

AdipoQ User Guide

Content

<u>GET STARTED</u>	<u>2</u>
INFORMATION	2
INSTALLATIONS	2
WORKFLOW OVERVIEW	2
<u>INPUT IMAGES</u>	<u>4</u>
<u>PREPROCESS IMAGES WITH ADIPOQ PREPARATOR</u>	<u>5</u>
ADIPOQ PREPARATOR: SETTINGS	5
ADIPOQ PREPARATOR: MANUALLY ENTER PREFERENCES - FOR "HISTOLOGY"	8
ADIPOQ PREPARATOR: MANUALLY ENTER PREFERENCES - FOR "CULTURED CELLS"	15
ADIPOQ PREPARATOR: OUTPUT.....	22
<u>SCRUTINIZING AND EVENTUALLY CORRECTING SEGMENTATION.....</u>	<u>23</u>
<u>QUANTIFICATION WITH ADIPOQ ANALYZER.....</u>	<u>31</u>
ADIPOQ ANALYZER: GENERAL SETTINGS.....	32
ADIPOQ ANALYZER: AUTOMATICALLY LOAD PREFERENCES	32
ADIPOQ ANALYZER: MANUALLY ENTER PREFERENCES	33
ADIPOQ ANALYZER: OUTPUT FILE.....	35
ADIPOQ ANALYZER: DESCRIPTION OF OUTPUT PARAMETERS	39
SURROUNDINGS PARAMETERS.....	41
EXAMPLE FOR THE SURROUNDINGS PARAMETERS.....	44
<u>EXPLORE DATA SETS IN A POST-HOC ANALYSIS</u>	<u>45</u>

Get started

Information

The AdipoQ workflow has been developed in the research group *Biophysical Imaging* at the *Institute of Innate Immunity*, Bonn, Germany (<https://www.iiibonn.de/dagmar-wachten-lab/dagmar-wachten-lab-science>). Before using AdipoQ, we recommend to read the AdipoQ publication. Please cite AdipoQ when presenting results obtained with AdipoQ:

A citation will be available here as soon as the AdipoQ publication is published - please visit <https://github.com/hansenjn/AdipoQ#how-to-cite> to get the latest information about how to cite AdipoQ!

For news on AdipoQ and updates of the plugins or the user guide, please check the central AdipoQ GitHub repository: <https://github.com/hansenjn/AdipoQ>. Alternatively, and for any other inquiries, feel free to contact jan.hansen@uni-bonn.de or dwachten@uni-bonn.de.

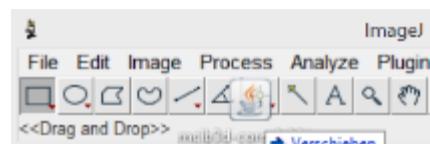
Installations

Download the latest releases of the following ImageJ plugins:

- AdipoQ_Preparator_-...-SNAPSHOT.jar
(https://github.com/hansenjn/AdipoQ_Preparator/releases)
- AdipoQ_Analyzer_-...-SNAPSHOT.jar
(https://github.com/hansenjn/AdipoQ_Analyzer/releases)

Unfortunately, AdipoQ Preparator has the most functionalities in FIJI while AdipoQ Analyzer runs fastest on ImageJ. Thus, it is recommended to install a FIJI and an ImageJ distribution (if not already installed) and use AdipoQ Preparator in FIJI and AdipoQ Analyzer in ImageJ. Briefly, perform it as follows:

Install FIJI (<https://fiji.sc/#download>) and open FIJI. Install the downloaded AdipoQ_Preparator_....jar file into you FIJI distribution by dragging and dropping the .jar files into the status bar of the FIJI window; If a dialog pops up, press save and restart FIJI.



Next, install ImageJ (<https://imagej.nih.gov/ij/download.html>) and open ImageJ. Install the downloaded AdipoQ_Analyzer_....jar file into you ImageJ distribution by dragging and dropping the .jar files into the status bar of the ImageJ window; If a dialog pops up, press save and restart ImageJ.

Note for Mac users: Mac OS performs a certain type of “Path randomization” as a security mechanisms and thus, in case you install ImageJ and FIJI to the Applications folder, you might encounter error messages when launching one of the AdipoQ plugins. For Mac users, it is recommended to place the FIJI or ImageJ program somewhere else than the Applications folder (e.g. on the desktop or in the documents folder), where you should not encounter these problems.

