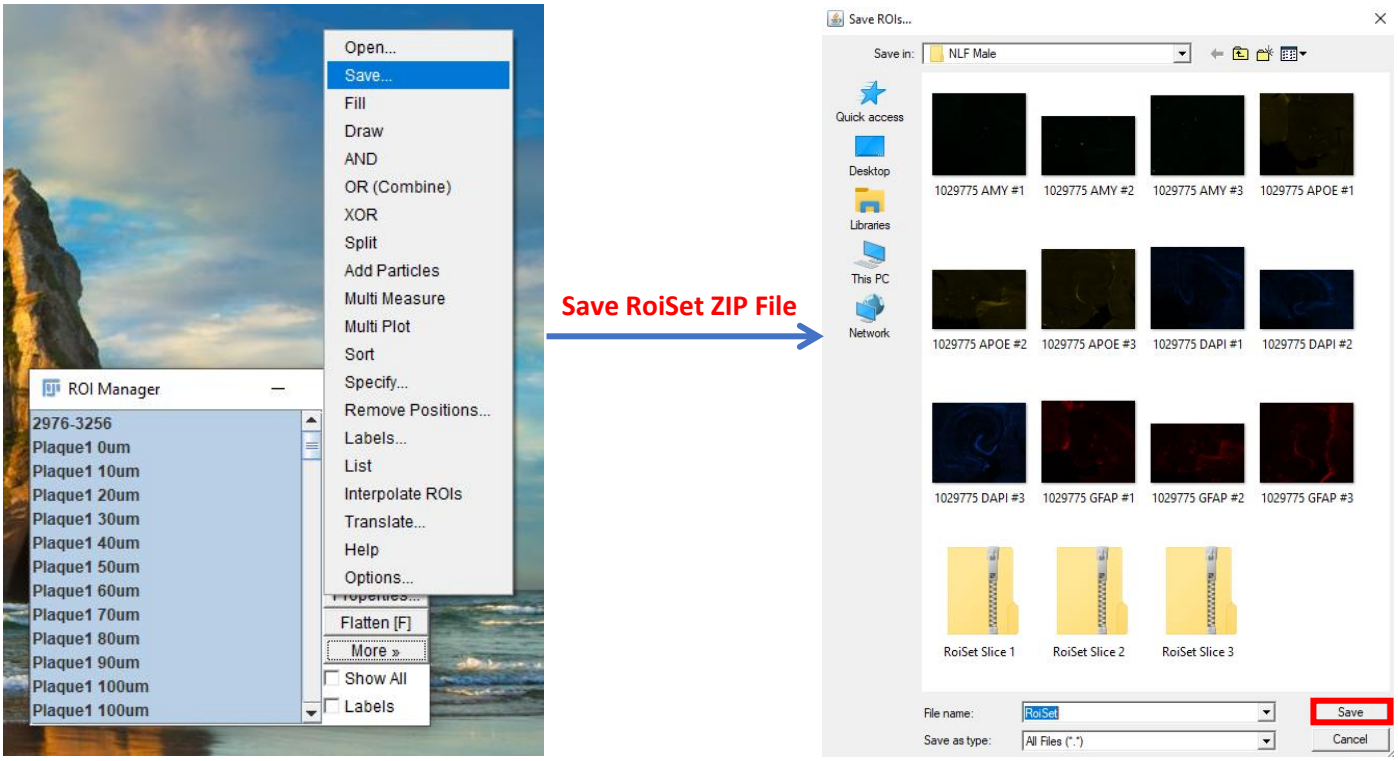
Instructions

1. Open FIJI
2. Load macro on FIJI – *Plugins* > *Macros* > *Run*
3. Open hippocampus (DAPI) image
4. Click on ROIs in the hippocampus to place pseudoplaques - click 'OK' after each selection



Pseudoplaques are distributed around defined regions of the hippocampus; occupying the dentate gyrus (DG), subiculum (SUB) and CA1 regions; including the stratum lacunosum moleculare (SLM) and stratum radiatum (SR).

5. Save RoiSet on ROI Manager to create a ZIP file - Ctrl+A > More > Save



Code

```
print('Please Open Hippocampus (DAPI) Image');
run("Open...");
run("8-bit");
setOption("BlackBackground", false);
run("Set Scale...", "distance=1 known=0.378 unit=um global");
for (i=0; i<10;i++)
{setTool("multipoint");
waitForUser("Please Select Your Hippocampus Region");
roiManager("add");
run("Set Measurements...", "area centroid stack limit redirect=None decimal=3");
run("Measure");
X=getResult("X");
Y=getResult("Y");
X=X/0.378;
Y=Y/0.378;
rad=27;
setTool("oval");
makeOval(X-rad, Y-rad, rad*2,rad*2);
um10_step =54;
um10=um10_step;
```

```
z=1;
while(z<12)
{
makeOval(X-(rad+um10/2), Y-(rad+um10/2), (rad*2)+um10,(rad*2)+um10);
run("Measure");
num = i+1;
string1 = "Plaque"+num+" "+(um10/54*10)-10+"um" ;
Roi.setName(string1);
roiManager("Add");
um10=um10+um10_step;
z=z+1;
}
}
```

