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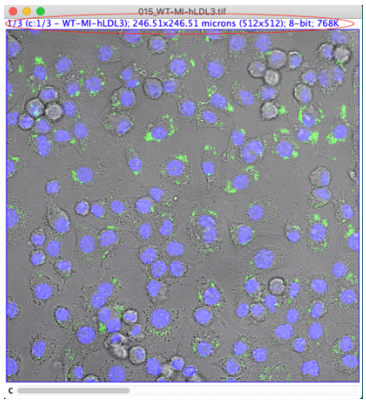
Javier Casas
BioImage Analysis Facility - IBGM

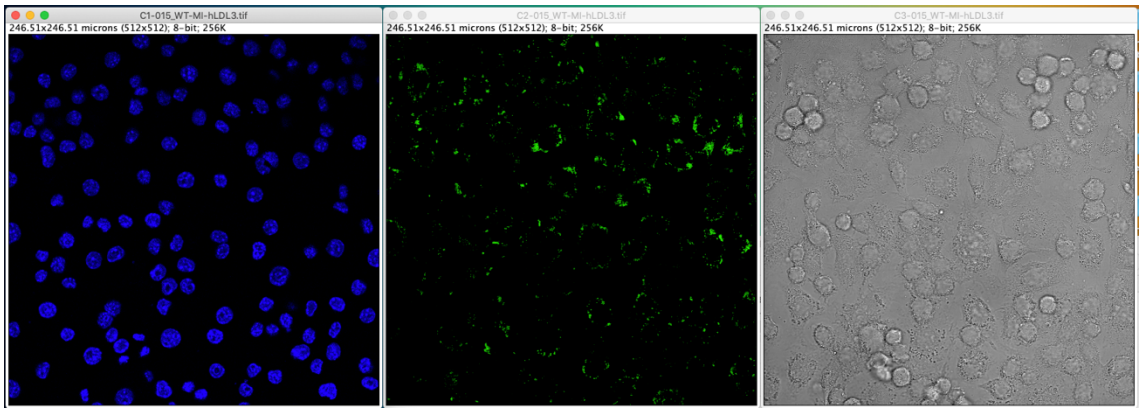
# *BATCH PROCESSING FOR LIPID DROPLET ANALYSIS IN ImageJ/FIJI*

Practically, any automatic analysis is based in the ability to separate objects by their fluorescence intensity levels from the background (black). Therefore, it is very useful to play with the detection parameters before jump into batch image analysis. Let's take a typical image: 3 Channels, Blue, Green and Transmission, directly from a Leica SP5X confocal microscope are calibrated so we can see the actual dimensions of the image (in this case 246.51 x 246.51 µm).



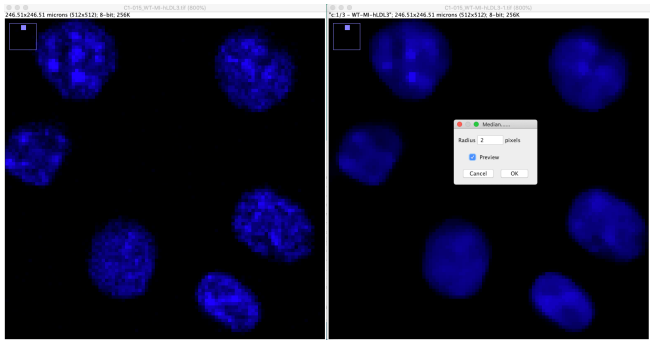

To separate the different channels:

Image > Color > Split Channels

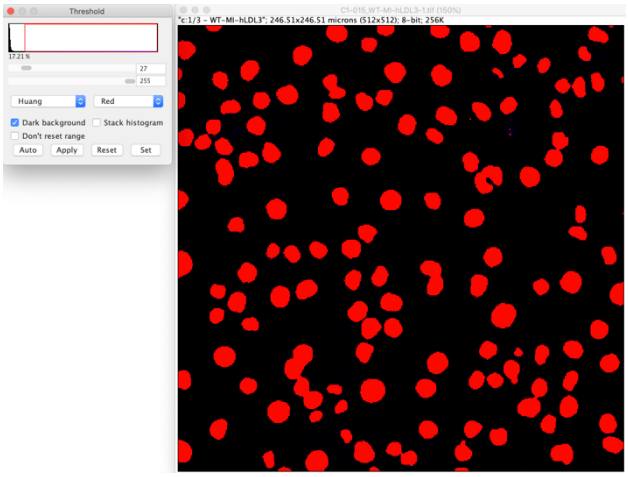



We'll focus on channel 1, blue, which shows nuclear staining. We will ALWAYS duplicate the images since ImageJ/FIJI has the funny manner of not let undoing changes, therefore: Image > Duplicate