Workflow overview

AdipoQ follows a two- to four-step workflow based on two ImageJ plugins and one R script:

0. Automatically preprocess images with AdipoQ Preparator to segment the channels containing adipocytes (for immunohistochemistry) or lipid droplets / nuclei (for fluorescent stainings)
1. *OPTIONAL:* scrutinize and correct the images output by AdipoQ Preparator.

2. Automatically quantify adipocytes / droplets / nuclei with AdipoQ Analyzer: Connects adjacent pixels to objects, quantifies objects, filters out noise using a size threshold.
3. *OPTIONAL:* Convolve analysis data from many analyzed images and explore the data set in R using one of our R-markdown templates (<https://github.com/hansenjn/AdipoQ/tree/main/R%20Scripts>).

Input images

AdipoQ allows to **(A)** analyze classical HE-stainings of adipose tissue or **(B)** analyze fluorescent images of samples where lipid droplets or nuclei were labeled with fluorescent labels. You may use different methods to record the images.

For HE-stainings, we have used a Zeiss Slide Scanner that allows to automatically scan huge adipose tissue sections embedded in Paraffin. The slide scanner delivers the images as .czi-files, that contains multiple images from one slide). From the .czi file, the images can be directly processed using AdipoQ Preparator. The images recorded from a HE-staining are usually RGB (Red Green Blue) images. Thus, in AdipoQ Preparator one of the three channels (Red, Green, or Blue) needs to be selected. Our pipeline is optimized to analyze the tissue based on the Green channel.

For fluorescent images, we used a Zeiss Cell Discoverer (an automated fluorescence microscope), as this allows to perform large-scale experiments with many conditions and high-throughput. The Zeiss Cell Discoverer stores all images from one run in one .czi file. Also, here, AdipoQ Preparator will allow to load a .czi file and process all (or selected) images of the whole experiment. Of note, AdipoQ Preparator analyzes 2D images and not 3D stack images. Thus, you may need to convert images recorded with such an automated microscope that records multiple planes of the sample into a 2D image. For example, we have acquired multiple z planes with a Zeiss Cell Discoverer for each image, because this allows to definitely feature a z plane with cells in focus, even when the auto-focus does not work perfectly. Additionally, it could be that nuclei and lipid droplets lie in different focal planes and thus require recording of multiple planes to get a sharp image of both. In such cases we would like to extract the sharpest planes from the stack to analyze them with AdipoQ. This can be a tedious job, when done manually for big data sets. We thus also have developed an ImageJ plugin that can help you by fully-automatically extracting and saving the sharpest plane of stack images (more information, see here: https://github.com/hansenjn/ExtractSharpestPlane_JNH).

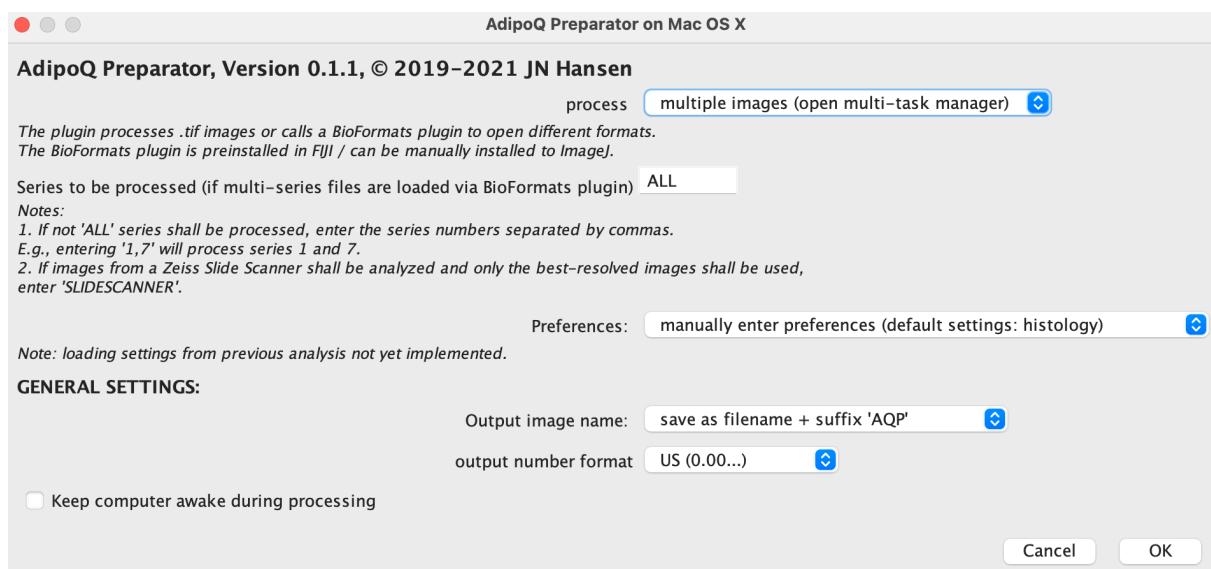
To load file formats like .czi, AdipoQ Preparator needs to be installed in FIJI, as in FIJI, a plugin called BioFormats for opening such microscope file-formats is pre-installed. However, when only .tif images should be processed, you can also use AdipoQ Preparator in ImageJ.