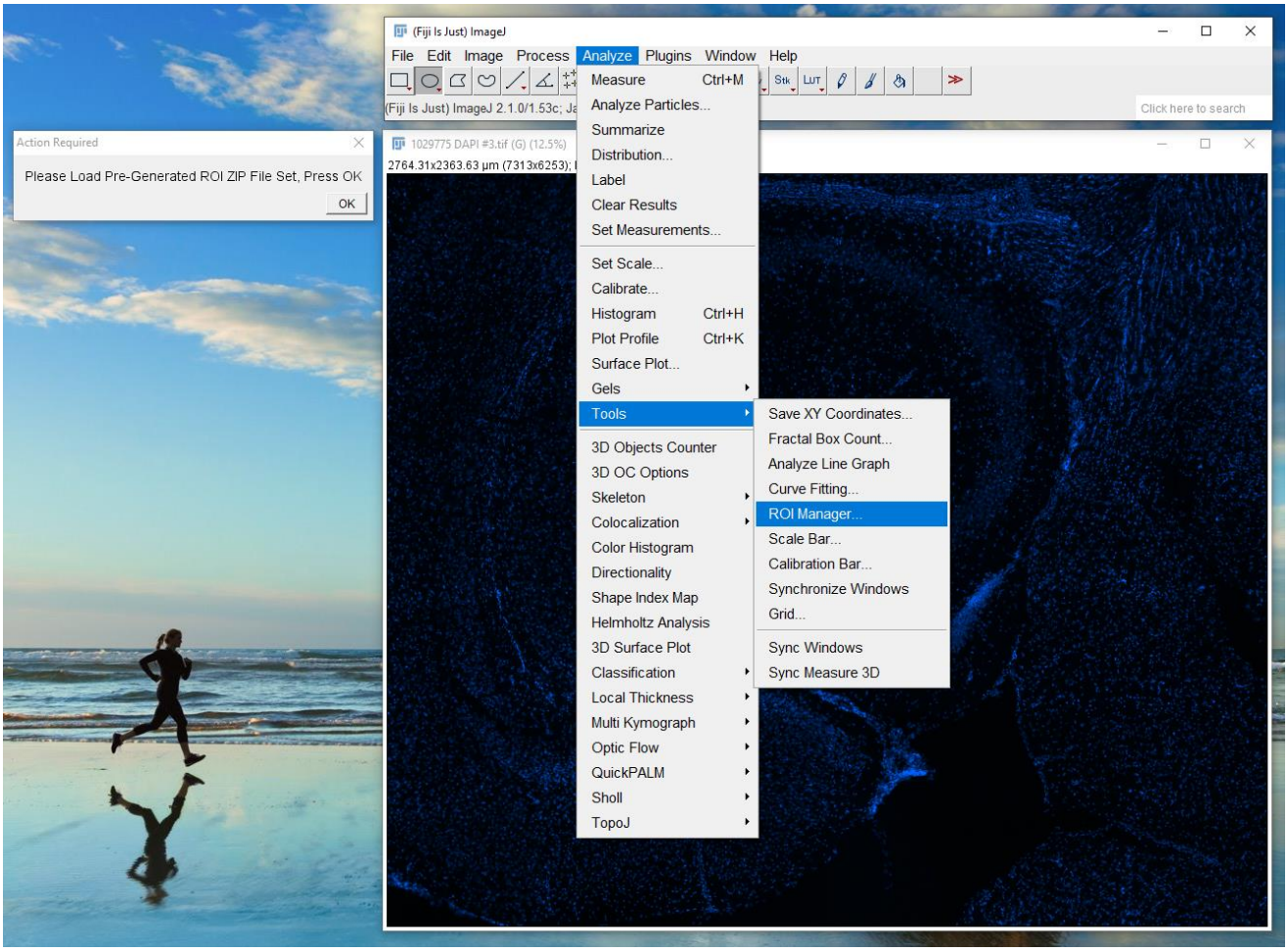
TREM2 Expression Macro

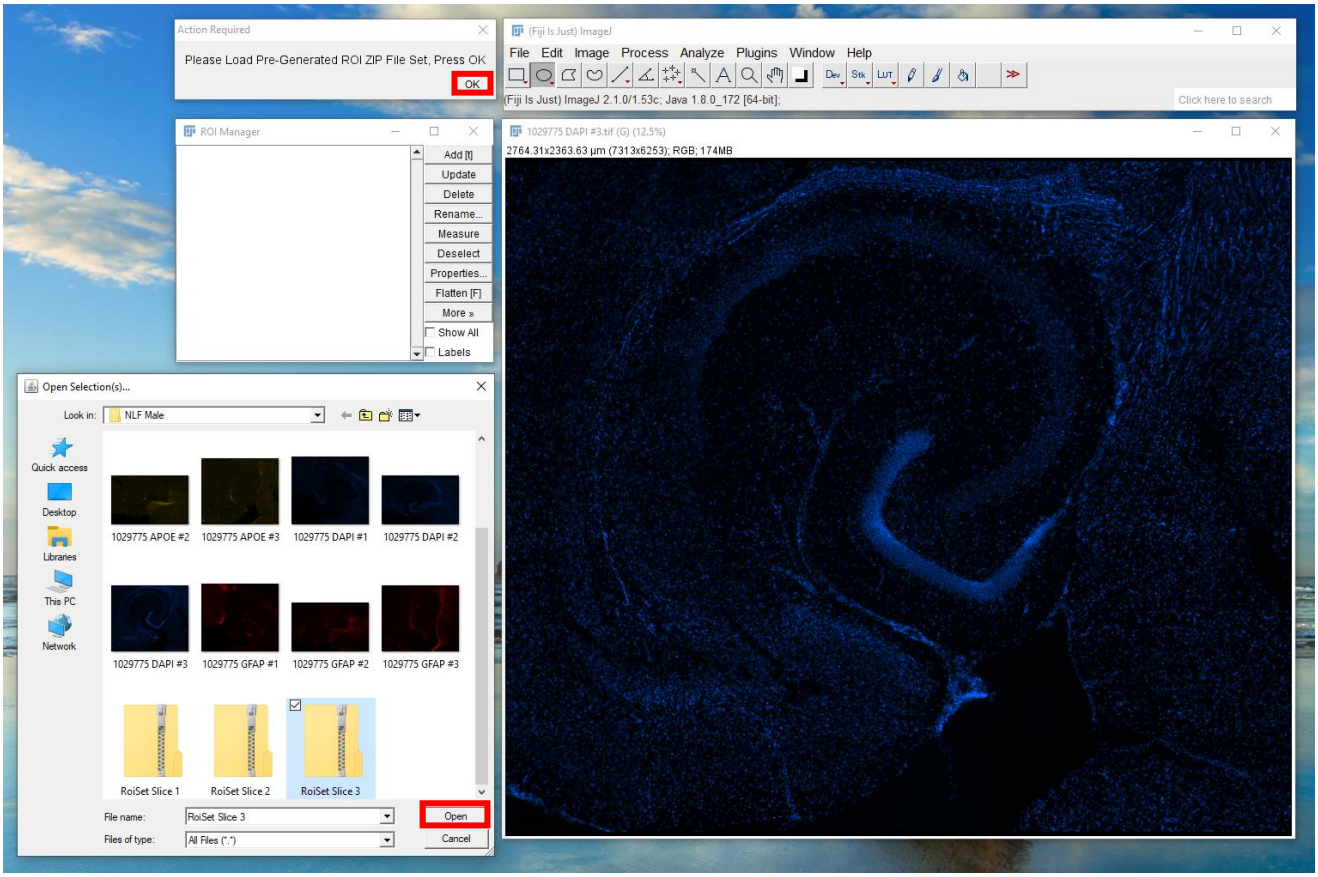
Using the RoiSet Zip files created, this macro will return the integrated density and area of the protein stain within the concentric circles and plaque area itself. From these values the mean grey value for the protein of interest can be calculated with distance from plaque.

Instructions

1. Open FIJI
2. Load macro on FIJI – *Plugins > Macros > Run*
3. Open hippocampus (DAPI) image
4. Load the pre-generated RoiSet pseudoplaque or actual plaque ZIP file on ROI Manager

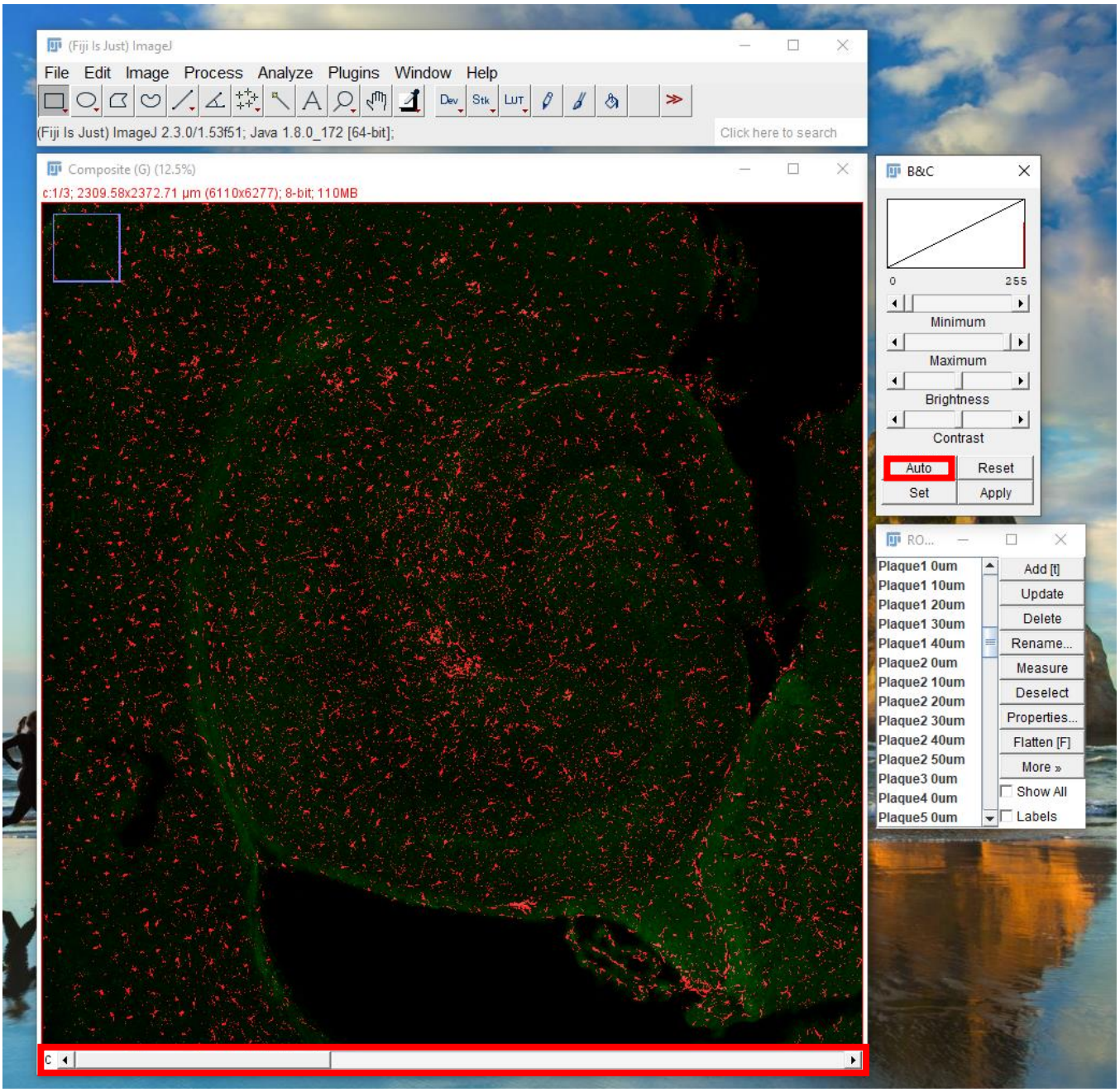
Analyse > Tools > ROI Manager > More > Open (Select ZIP File) > Press 'OK'



