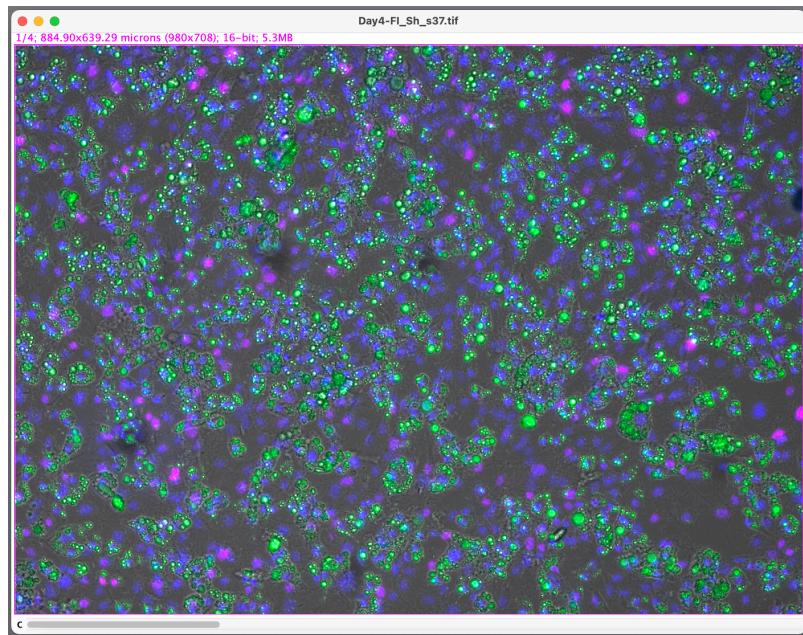


AdipoQ – a walk-through guide for an exemplary image

Input – For fluorescently labeled cultured cells

Example image: WAT- SVF cells, cultured *in vitro* and treated with Full Induction (FI) cocktail, fixed on day 4, stained with Ki-67 (magenta), DAPI (blue), and nuclei (green)



Note: For the walk-through guide, only a single image was analyzed. However, AdipoQ is designed for processing in a parallel manner.

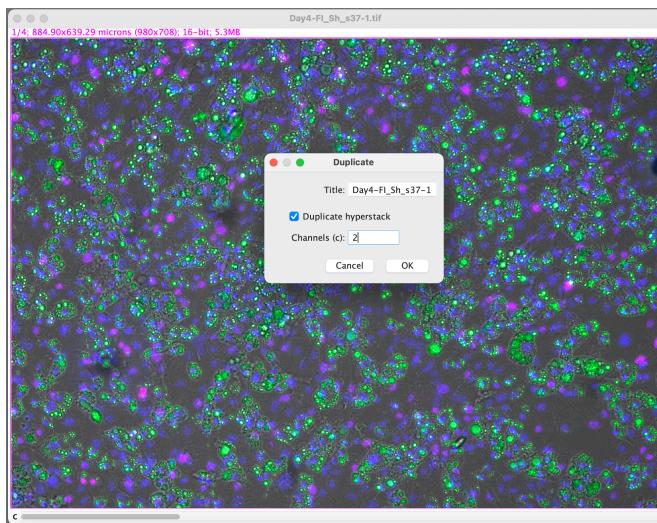
Channel 1	Channel 2
Ki-67	DAPI
A fluorescence image showing Ki-67 staining in magenta. The image is titled 'Day4-FI_Sh_s37.tif' and has dimensions of 884.90x639.29 microns (980x708); 16-bit; 5.3MB.	A fluorescence image showing DAPI staining in blue. The image is titled 'Day4-FI_Sh_s37.tif' and has dimensions of 884.90x639.29 microns (980x708); 16-bit; 5.3MB.
Channel 3	Channel 4
LD540	Transmitted
A fluorescence image showing LD540 staining in green. The image is titled 'Day4-FI_Sh_s37.tif' and has dimensions of 884.90x639.29 microns (980x708); 16-bit; 5.3MB.	A transmitted light image showing the cellular morphology. The image is titled 'Day4-FI_Sh_s37.tif' and has dimensions of 884.90x639.29 microns (980x708); 16-bit; 5.3MB.

1. Finding the best parameters to analyze your image

It is recommended that you manually apply the settings to an exemplary image first, to optimize the parameters. Changing and optimizing the parameters is necessary if you use a different input file format, a different dye or a different magnification. See the example for an in-depth workflow.

1.1 Nuclei detection (DAPI):

1. Duplicate image of the channel of interest so you have *two* copies of your channel of interest:
IMAGE > DUPLICATE here: channel 2



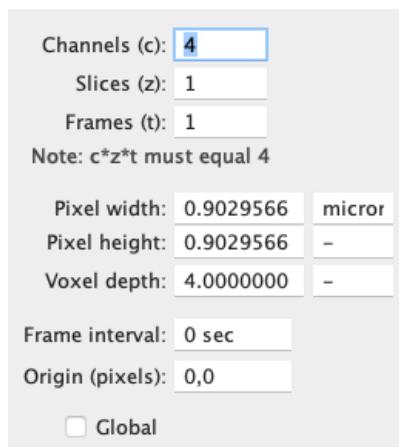
2. Duplicate the DAPI channel again, so you end up with two copies of the DAPI channel image.

Notes on finding the gaussian blur:

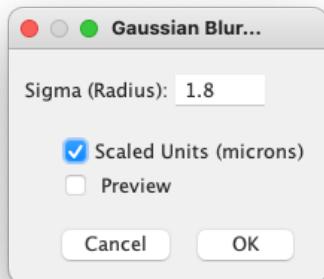
A gaussian blur can reduce detection/camera noise in the image and blurs the objects. Beyond that, it allows to blur-away small intensity differences in the object. This is particularly useful for segmenting a DAPI channel as otherwise these small points may be detected as objects instead of the whole DAPI nucleus.