Preprocess images with AdipoQ Preparator

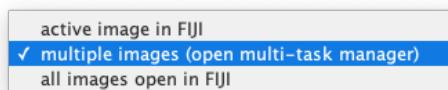
For segmenting the channel that allows to separate the object of interest (i.e., the green channel of HE-stained adipose tissues, or the lipid droplet and nuclei channels of fluorescently-labeled adipocytes) from background, use the ImageJ plugin *AdipoQ Preparator*. Launch it via [PLUGINS > ADIPOQ > ADIPOQ PREPARATOR V....](#)

AdipoQ Preparator: Settings

Upon launching the plugin, the following dialog pops up:



The **process** setting offers three possibilities to load data into the plugin and process these as a batch



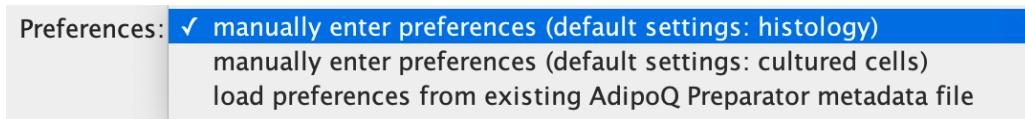
- Multiple images: a dialog will open after pressing ok (see below). Here you can generate a list of images to be processed. Add files to the list (select files individually) and press start processing. Alternatively, files can be added to the list based on their file names (select files by pattern)



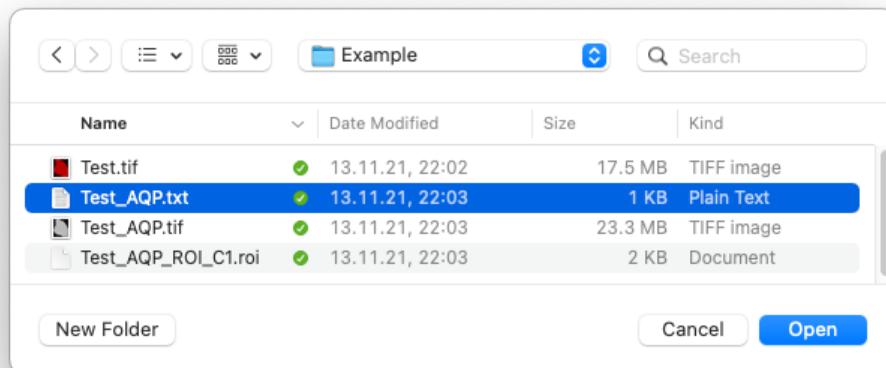
- Active image in FIJI: the currently front-most image opened in FIJI will be processed
- All images open in FIJI: all images that are open in FIJI will be processed

Note that the software can process only saved images – every image to be processed needs to be saved on the hard disk before it can be processed. This is required because AdipoQ_Preparator needs a path where it shall store the output image and metadata. Only saved images can provide that path in FIJI. If you process the image in FIJI and subject an unsaved image to AdipoQ_Preparator, it produces errors.