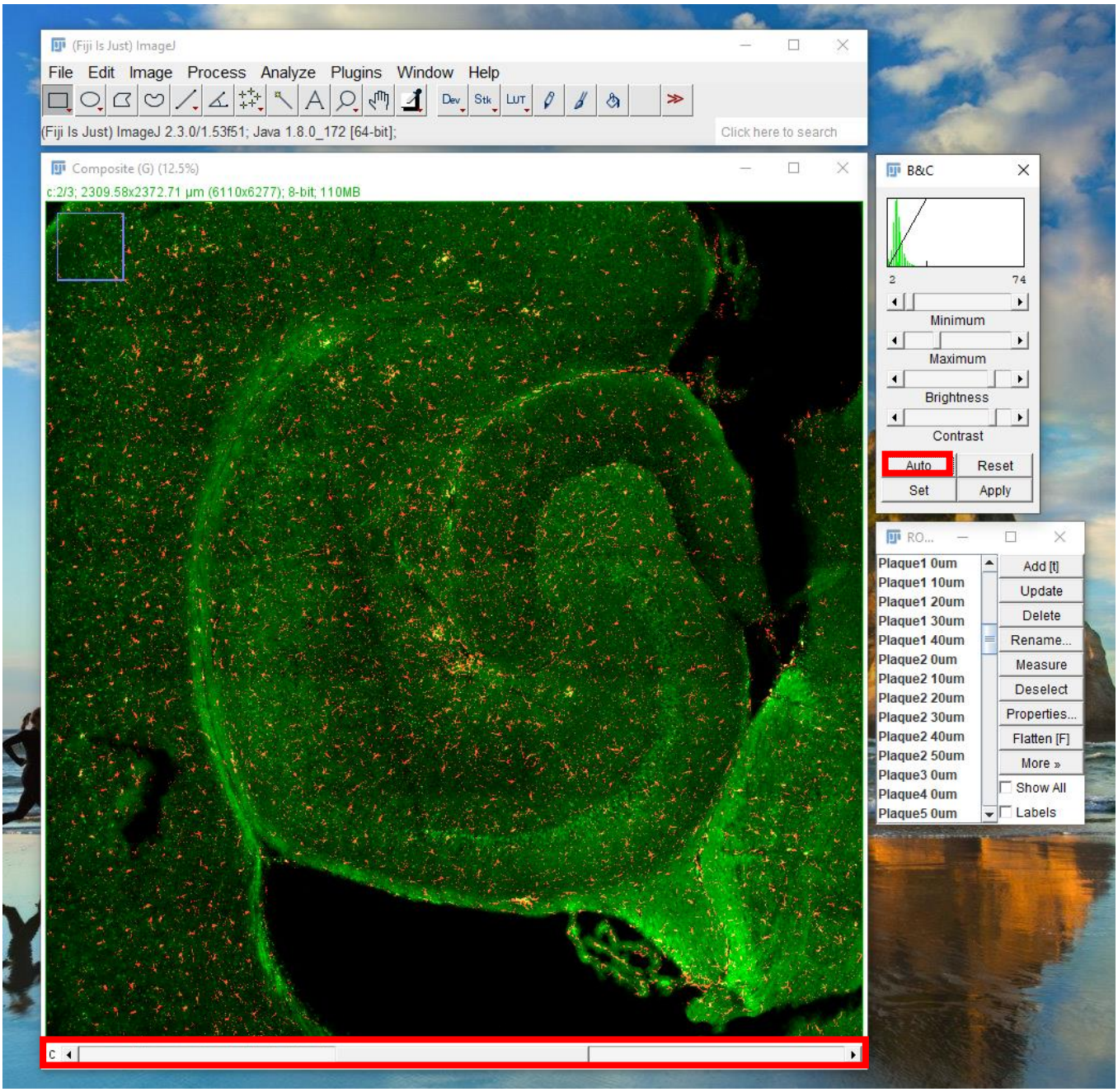


5. Open microglia (IBA1) image
6. Open TREM2 image – *for quantifying APOE expression microglia*
7. To visualise TREM2 expression on the merged image - Shift+Ctrl+C > Press Auto n Each Channel On B&C Window

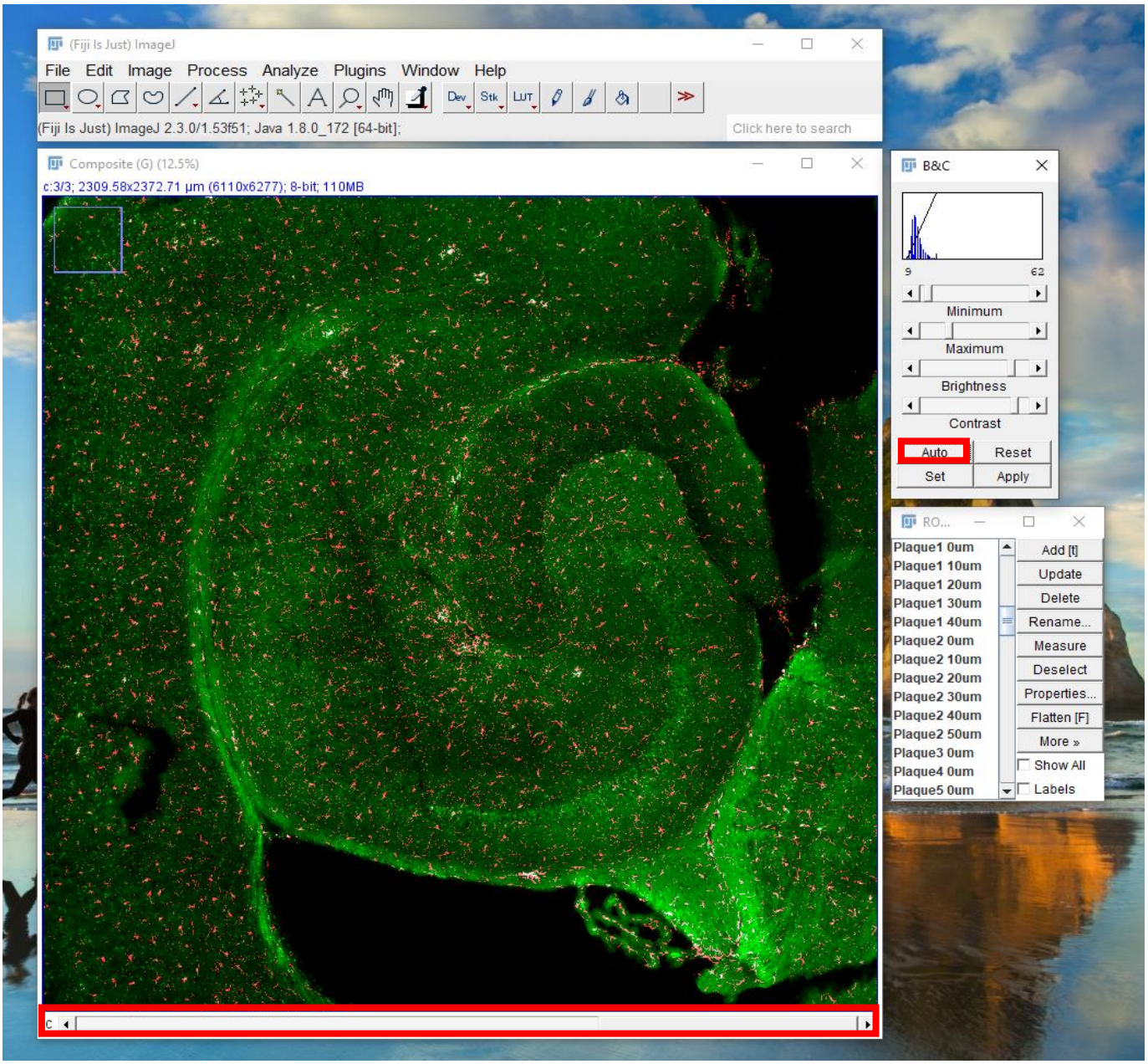
Channel 1


(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
Composite (G) (12.5%)
c:1/3; 2309.58x2372.71 µm (6110x6277); 8-bit; 110MB
B&C
0
255
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply
RO...
Plaque1 0um
Plaque1 10um
Plaque1 20um
Plaque1 30um
Plaque1 40um
Plaque2 0um
Plaque2 10um
Plaque2 20um
Plaque2 30um
Plaque2 40um
Plaque2 50um
Plaque3 0um
Plaque4 0um
Plaque5 0um
Add [t]
Update
Delete
Rename...
Measure
Deselect
Properties...
Flatten [F]
More »
Show All
Labels

