Standard Operating Procedure: IF_particles_analysis_macro

This is the SOP for the IF_particles_analysis_macro created by Arya Akkus, Frederique Larroquette and Eddie Cai for the Fon Lab at the Montreal Neurological Institute of McGill University.

User Guide

Purpose and Output

This macro in Python is designed to perform particle and colocalization analysis on 3 channeled RGB images. It is meant to be run on FIJI (ImageJ) platform. The output of the analysis is saved to a .csv file in the output directory chosen by the user.

This macro takes images from the input folder and its subfolders, then it detects distinct particles in each channel (regions of interests – ROI) and measures the tissue area, number of particles and cells, total area of particles and cells, staining ratio and colocalization percentage.

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Manual

Getting Started

To be able to run this macro, you should have FIJI (ImageJ) installed on your computer. Once you open FIJI, select File>Open> or simply drag the macro to FIJI menu. Then you can run the macro from the editor. Before analyzing, the user is asked to choose the threshold mode, input and output directories as well as Min and Max ROIs. For further information see following sections: Thresholds and Threshold mode*Thresholds and Threshold mode* and Particle Analysis and ROIs

Thresholds and Threshold mode

Thresholding is a technique for dividing an image into two (or more) classes of pixels, which are typically called "foreground" and "background". This will help separate the specific signal in each channel from the background. If the threshold mode is NOT enabled, the user must define default thresholds for each channel beforehand.
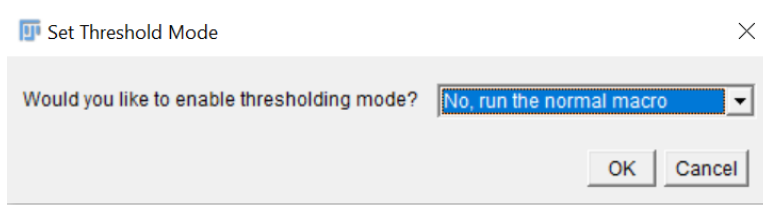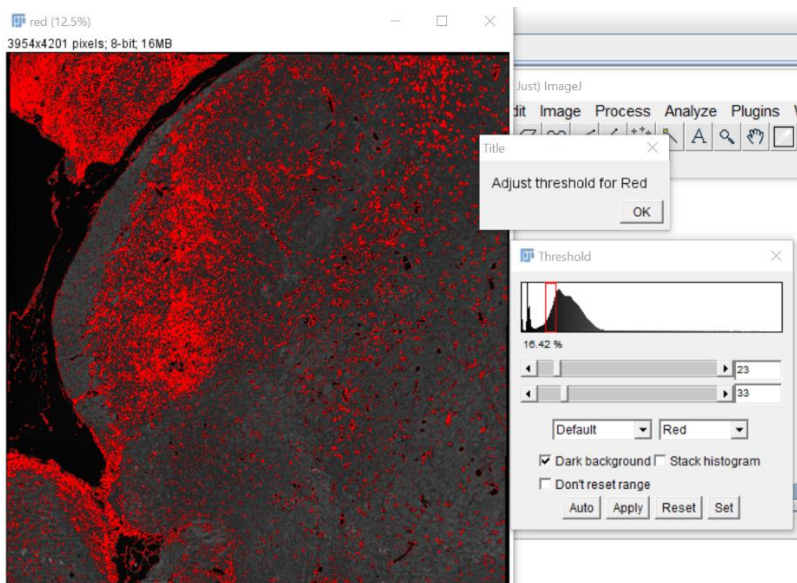

Setting the threshold mode
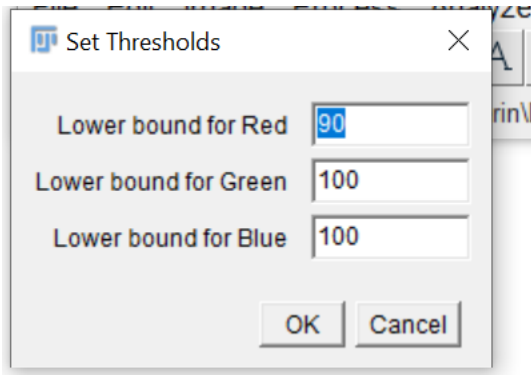
Setting the default thresholds

If the threshold mode is enabled, user can adjust and change the threshold after seeing the ROIs until a correct value is found. In the threshold mode, there is a loop where the user can choose the threshold as many times they want and see the ROIs until they decide to move on to analysis.