Finding the optimal blur depends on the size of the object you are interested in (here: nuclei). Make sure that the image is calibrated in a metric unit – this allows to relate the distance in the image to a distance in the real world. It is required for correctly setting the blur in AdipoQ Preparator and also later on for outputting correct size estimates of the objects, i.e., nuclei.

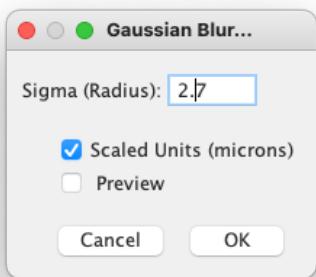
You can find out the calibration (pixel size) of your image by clicking **IMAGE > PROPERTIES**, e.g., my image has a pixel size of ~0.9 microns. The fact that the unit is specified in micron (= μm) shows me that the image is already calibrated. If it is set in pixel, try to find out the pixel size and enter it here, press OK, and save the image (This, unfortunately, needs to be done for all images you want to process).



3. Apply gaussian blur with a sigma of 1.8 μm for the first DAPI image (Day4-Fl_Sh_s37-1-1.tif)
[PROCESS > FILTERS > GAUSSIAN BLUR...](#)

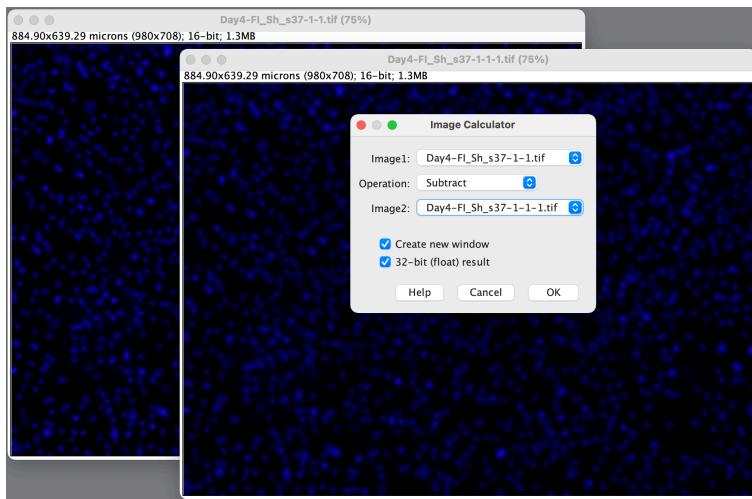


4. Click on the second image and apply a gaussian blur with a *sigma of 2.7 μm* for the second DAPI image (Day4-Fl_Sh_s37-1-1-1.tif)
[PROCESS > FILTERS > GAUSSIAN BLUR...](#)



Note that your second blur should always be larger than your first blur.

5. Subtract the second image from the first image and create a 32-bit (float) result.
[PROCESS > IMAGE CALCULATOR > GAUSSIAN BLUR](#)



6. On the blurred result image, you can try different threshold algorithms provided by ImageJ

Output of blur subtraction:

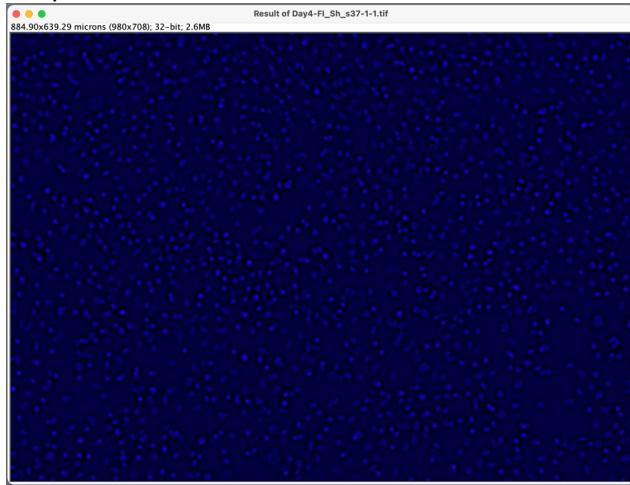
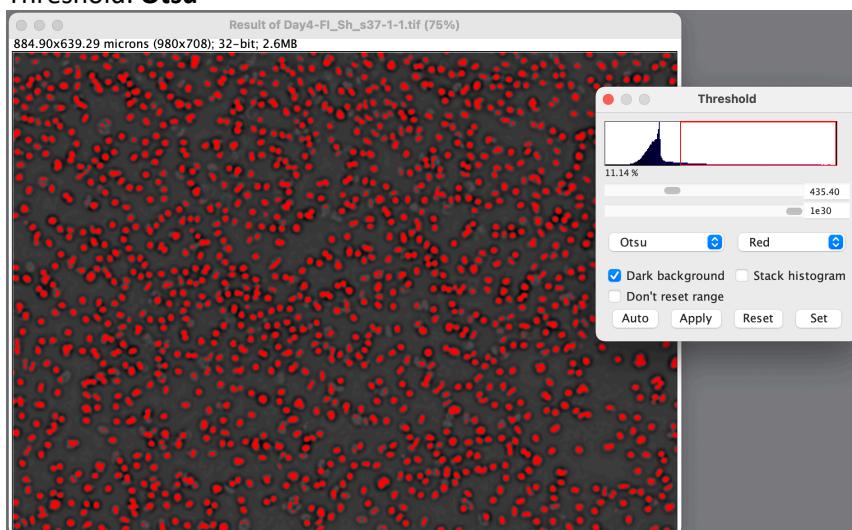
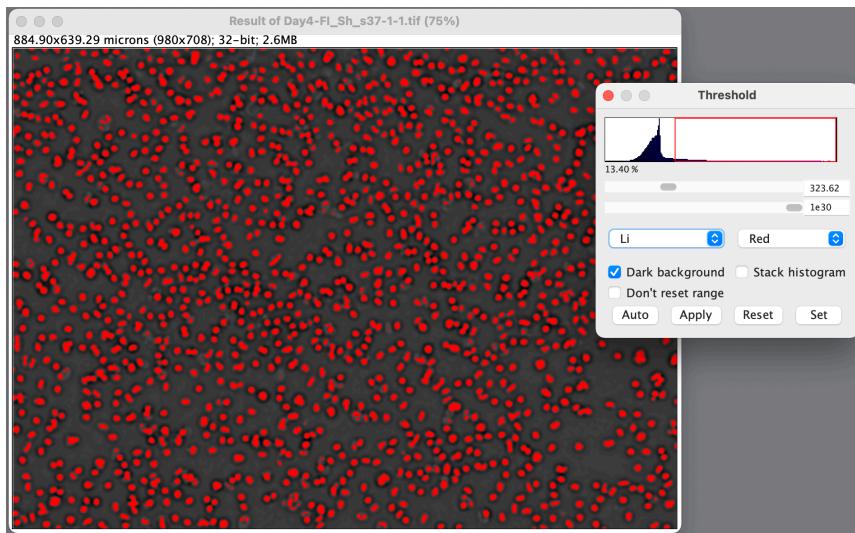