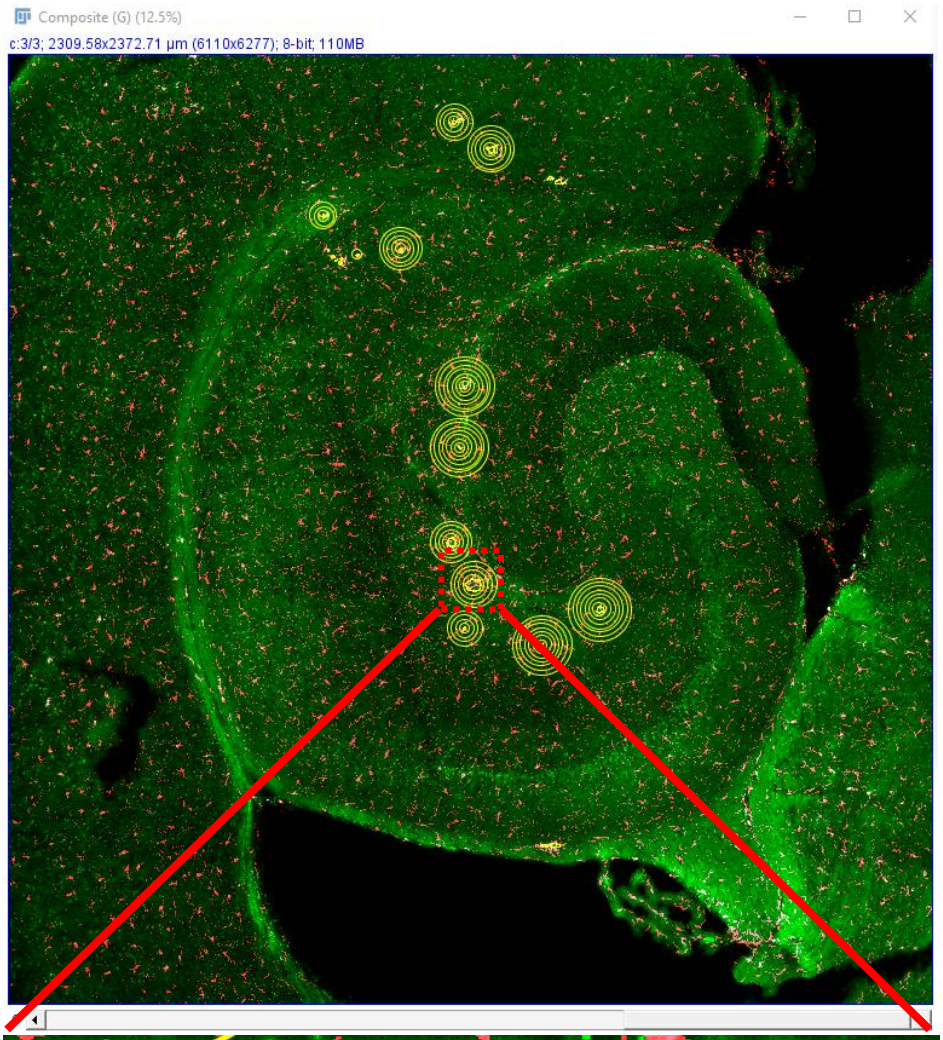
Channel 2

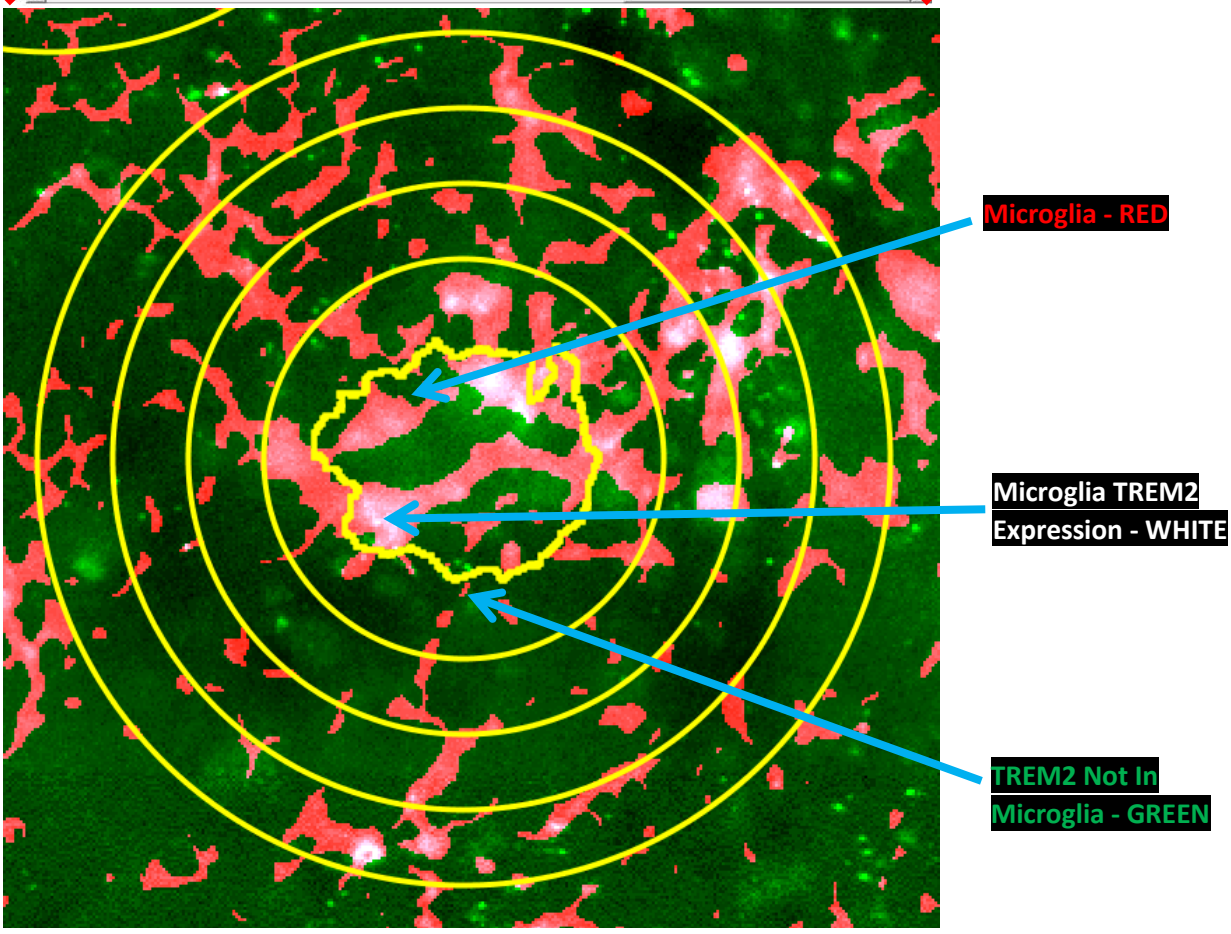

(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
Composite (G) (12.5%)
c:2/3; 2309.58x2372.71 µm (6110x6277); 8-bit; 110MB
B&C
2
74
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply
RO...
Plaque1 0um
Plaque1 10um
Plaque1 20um
Plaque1 30um
Plaque1 40um
Plaque2 0um
Plaque2 10um
Plaque2 20um
Plaque2 30um
Plaque2 40um
Plaque2 50um
Plaque3 0um
Plaque4 0um
Plaque5 0um
Add [t]
Update
Delete
Rename...
Measure
Deselect
Properties...
Flatten [F]
More »
Show All
Labels

Channel 3


(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
Composite (G) (12.5%)
c:3/3; 2309.58x2372.71 µm (6110x6277); 8-bit; 110MB
B&C
9
62
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply
RO...
Plaque1 0um
Plaque1 10um
Plaque1 20um
Plaque1 30um
Plaque1 40um
Plaque2 0um
Plaque2 10um
Plaque2 20um
Plaque2 30um
Plaque2 40um
Plaque2 50um
Plaque3 0um
Plaque4 0um
Plaque5 0um
Add [t]
Update
Delete
Rename...
Measure
Deselect
Properties...
Flatten [F]
More »
Show All
Labels

Example image with actual plaque ZIP file uploaded





8. The results table will have the values outputted automatically. No need to measure from ROI Manager

Interpreting values – N.B. Integrated Density is Abbreviated to IntDen

Microglial (IBA1) Area

Results

File Edit Font Results

	Label	Area	Mean	Mode	IntDen	Median	RawIntDen	MinThr	MaxThr
24	NLFTREM2 957317 18M M IBA1 #3.tif:Plaque1 0um	22.861	255	255	5829.667	255	40800	255	255
25	NLFTREM2 957317 18M M IBA1 #3.tif:Plaque1 10um	256.048	255	255	65292.273	255	456960	255	255
26	NLFTREM2 957317 18M M IBA1 #3.tif:Plaque1 20um	463.801	255	255	118269.373	255	827730	255	255
27	NLFTREM2 957317 18M M IBA1 #3.tif:Plaque1 30um	668.126	255	255	170372.024	255	1192380	255	255
28	NLFTREM2 957317 18M M IBA1 #3.tif:Plaque1 40um	801.436	255	255	204366.271	255	1430295	255	255

Total TREM2 Expression (IntDen) and Plaque Area *(same value for IBA1 specific TREM2 expression)*