Adjusting the threshold in case of threshold mode

Particle Analysis

Tissue Area and Intensity

Before proceeding to particle detection and analysis, this macro measures the tissue area. The total tissue area value will be used in the output to normalize the number of particles detected or total staining area to the tissue area observed. Then, each original channel's intensity is also calculated. These values are added to the output table.

Particle Analysis and ROIs

In this macro, maximum and minimum size of ROIs detected are set before the particle analysis. This means that the user have to choose their minimum and maximum ROI sizes at the beginning of the macro in the 'Other Thresholds' window.Hence, it is important to have an idea of your desired particle size interval for each channel before running the macro.
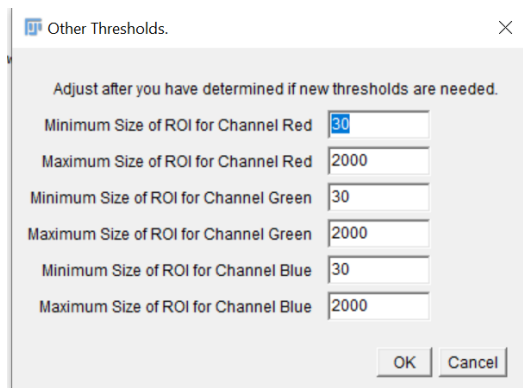
Setting Max and Min sizes of ROIs

Once the user has chosen the correct ROIs size exclusion parameters and thresholding values, image processor finds all the ROIs. Then, it counts the number of particles, total area of particles, number of cells and total area of cells. A cell is assumed to be a particle with size between 100 and 3000 for this macro. However, it is possible to change it. For this macro, the cell analysis is only output for the red channel, similarly, it is also very possible to extend it to other channels.

Colocalization

This macro focuses on the colocalization between particles detected in the green and red channels. It simply measures the ratio of common particle area and the channels (green and red) particle area. So there are two colocalization ratio at the end: red /green colocalization percentage and green/red colocalization percentage. For visualization, the new composite image is created and saved to output directory with the name "coloc_<yourimagename>".
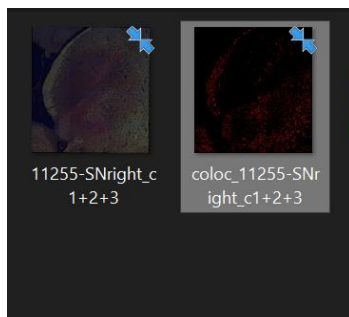
Composite image next to the original image

More details on Colocalization

For measuring colocalization percentage, we work on a duplicate image and get its green and red channels. Then, both channels are thresholded (user's chosen threshold taken here) and converted to a mask and measured for their integrated density. Finally, both images are multiplied measured for their integrated density (coloc. intensity) again.

The red green colocalization percentage is equal to (coloc. density/red density)*100.

The green red colocalization percentage is equal to (coloc. density/green density)*100.

Output file

Output file is basically a csv table named "output". It has information about:

- Image name
- Tissue area
- Big area (total image area)
- Used thresholds and ROIs
- Cell Areas and Cell Count
- Particle Areas and ROI count
- Ratio of Areas and Counts/ Tissue Area
- Colocalization percentages

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Filename	Tissue-a	Big-area	Red-inter	Red-thre:	Red-ROI	Red-Max	Red-Min:	Red-Total-area	Red-Cell	Red-Cell-Area	Red-ratio	Red-ratio	Red-ratio	Green-in	Green-th	Green-R(
11255-SN	1.5E+07	1.7E+07	40.1751	63	11558	2000	30	991031	2837	524976	0.00079	0.06762	0.00019	33.3002	0	19011

A view from the output file

Questions and answers

What does it mean if I get "NA" for my ROI count or in the colocalization values?

It means that the macro was not able to find any particles in that channel or process your image. Try running the macro with new ROI and threshold settings.

The macro is not counting some of my particles even though I highlight them when choosing a threshold, what can I do?

It is normal for the macro to not count all your particles; however, you can try to change your ROI settings.