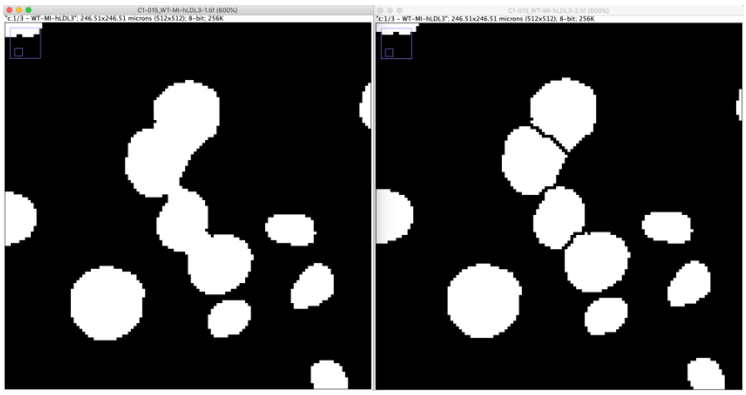
The first thing we're going to do before segmenting the image is to apply a median filter to round pixel roughness and make detection easier: Process > Filter > Median
You can play with the size of the filter radius and the preview tab until you consider the edges to be uniform.

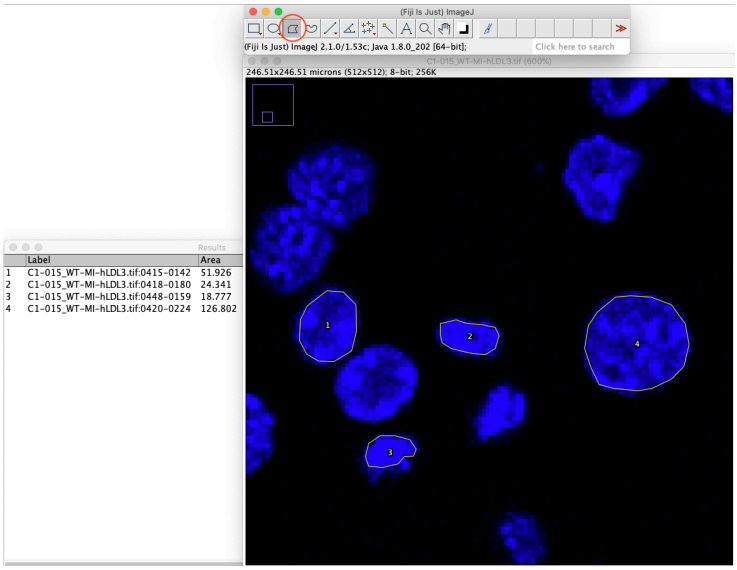


Now the real segmentation begins, the process of converting an image with a lot of gray levels into one with just two, that is, binary, so that there are only two levels either there is pixel or there is no pixel. To do this Image > Adjust > Threshold
Here you can play with the different methods or even adjust it manually by moving the sliders. Once done... Apply



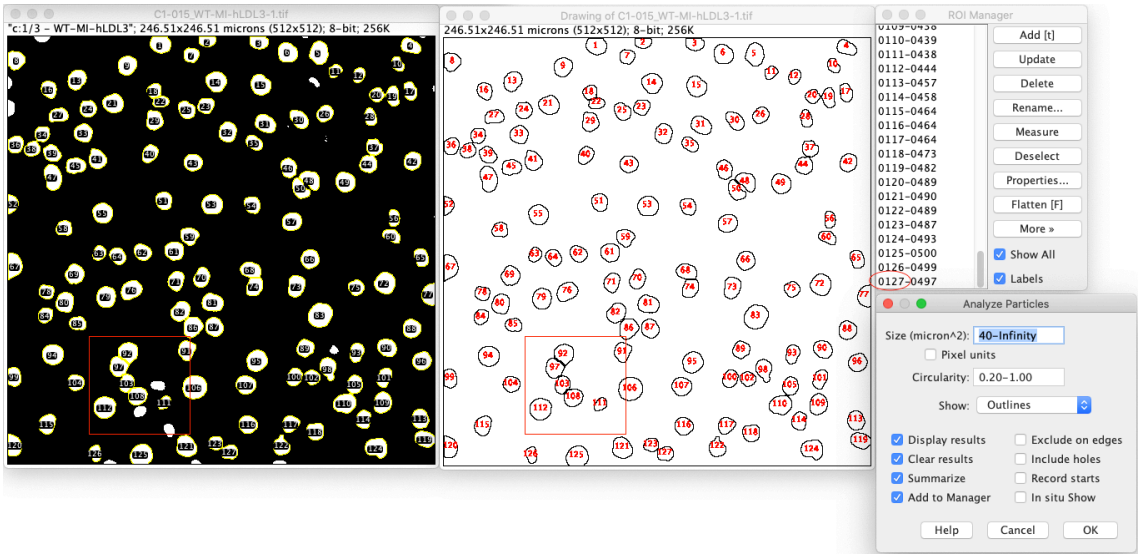

Another very useful tool for separating adjacent nuclei is obtained using Process > Binary > Watershed






With the selection tool, we select nuclei of different sizes, adding them to the ROI Manager by pressing the letter t after each selection and measure their area in Analyze > Measure
So we can later filter the smaller sizes that we are not interested. In this case 20 $\mu m^2$ or less do not indicate healthy cells.