IMAGE > ADJUST > THRESHOLD

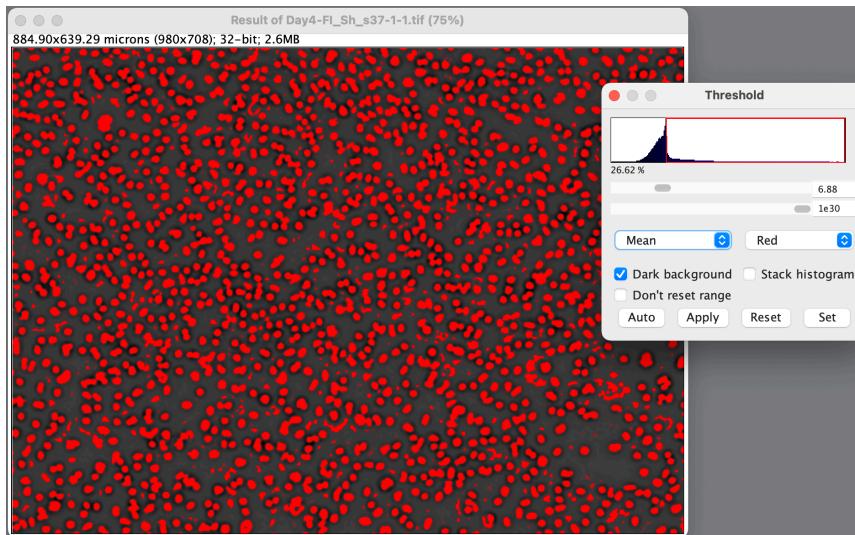
Threshold: Otsu



Threshold: Li



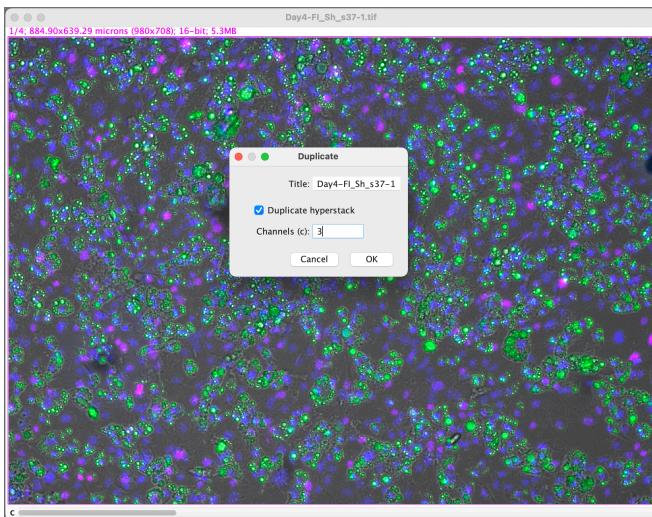
Threshold: Mean



For detecting nuclei, I prefer one of these three threshold algorithms. Based on these images I choose the Mean algorithm, as it covers all nuclei. Although it also covers some small particles (probably dirt), I prefer this algorithm as the others miss some nuclei. I also know that I can get rid of very small particles later in the using the Analyzer, as there is an option to filter for small particles.

1.2 Lipid droplet detection (LD540):

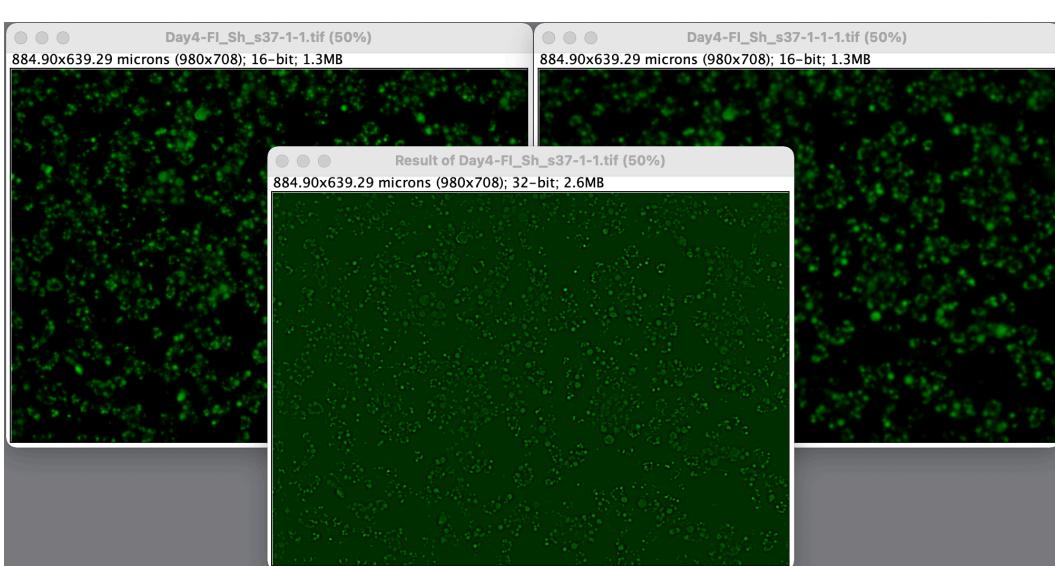
1. Start again by duplicating the image of the channel of interest so you have two copies of your channel of interest: **IMAGE > DUPLICATE** here: channel 3