Results

File Edit Font Results

	Label	Area	Mean	Mode	IntDen	Median	RawIntDen	MinThr	MaxThr
128	NLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 0um	66.155	33.346	28	2205.986	32	15439	0	255
129	NLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 10um	700.846	38.275	28	26825.185	32	187741	0	255
130	NLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 20um	1987.374	32.187	23	63967.309	28	447687.000	0	255
131	NLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 30um	3926.024	27.803	23	109154.517	25	763938	0	255
132	NLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 40um	6518.511	25.277	19	164769.542	23	1153170	0	255

Microglial (IBA1) Specific APOE Expression (IntDen) and Plaque Area *(same value for total TREM2 expression)*

Results

File Edit Font Results

	Label	Area	Mean	Mode	IntDen	Median	RawIntDen	MinThr	MaxThr
232	NLFTREM2 957317 18M M IBA1 #3.tifNLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 0um	66.155	13.780	0	911.600	0	6380	0	255
233	NLFTREM2 957317 18M M IBA1 #3.tifNLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 10um	700.846	19.345	0	13557.548	0	94885	0	255
234	NLFTREM2 957317 18M M IBA1 #3.tifNLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 20um	1987.374	11.714	0	23280.233	0	162931	0	255
235	NLFTREM2 957317 18M M IBA1 #3.tifNLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 30um	3926.024	7.615	0	29896.048	0	209233	0	255
236	NLFTREM2 957317 18M M IBA1 #3.tifNLFTREM2 957317 18M M TREM2 #3.tif:Plaque1 40um	6518.511	5.176	0	33737.056	0	236115.000	0	255

9. To obtain the area within the boundaries from plaque subtract smaller from larger circle

Example – Microglial (IBA1) Area – Same applies for plaque area

Since the area values are cumulative the larger areas must be subtracted from the smaller ones beneath! As an example, looking at the table above, the IBA1 (microglia) area for plaque 1 at 0µm will be 22.861µm^2 since no boundaries are present beneath. However, for plaque 1 at 10µm the IBA1 area will be 256.048µm^2 (larger circle) – 22.861µm^2 (smaller circle) = 233.187µm^2 (IBA1 area for plaque 1 at 10µm from plaque). Similarly, the IBA1 area for plaque 1 at 30µm will be 668.126µm^2 – 463.801µm^2 = 204.325µm^2 (IBA1 area for plaque 1 at 30µm from plaque.

Code

```
print('Please Open DAPI Channel');

run("Open...");

waitForUser("Please Load Pre-Generated ROI ZIP File Set, Press OK")

close();

print('Please Open Red (IBA1) Channel');

run("Open...");

run("8-bit");

run("Despeckle");

run("Gaussian Blur...", "sigma=1");

run("Subtract Background...", "rolling=11");

setAutoThreshold("Huang dark no-reset");

setOption("BlackBackground", true);
```

```
run("Convert to Mask");
run("Options...", "iterations=1 count=2 do=Nothing");
setOption("BlackBackground", true);
run("Erode");
run("Set Measurements...", "area mean modal integrated median limit display redirect=None decimal=3");
numROIs = roiManager("count");
array1 = newArray("0");;
for (i=1;i<roiManager("count");i++){
array1 = Array.concat(array1,i);
Array.print(array1);
}
run("Set Scale...", "distance=1 known=0.378 unit=um global");
roiManager("select", array1);
roiManager("Measure");
run("8-bit");
run("Multiply...", "value=0.00392");
name1 = getTitle();
print('Please Open Yellow (TREM2) Channel');
run("Open...");
run("Brightness/Contrast...");
setMinAndMax(2, 50);
run("8-bit");
name2 = getTitle();
current_name = getTitle();
selectWindow(name2);
selectWindow(current_name);
rename(name2);
run("Set Scale...", "distance=1 known=0.378 unit=um global");
roiManager("select", array1);
roiManager("Measure");
imageCalculator("Multiply create", name2,name1);
rename(name1+name2);
run("8-bit");
run("Set Scale...", "distance=1 known=0.378 unit=um global");
roiManager("select", array1);
```

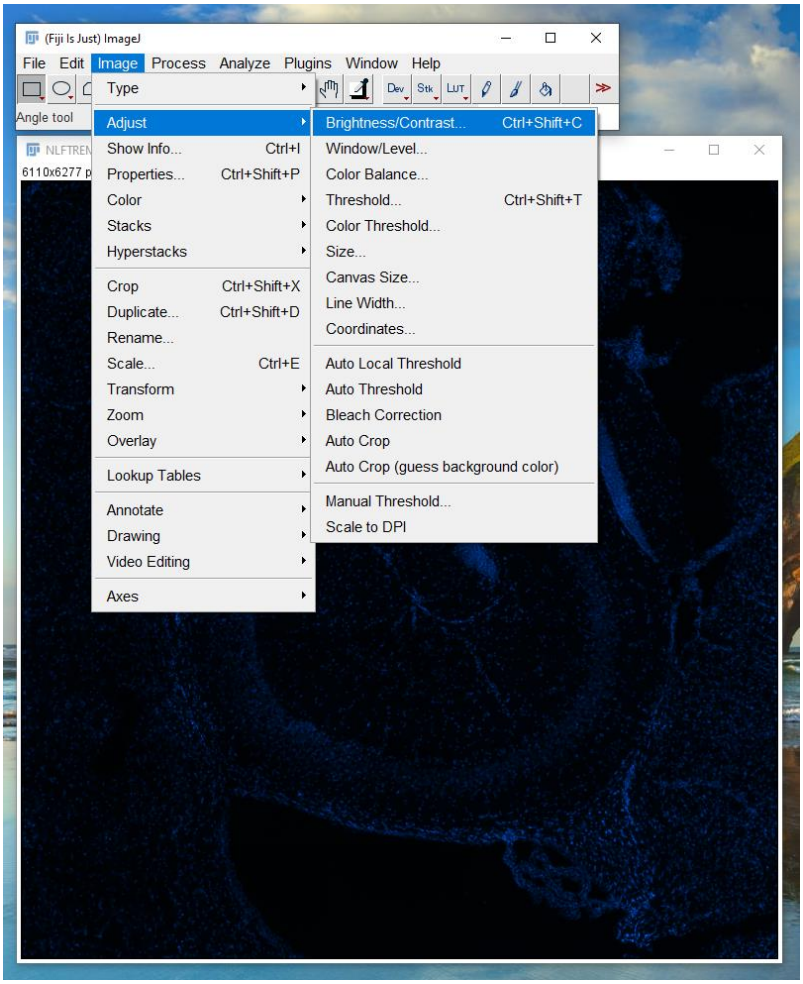
```
roiManager("Measure");
selectWindow(name1);
run("Multiply...", "value=255");
k = name1+name2;
line_merge = "c2=["+name1+"] c3=["+name2+"] c6=["+k+"] keep"
run("Merge Channels...", "c1=["+name1+"]c2=["+name2+"]c3=["+k+"] keep create");
run("Set Scale...", "distance=1 known=0.378 unit=um global");
print('RED: IBA1, GREEN: TREM2, WHITE: overlap ');
```

Microglial Density Analysis

Using the RoiSet Zip files created, microglial number can be manually counted. With this, microglial density with distance from plaque can be calculated. This step is manual and does not require a macro.