Finally, to count the number of cells based on nuclei count: Analyze > Analyze Particles
And in the drop-down we can select the minimum and maximum size (in this case particles between 40 $\mu m^2$ and infinite) and its circularity where 1.00 is a perfect circle and 0.00 not (that's why we select 0.20 minimum so that it has some circularity and does not detect amorph shapes that do not correspond to nuclei)




We can see how in this image there were about 127 cells. In the same way we can check the minimum size of lipid droplets. The handling of this small protocol in the first image is very useful to find out the parameters that we must introduce in automatic analysis. In essence this is what does the macro "LD Analysis_1.0.ijm" first detects the number of cells and then does the same with the amount of lipid *droplets*.

BEFORE **RUNNING THE MACRO FOR THE FIRST TIME!!!**

***On Windows***

Open it with FIJI and change the lines 87 and 91 of the Macro "LD Analysis_1.0.ijm" to save the results in the desired folder:

87 saveAs("results", "C:\\Users\\YourUser\\Desktop\\Folder\\" + title + "_results.csv");

91 saveAs("Results", "C:\\Users\\YourUser\\Desktop\\Folder\\Summary.csv");

In the line where we select the threshold method for lipid droplet detection

69 setAutoThreshold("Huang dark");

We can change for:

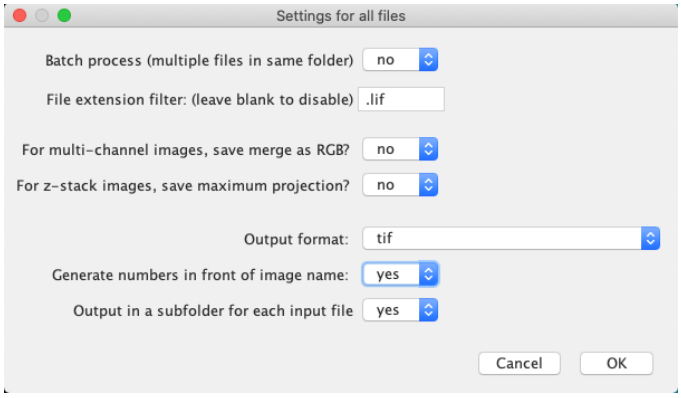
69 setAutoThreshold("Default dark"); in the case that will be our preference

The main goal of this automated analysis is the analysis of various parameters (fluorescence intensity, area, etc.) of a BODIPY stain to detect lipid droplets based on detection of the number of cells and number of lipid droplets bysize.

First you´ll need to export all the images contained in the proprietary format Leica file (*. lif) to a universal manageable format such as *. tif in the same directory

- Open in FIJI "Export-as-individual-images_1.6.ijm" and run *"Run"*. If ImageJ is used then: Plugins > Macro > Install and then Plugins > Macro > Export-as-individual-images_1.6

- Filter by Leica File Extension (*.lif)



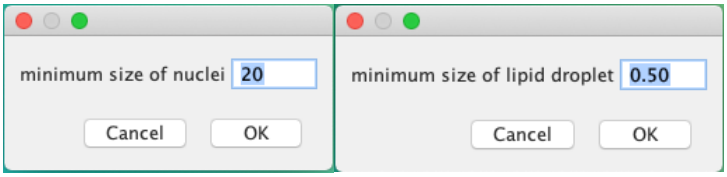

- Select folder where the *.lif file is located. The program will create a directory with the same name and save the * files. tif newly created and numbered

To automatically analyze images:

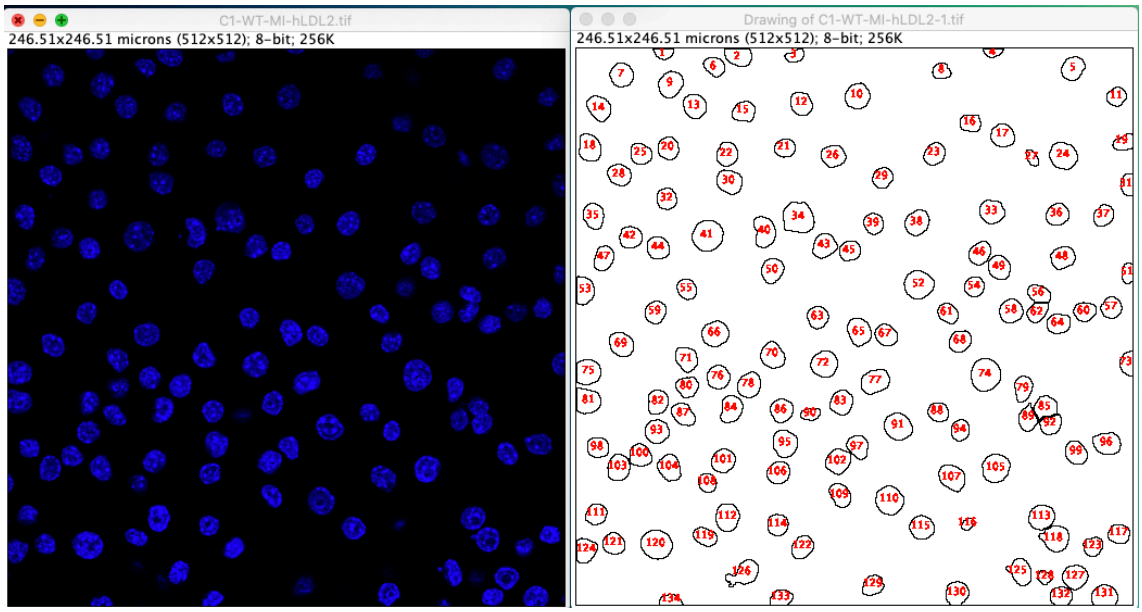
- Open LD Analysis_1.0.ijm and run *"Run"*. If ImageJ is used then: Plugins > Macro > Install and then Plugins > Macro > LD Analysis_1.0

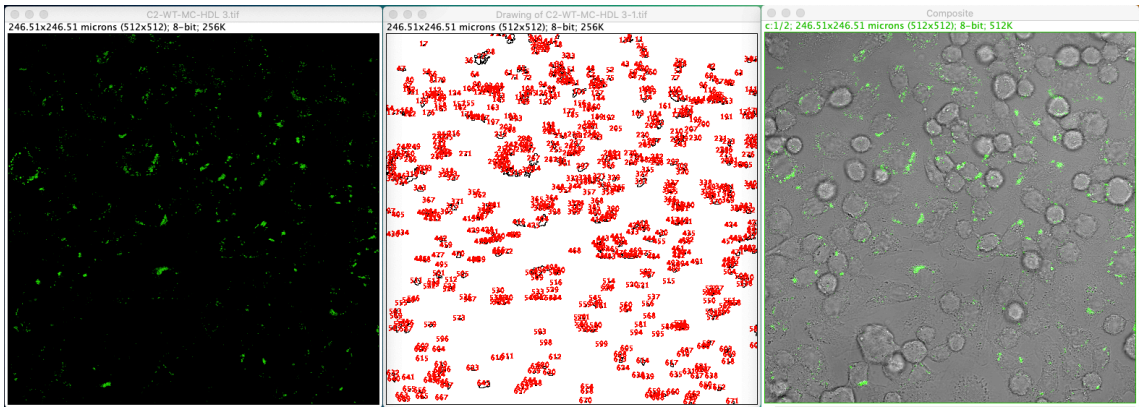
- Select Folder where the converted *.tif are located

- Enter the minimum core detection size (in $\mu m^2$) (size we already know when we have been fiddling with a sample image of the experiment) and lipid droplets. You will only ask for it in the first image and apply it the same for all. The size to select depends on the cell type and the lens used.



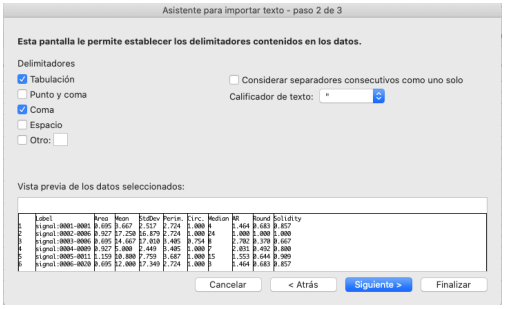

With a minimum size of 20-40 $m^2$ per nucleus we make sure to analyze all cells and not cell remains. For *lipid droplets* what we want is to analyze everything and regardless of circularity, hence the minimum size must be small (not 0, but almost, let´s say 0.15-0.20) and the circularity is programmed between 0.00 and 1.00.






Enjoy the analysis...

- Import *.csv files into Microsoft Excel




To get the number *of* lipid droplets per cell you would have to divide the total number by the number of cells in each file located in the corresponding count column of the Summary file.csv