2. Apply gaussian blur with a sigma of 0.9 µm for the first LD540 image (Day4-Fl_Sh_s37-1-1.tif)
[PROCESS > FILTERS > GAUSSIAN BLUR...](#)

As mentioned before, choosing the correct blur depends on the object size. When analyzing lipid droplets, you want to identify also very small droplets (smaller than nuclei). Thus, it is useful to take smaller sigma values than for the nuclei, as it enables a better representation of smaller structures due to lesser blurring.

3. Click on the second image and apply a gaussian blur with a sigma of 1.8 µm for the second LD540 image (Day4-Fl_Sh_s37-1-1-1.tif)
[PROCESS > FILTERS > GAUSSIAN BLUR](#)
4. Subtract the second image from the first image and create a 32-bit (float) result.
[PROCESS > IMAGE CALCULATOR > GAUSSIAN BLUR](#)



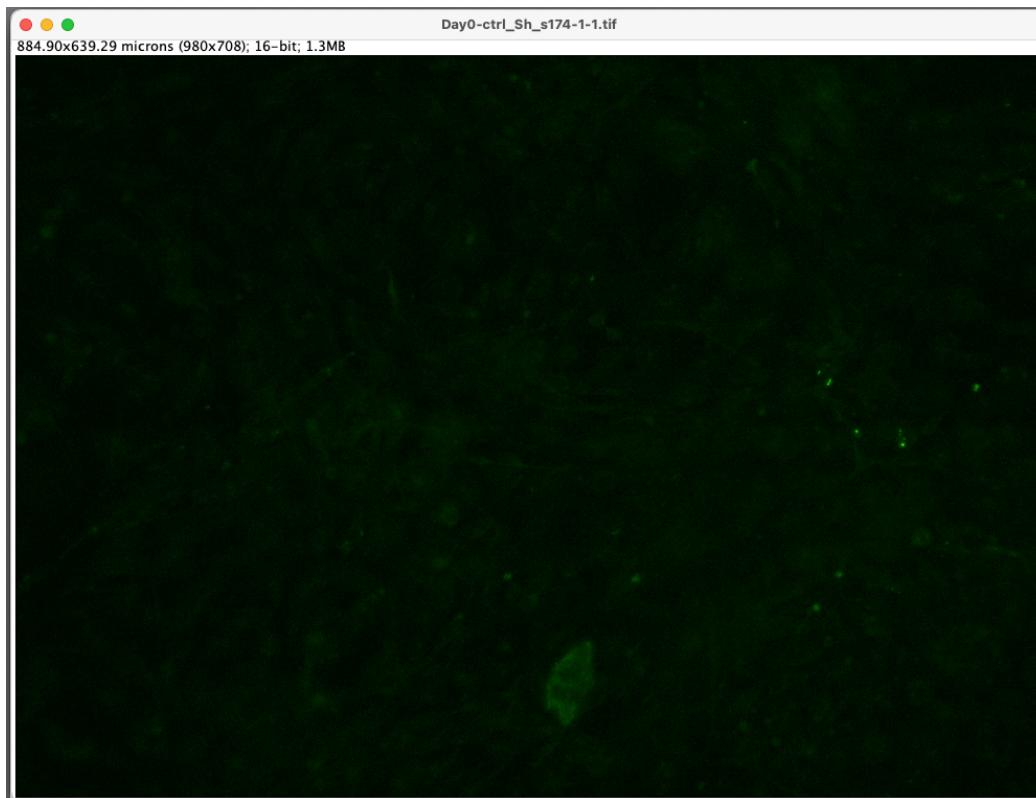
5. On the blurred result image, you can try different threshold algorithms provided by ImageJ

Run on the results image:

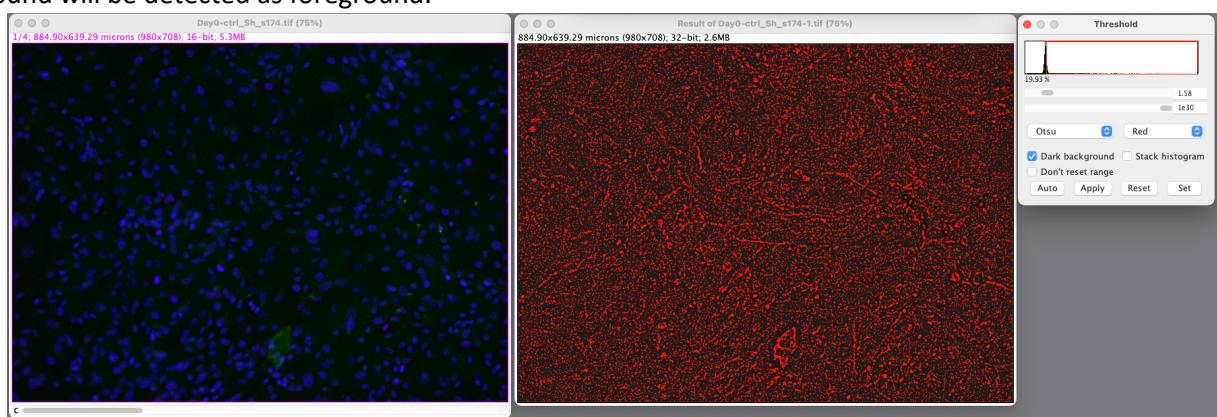
[IMAGE > ADJUST > THRESHOLD](#)