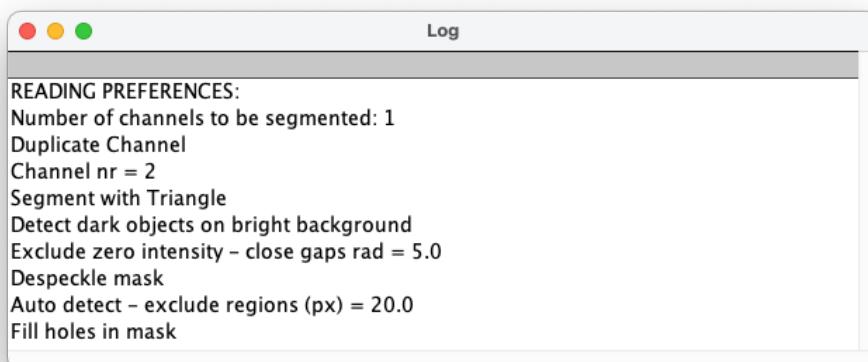
Preferences can be either manually entered or loaded from the meta-data file generated by a previous AdipoQ Preparator run (ending “..._AQP.txt”).



If load preferences from existing AdipoQ Preparator metadata file is selected, a dialog will open that requests you to select a AdipoQ Preparator metadata file to import settings – no settings need to be manually entered. Navigate to the folder where the pre-existing AdipoQ Preparator run was stored and select the AQP.txt file from it to load the settings from that run.



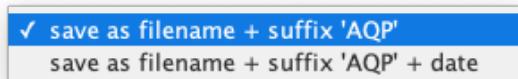
In the Log windows that pops up next, you can check the loaded preferences.



Next, either AdipoQ Preparator directly launches or you will be directed to the dialog to load the input files.

If manually enter preferences is selected, further dialogs are displayed to enter the preferences. Here, two options can be selected to pre-set the preferences that can be set in the next dialog - settings tailored to an analysis of HE stained tissue (“default settings: histology”) or to an analysis of cultured cells (“default settings: cultured cells”).

The Output image name option allows to add the date and time of processing for each output file. This is particularly useful when you reanalyze files, because commonly AdipoQ Preparator overrides the output file when you run it again. If you however select to add the date and time to the file name, output file names will be unique and you will not override a previous run.



The Output number format option defines in which format numbers will appear in the metadata text file - this is usually not relevant for AdipoQ Preparator runs as you never read in this numbers. It is more important when you run AdipoQ Analyzer (see below).



The option Keep computer awake during processing allows to, when selected, prevent your computer from going to sleep mode during processing, which could pause the analysis. It is useful, when you run a large batch and have no control on a computer to prevent going to sleep (e.g., on Mac Books this can be difficult and thus select this option definitely if you are running the program on a Mac book).

AdipoQ Preparator: Manually enter preferences - for “histology”

Initially, a dialog pops up that allows you to select how many channels shall be segmented and whether the original channel and additional channels shall be kept in the image.

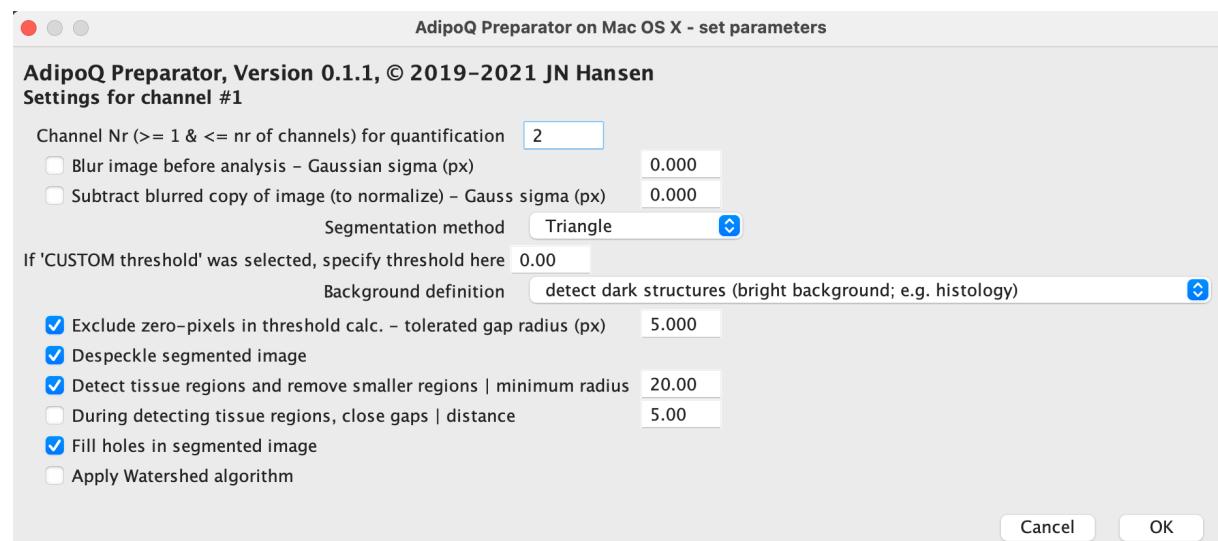
For HE stainings, only one channel needs to be analyzed (we usually analyze just the green channel) and the other channels are not relevant for the analysis results. Accordingly, leave the settings as pre-defined:



More detailed information:

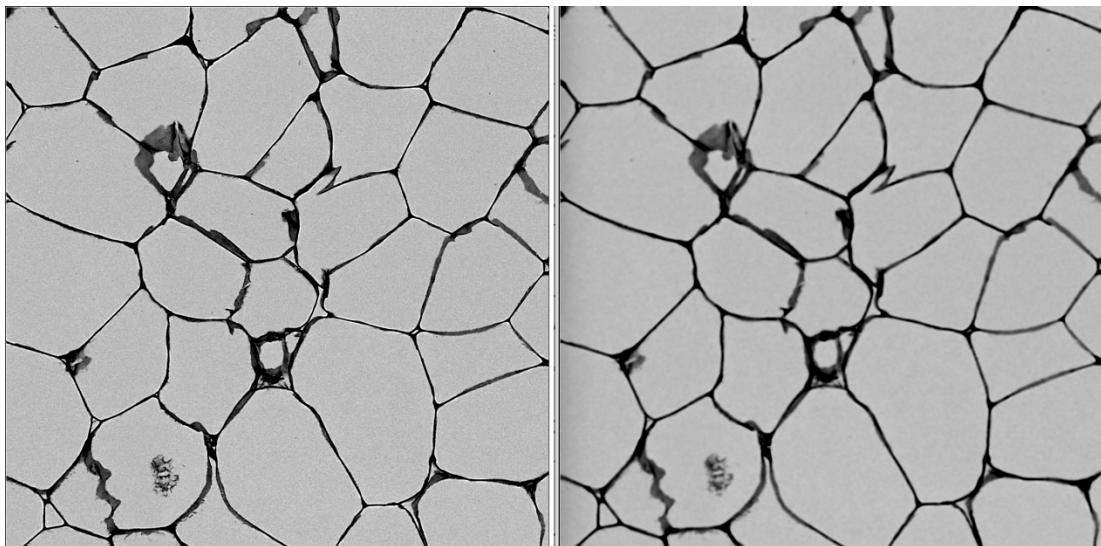
- Include raw copy of the channel in output image:** If you select this option, the channel is duplicated before segmentation. In the output file you will find two versions of the channel, one segmented version and next to it the raw unsegmented version. This option is particularly helpful to check in the output image, whether the segmentation worked well and to eventually correct segmentation errors, as the output image will show an overlay of segmentation and raw channel.
- Delete all non-segmented channels:** In the output image, all channels except for the segmented one will be removed. Usually, for HE staining images, these are obsolete as they do not need to be analyzed. Accordingly, removing them, reduces the file size of the output image.

When confirming the dialog, another dialog pops up that allows you to set the settings more specifically:



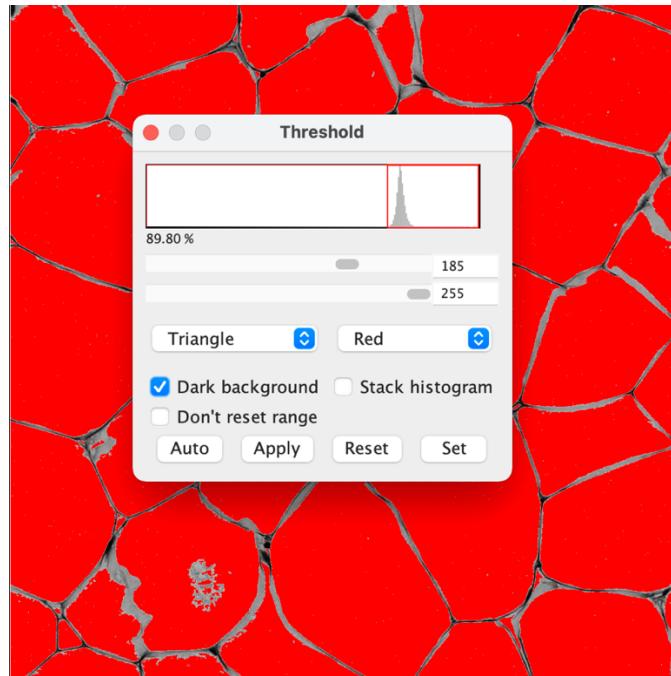
- Channel Nr:** the number of the channel that needs to be processed (e.g. first channel = 1, second channel = 2, etc.). In an RGB image, channel 1 represents the red, channel 2 the green, and channel 3 the blue channel. As we usually select the green channel for HE-staining images, we select channel 2.