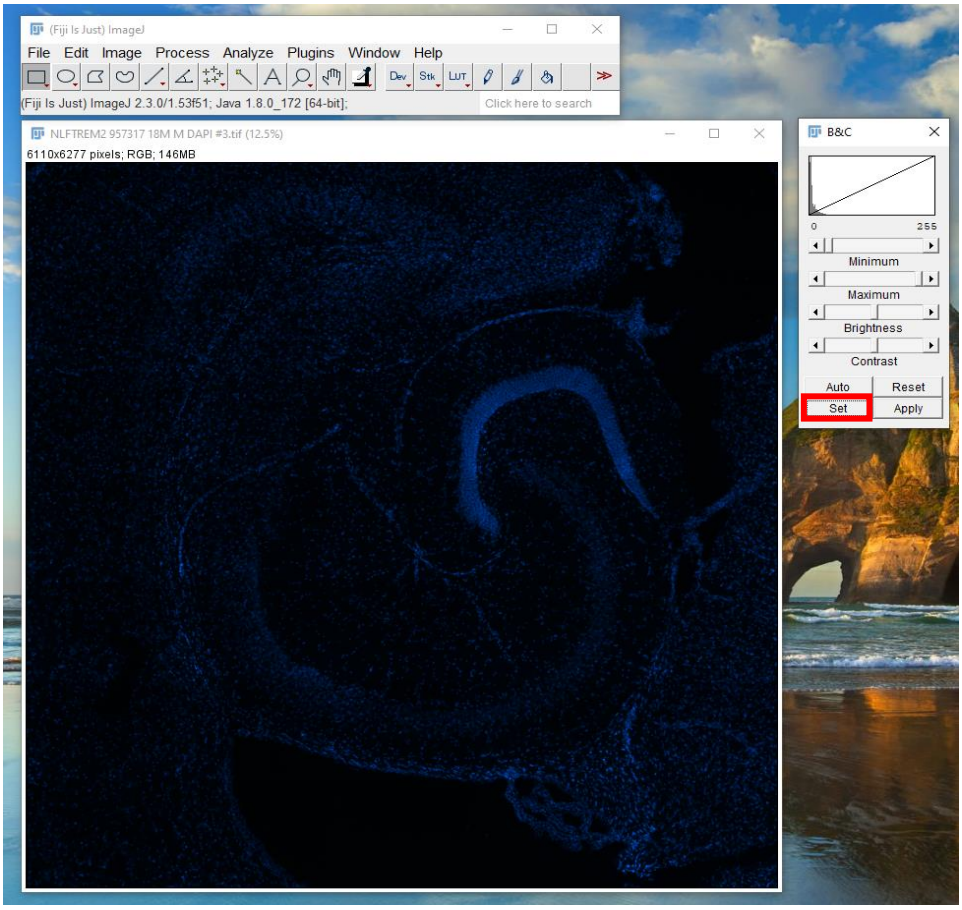
Instructions

1. Open FIJI
2. Drag and drop hippocampus (DAPI) and microglia (IBA1) image into FIJI
3. On each image adjust brightness and contrast to improve visualisation – Image > Adjust > Brightness/Contrast

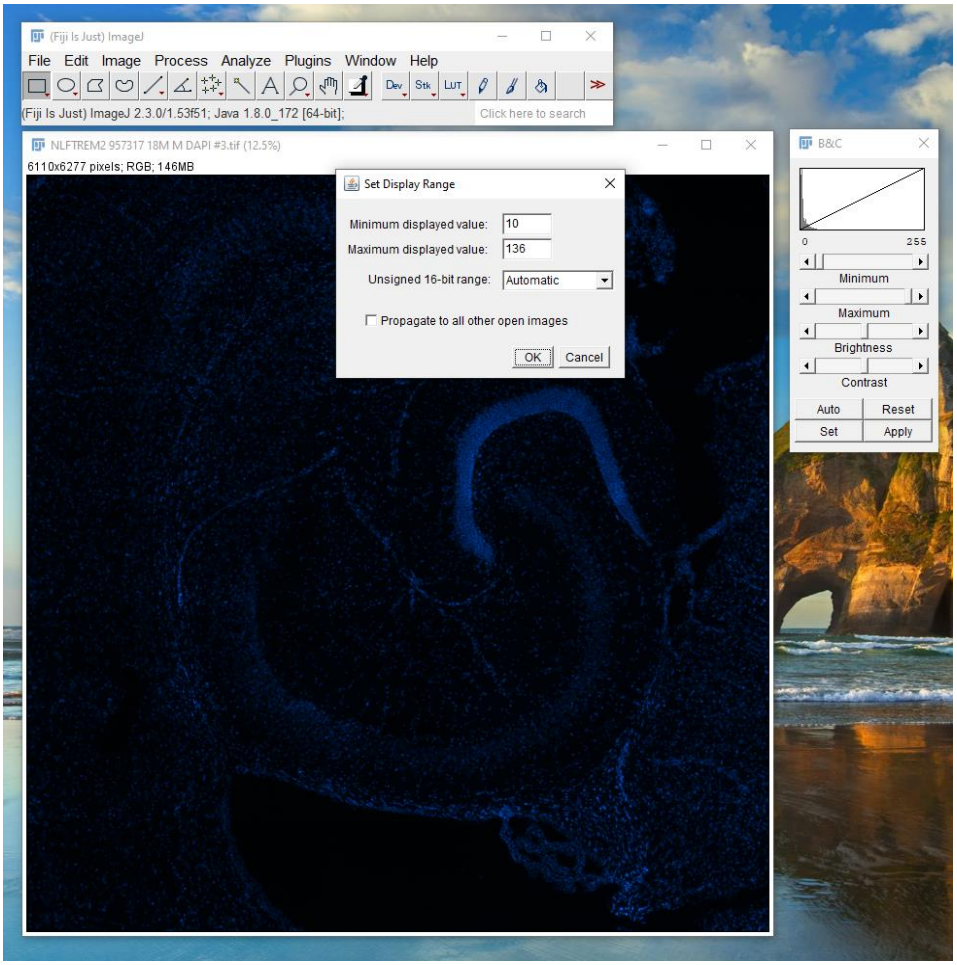


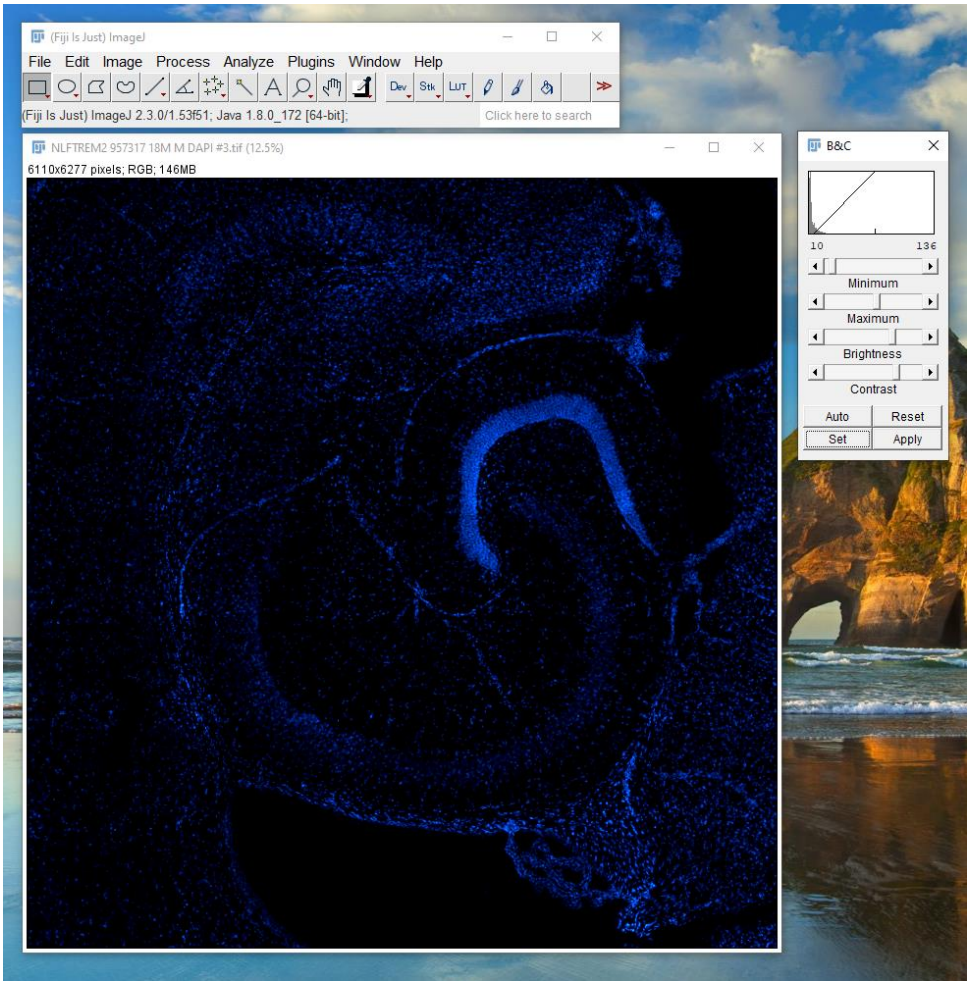
Adjust brightness/contrast via slider or enter values manually by clicking ‘set’.

In this example, for the DAPI image, the ‘minimum displayed value’ was set to 10 and the ‘maximum displayed value’ to 136. For the IBA1 image, the ‘minimum displayed value’ was set to 9 and the ‘maximum displayed value’ to 165.