Notes on finding the correct threshold for lipid droplets:

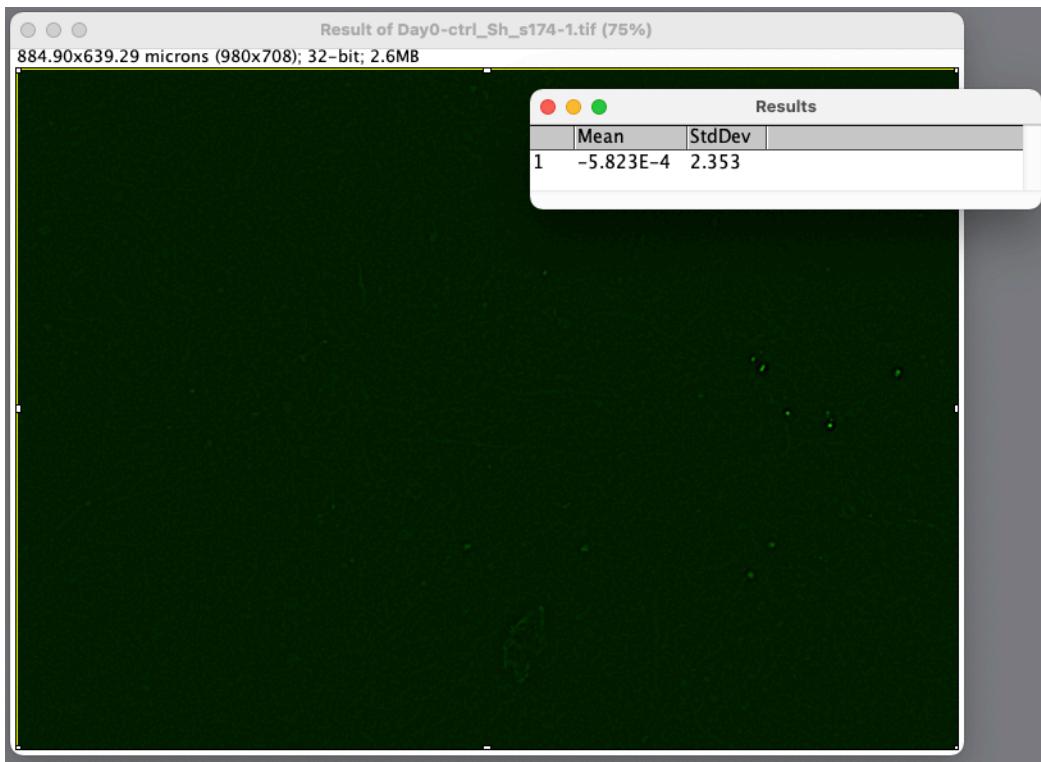
Be careful when choosing a threshold for lipid droplets: Intensity threshold algorithms can only correctly segment an image if it contains foreground and background structures. When you will include images into your analysis pipeline that are controls and do not show any lipid droplets, it may not be wise to use an automated intensity threshold in this pipeline.



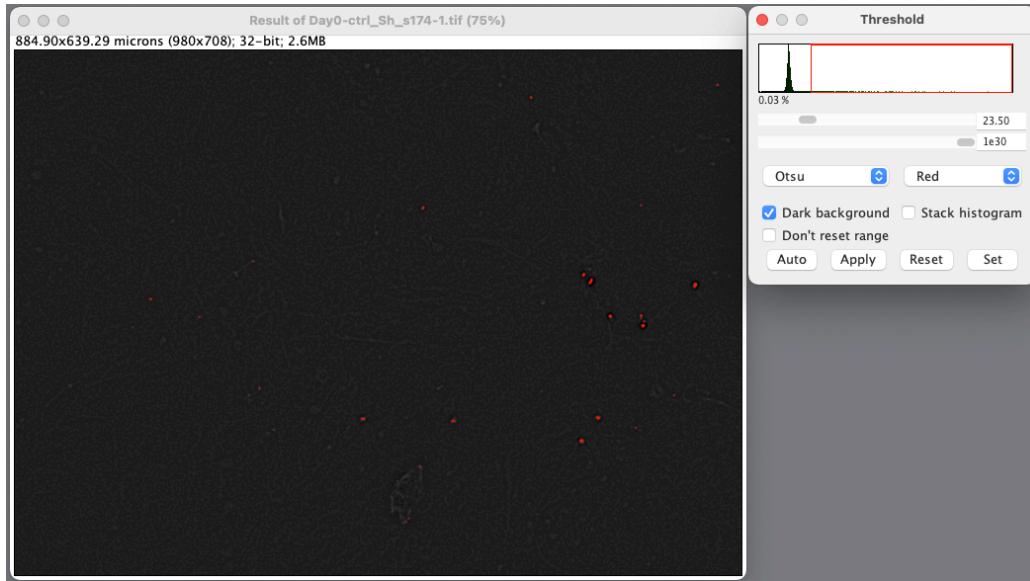
As can be seen, when the image does not contain any foreground structures (i.e., droplets), the background will be detected as foreground.



To circumvent this problem, we usually use a fixed threshold for the images from one experiment (that were stained at the same time). To determine the threshold, we use an image not showing any lipid droplets, blur and subtract the images, and next, measure the intensities in the image by selecting all with a ROI (press Control + A) and pressing M on the keyboard (or go to **ANALYZE > MEASURE** in the ImageJ menu).