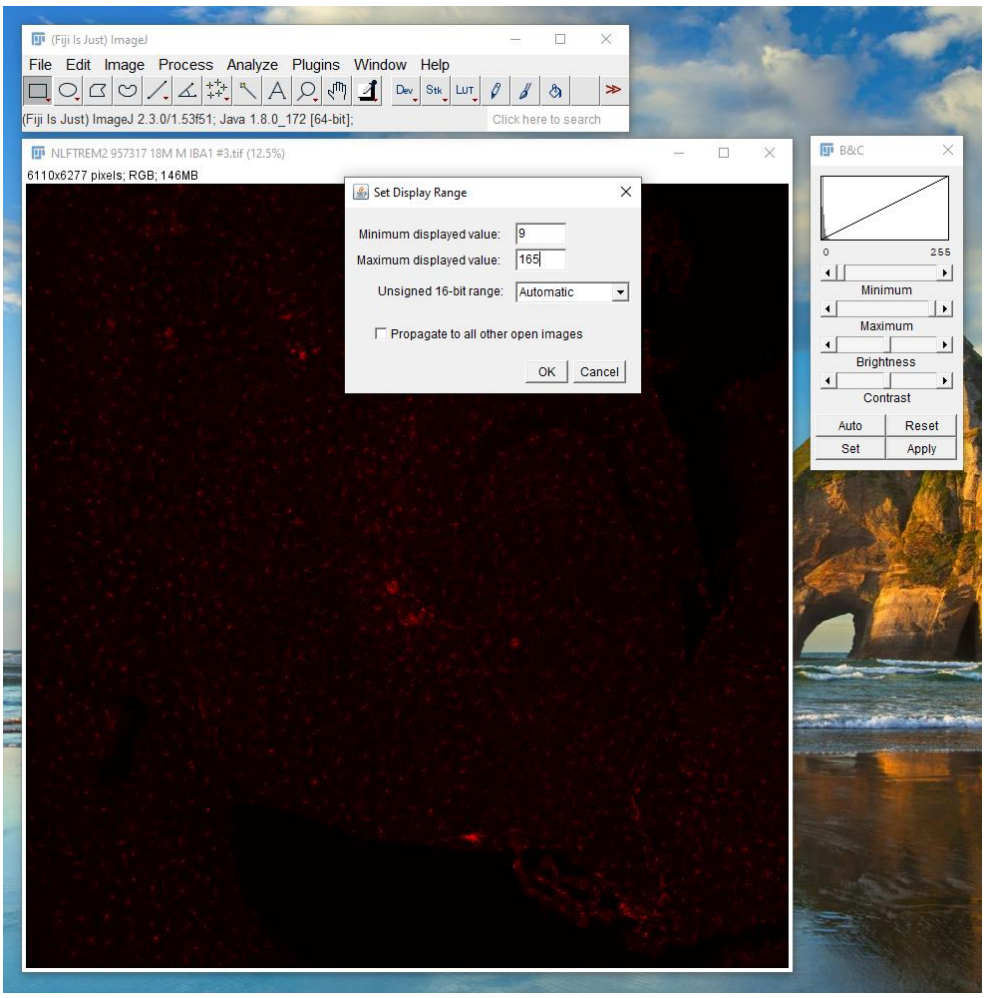

(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
NLFTREM2 957317 18M M DAPI #3.tif (12.5%)
6110x6277 pixels; RGB; 146MB
B&C
0
255
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply

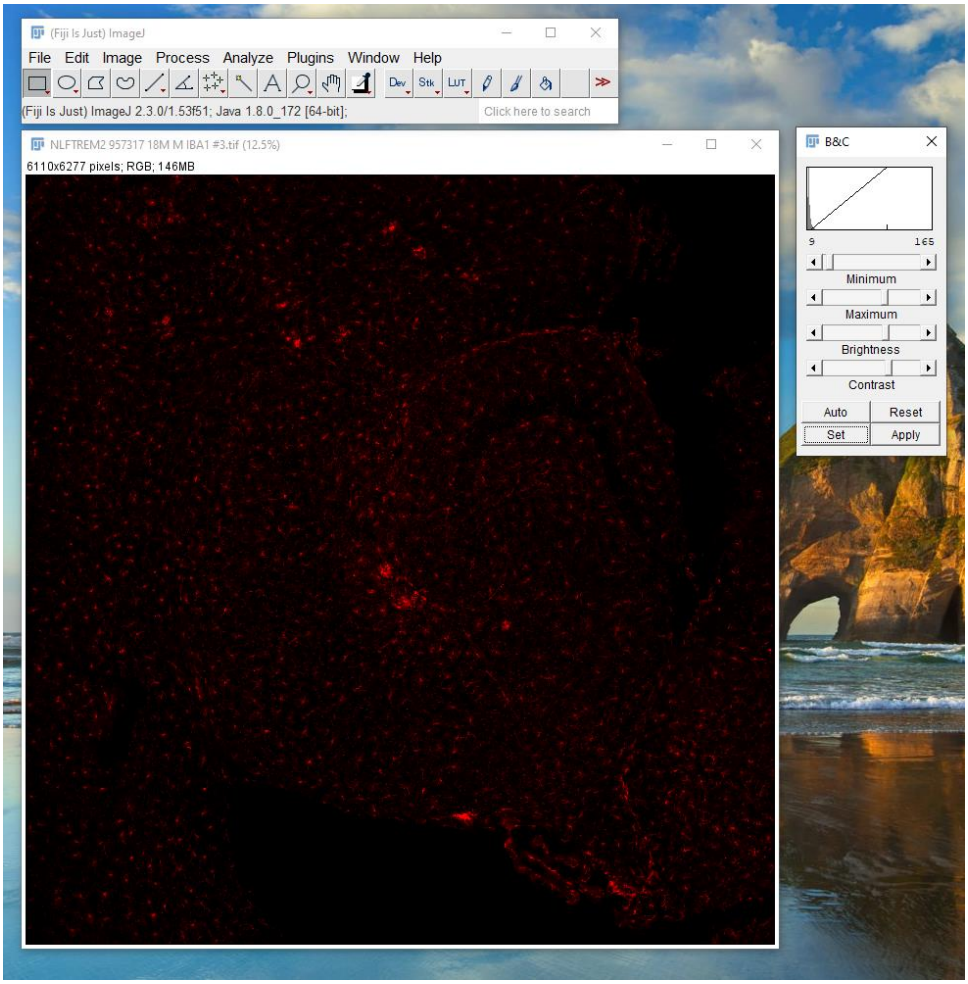
DAPI


(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
NLFTREM2 957317 18M M DAPI #3.tif (12.5%)
6110x6277 pixels; RGB; 146MB
Set Display Range
Minimum displayed value: 10
Maximum displayed value: 136
Unsigned 16-bit range: Automatic
Propagate to all other open images
OK
Cancel
B&C
0
255
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply

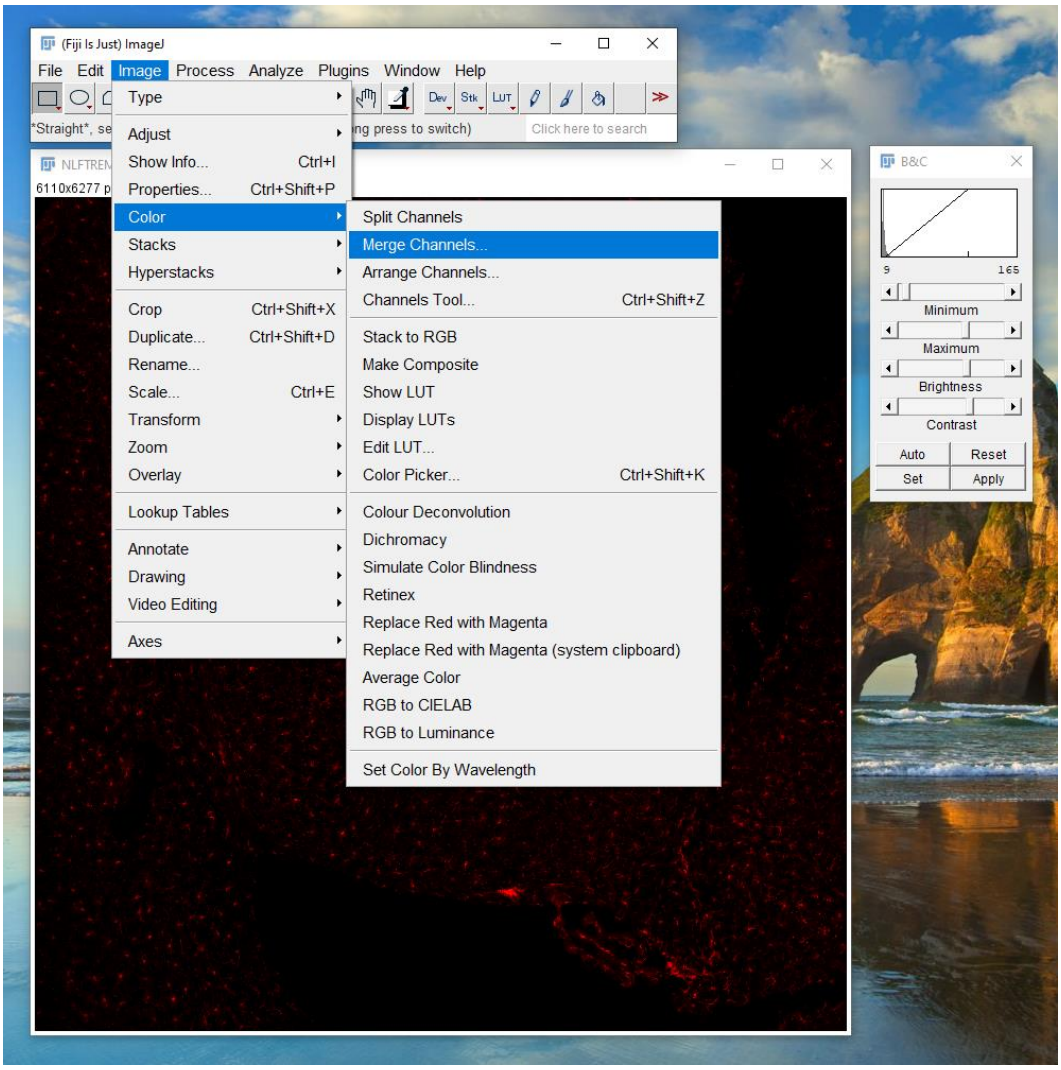

(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
NLFTREM2 957317 18M M DAPI #3.tif (12.5%)
6110x6277 pixels; RGB; 146MB
B&C
10
136
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply

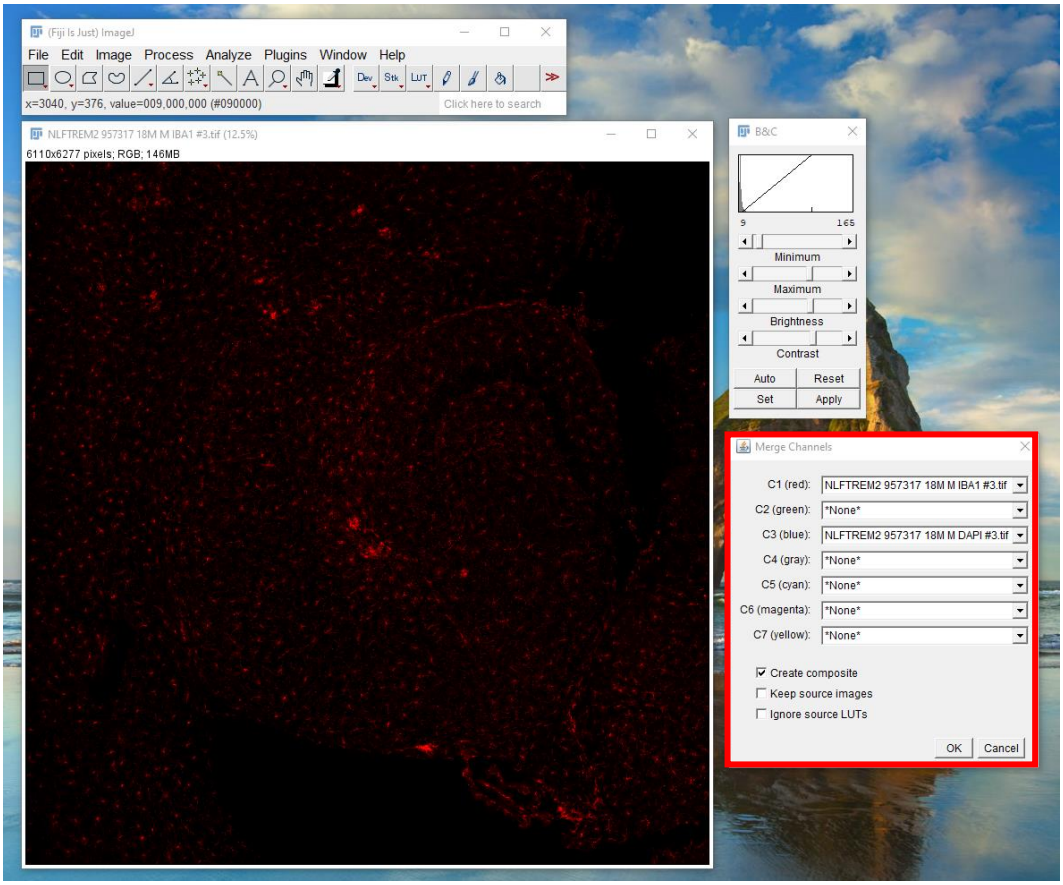
IBA1

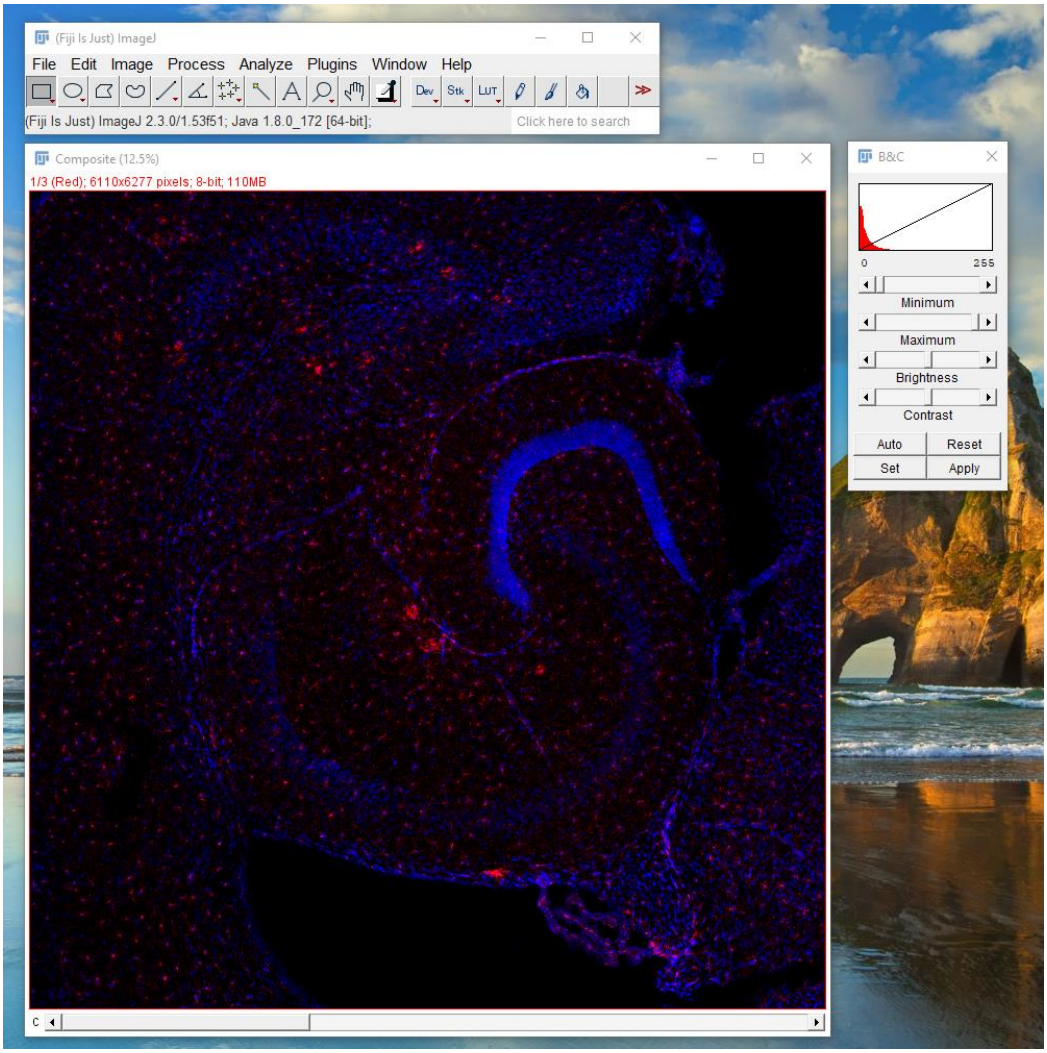

(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
NLFTREM2 957317 18M M IBA1 #3.tif (12.5%)
6110x6277 pixels; RGB; 146MB
Set Display Range
Minimum displayed value: 9
Maximum displayed value: 165
Unsigned 16-bit range: Automatic
Propagate to all other open images
OK
Cancel
B&C
0
255
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply


(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
(Fiji Is Just) ImageJ 2.3.0/1.53f51; Java 1.8.0_172 [64-bit];
Click here to search
NLFTREM2 957317 18M M IBA1 #3.tif (12.5%)
6110x6277 pixels; RGB; 146MB
B&C
9
165
Minimum
Maximum
Brightness
Contrast
Auto
Reset
Set
Apply

4. Merge images (channels) – Image > Colour > Merge Channels *(In this example, set blue – DAPI and red – IBA1)*







5. Drag and drop RoiSet ZIP file onto FIJI – Uncheck ‘Labels’
6. Zoom into plaque of interest and count microglial cell

For consistency and to eliminate bias, if a cell touches a ring, and it is not clear whether the cell is inside or outside the ring, it is recommended to always consider it either inside or outside throughout the entirety of the analysis.