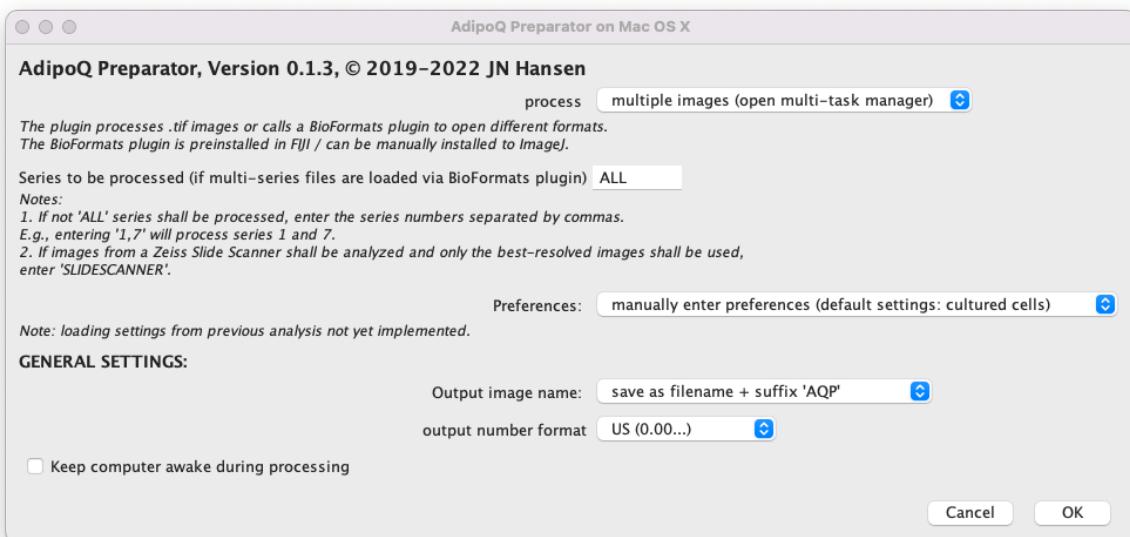
We then calculate the threshold as $10 * \text{STDDEV}$. For the example shown above, this would be $10 * 2.353 = 23.53$. As can be seen in the following example images, the segmentation works well when applying a threshold of 23.5.



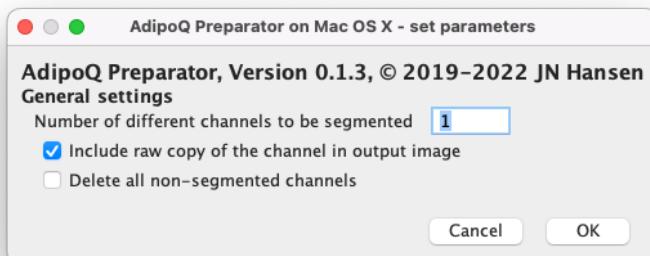
Now that all parameters have been tested and optimized, the AdipoQ preparator can be run with the ideal settings.

2. Set up the AdipoQ Preparator

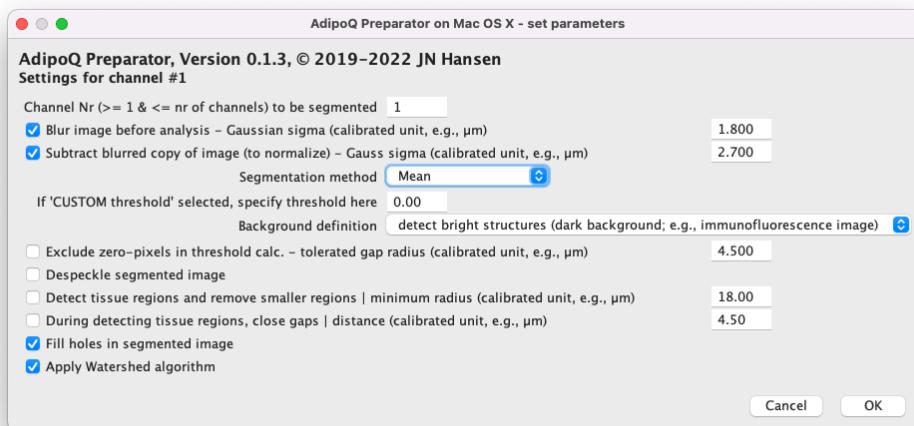
1. Launch the preparator and chose the default settings for preferences for cultured cells.



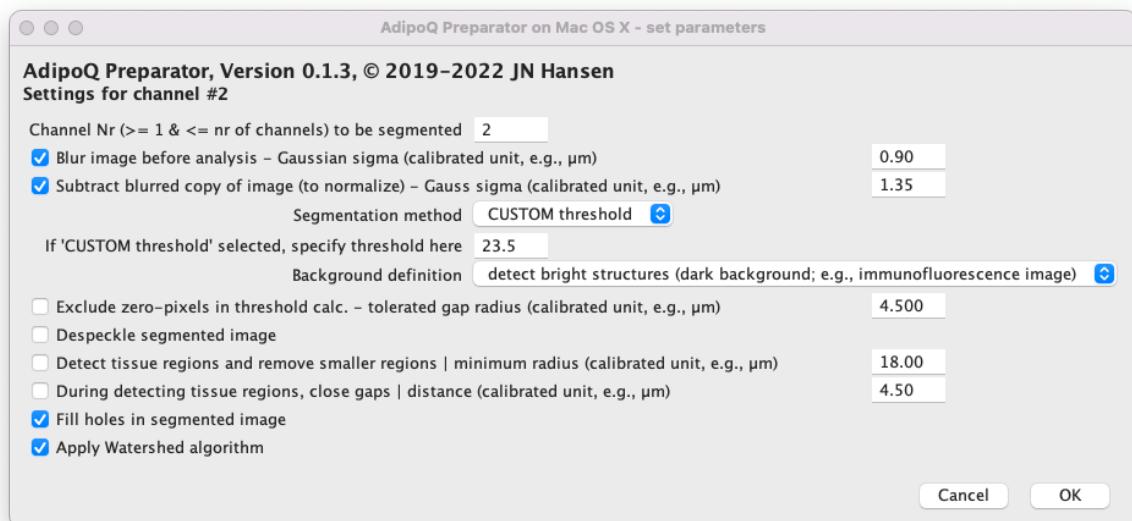
2. A dialog pops up that asks me how many channels should be segmented. As my image file contains both channels of interest (nuclei and lipid droplets) I will want to analyze them simultaneously. Therefore, I set the number for channels to be segmented to 2.



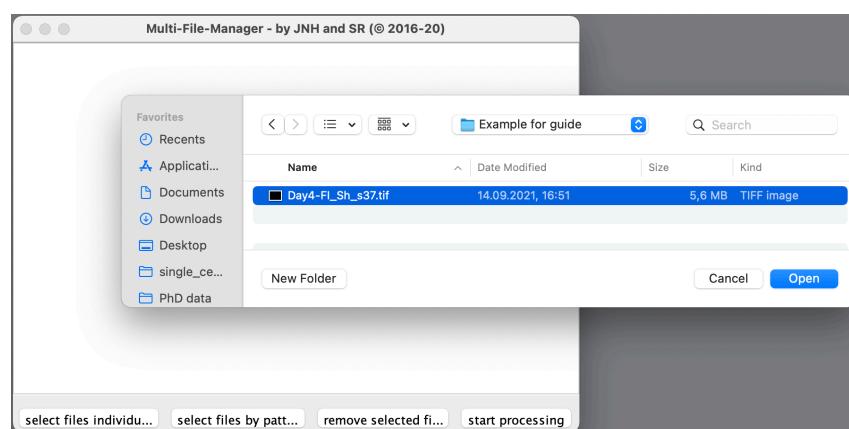
3. The first channel I want to analyze is the **DAPI channel**. Caution: Although the DAPI channel is the first one I want to analyze (channel #1), it is the second channel within my image file, as the first channel shows the Ki-67 signal. Therefore, I chose Channel Nr 2 for quantification.



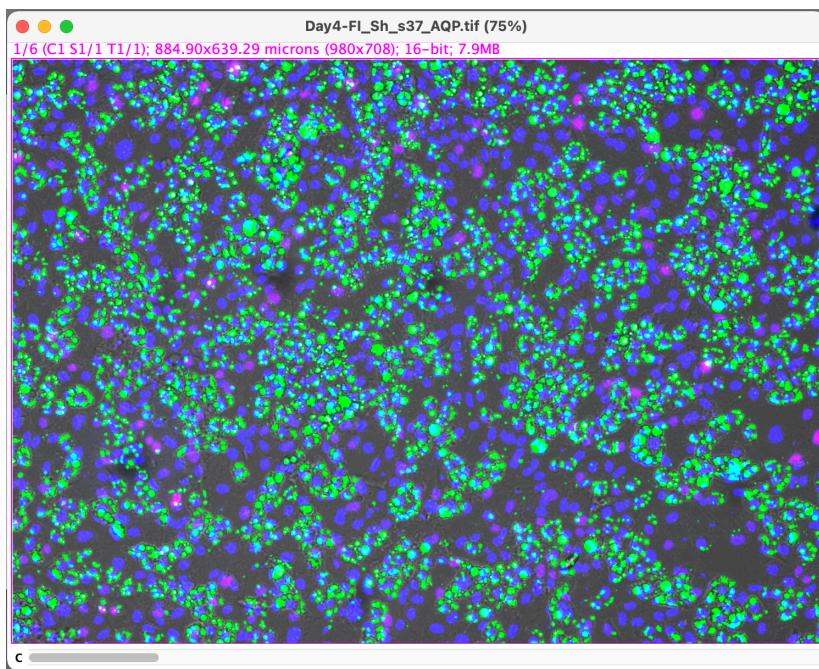
4. After pressing OK, the dialog pops up again for my second channel to be segmented. As my lipid droplets are in the third channel of my image data file, I chose channel Nr. 3 for quantification accordingly. Here, I change the sigma values for the Gaussian blur. I select a CUSTOM threshold as my segmentation method and enter the value I calculated above.



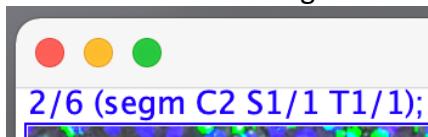
5. A dialog will pop up allowing you to load in your data file



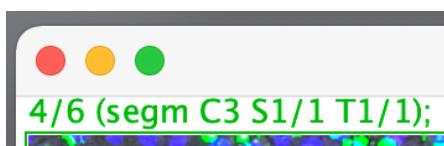
The output image file (_AQP.tif) will contain the new segmented channels as well as the original channels:



Channel 2 holds the segmented nuclei image:

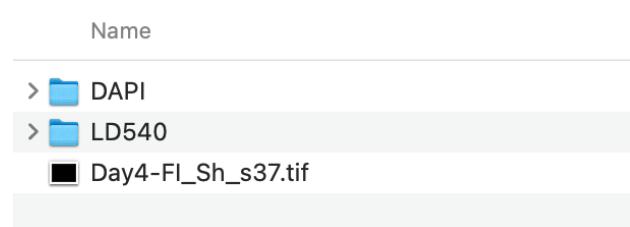


Channel 4 holds the segmented lipid droplet image:

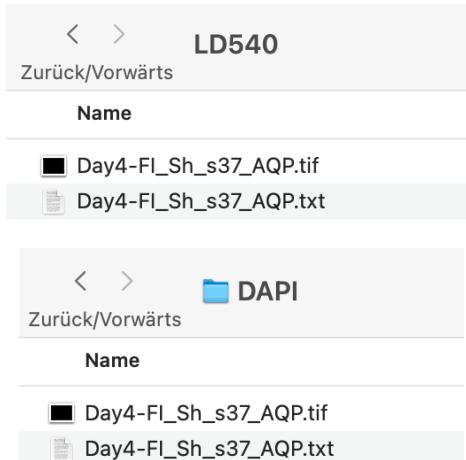


3. Set up the AdipoQ Analyzer

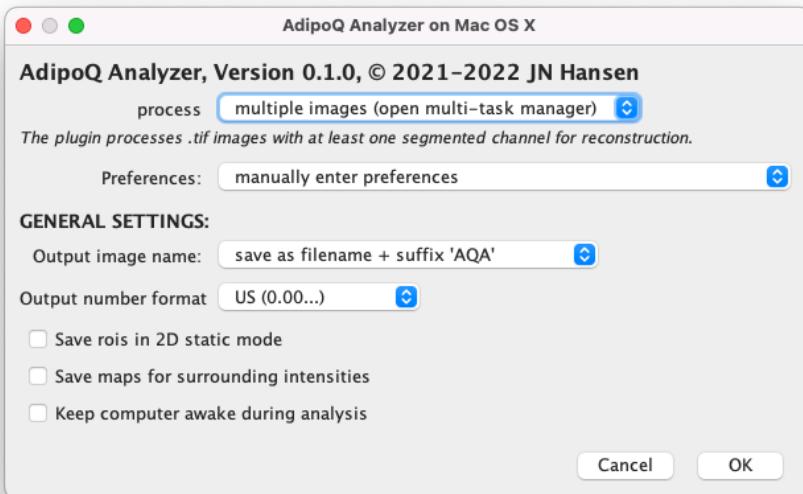
1. For quantification, I will copy the AQP output file to a folder for my nuclei quantification (DAPI) and to a folder for my lipid droplet quantification (LD540).



The LD540 folder and the DAPI folder contain the same files (_AQP.tif and .txt).

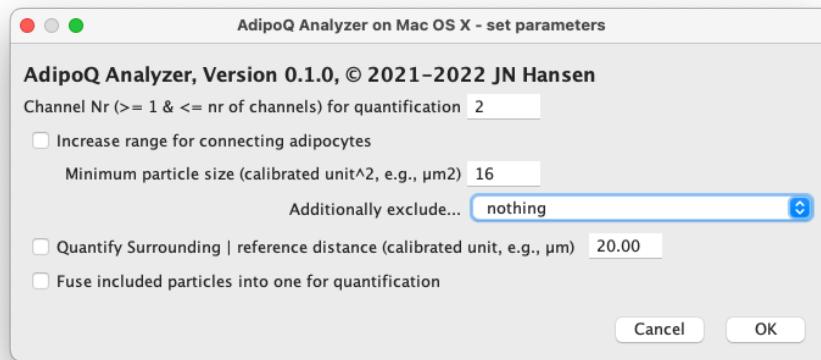


2. I will launch the Analyzer and will manually enter the preferences:

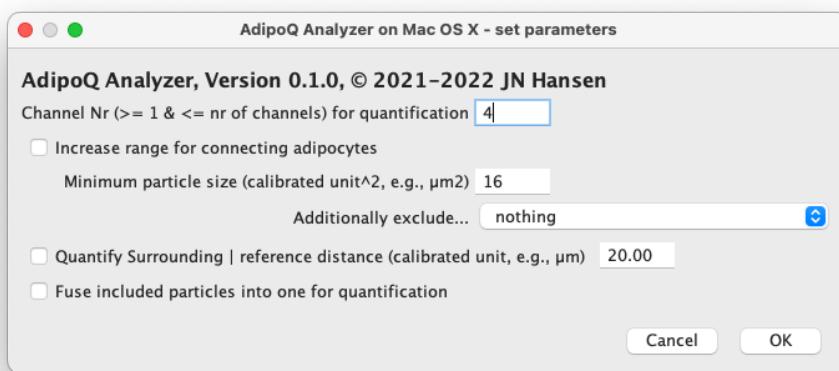


3. First, I will want to analyze my DAPI channel and chose channel Nr 2 (the segmented channel of DAPI).

As mentioned earlier I want to get rid of any small dirt particles so I filter for objects larger than $16 \mu\text{m}^2$. Consequently, the Analyzer will only consider the larger objects for quantification. When first using the tool, it might be helpful to run the Analyzer several times on the same image with different values for the minimum particle size and find the optimal value, as it is dependent on the image resolution and magnification. I do not choose to additionally exclude structures here.



4. After running the analysis on my DAPI channel, I will apply the same setting for the LD540 channel (indicated as channel number 4).



Output files:	_AQP_AQA_IDs.zip	IDs of objects (ROIs) in a zip file (can be loaded to ImageJ to overlay on an image by drag and drop)
	_AQP_AQA_RP.tif	A copy of the input image also containing labels of the detected objects
	_AQP_AQA.txt	A text file containing the analysis settings and results
	_AQAs.txt	A text file containing only the results lines from the file, including the table caption

4. Post-hoc analysis

Post-hoc analysis for calculating the adipogenic index, the number of nuclei, or the number of Ki-67 positive cells. Usually, the analysis will be done in R (script provided on GitHub).

However, for only one file the analysis can be easily done in excel.

Import the _AQAs.txt file, a tab-delimiter file containing the values of many parameters for each object.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Image name	ID	Custom	Frame	Total frames	Center X [micron]	Center Y [micron]	Center Z [micron]	Voxels	Area [micron ²]	Outline [micron]	2D-Asphericity Index

Calculating the sum of the Area (micron²) for all objects will give the total area of DAPI.

Counting the rows of the table or looking at the last ID number of all objects will give the total number of nuclei in an image.

Further information:

Please refer to the AdipoQ User Guide for a detailed and in-depth explanation of the AdipoQ Preparator and Analyzer tool.

Please refer to the example file for a walk-through guide for an exemplary image.