- AdipoQ Preparator allows two options to preprocess the image before threshold calculation. These options allow to reduce the noise in the image and normalize the image to the background. For HE staining images, they are usually not necessary, but could be applied in case the image is not of high quality.
 - **Blur image before analysis - Gaussian sigma (px):** A gaussian blur can reduce detection / camera noise in the image and smooth the lines that surround the adipocyte. Image below: raw image on left, blurred image on right (Gaussian sigma 2 px).

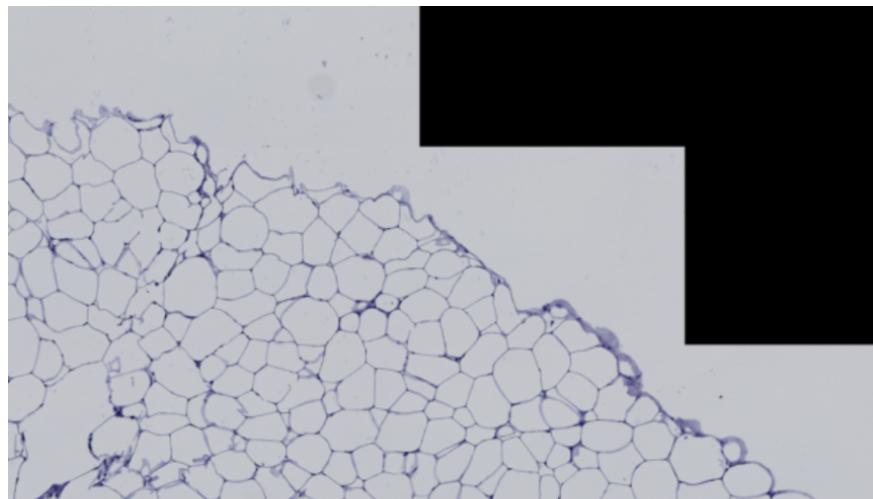


- **Subtract Blurred copy of image (to normalize)...**: A function that subtracts the local background and thereby, can normalize overall intensity differences in the image. This function may be mostly useful for fluorescent images. For HE staining images, it might be useful to apply this function with a radius larger than several adipocytes, in case the staining is unequally pronounced in different regions of the image.
- **Segmentation method:** Here you can either select one of ImageJ's common intensity threshold algorithms (such as RenyiEntropy, Huang, Triangle, Li, Huang ...), or specify a CUSTOM threshold value that you then could enter in the number field below this settings. ImageJ's threshold algorithms separate the objects from the background based on the histogram of the image. The algorithms differ in how they calculate the threshold. Thus, there are less harsh thresholds (that define rather more as background, e.g. MinError, Huang, Triangle, Li) and more harsh thresholds (that define rather less as foreground, e.g. Otsu, Renyi Entropy). If the segmentation does not work well, you should think about switching the threshold method. You can try the effect of different threshold methods in ImageJ as follows.
 - Open an example image
 - Preprocess the image as you would do automatically (see above).
 - Eventually, if you had selected this in AdipoQ Preparator, perform a Gaussian Blur by going to "Process > Filters > Gaussian Blur",
 - Eventually, if you had selected this in AdipoQ Preparator, manually subtract a blurred copy of the image as follows:
 1. Duplicating the image ("Image > Duplicate")
 2. Blurr it "Process > Filters > Gaussian Blur"
 3. Subtract it from the original image ("Process > Image calculator", select original image as Image 1, Operation "Subtract", and the duplicated blurred image as Image 1)

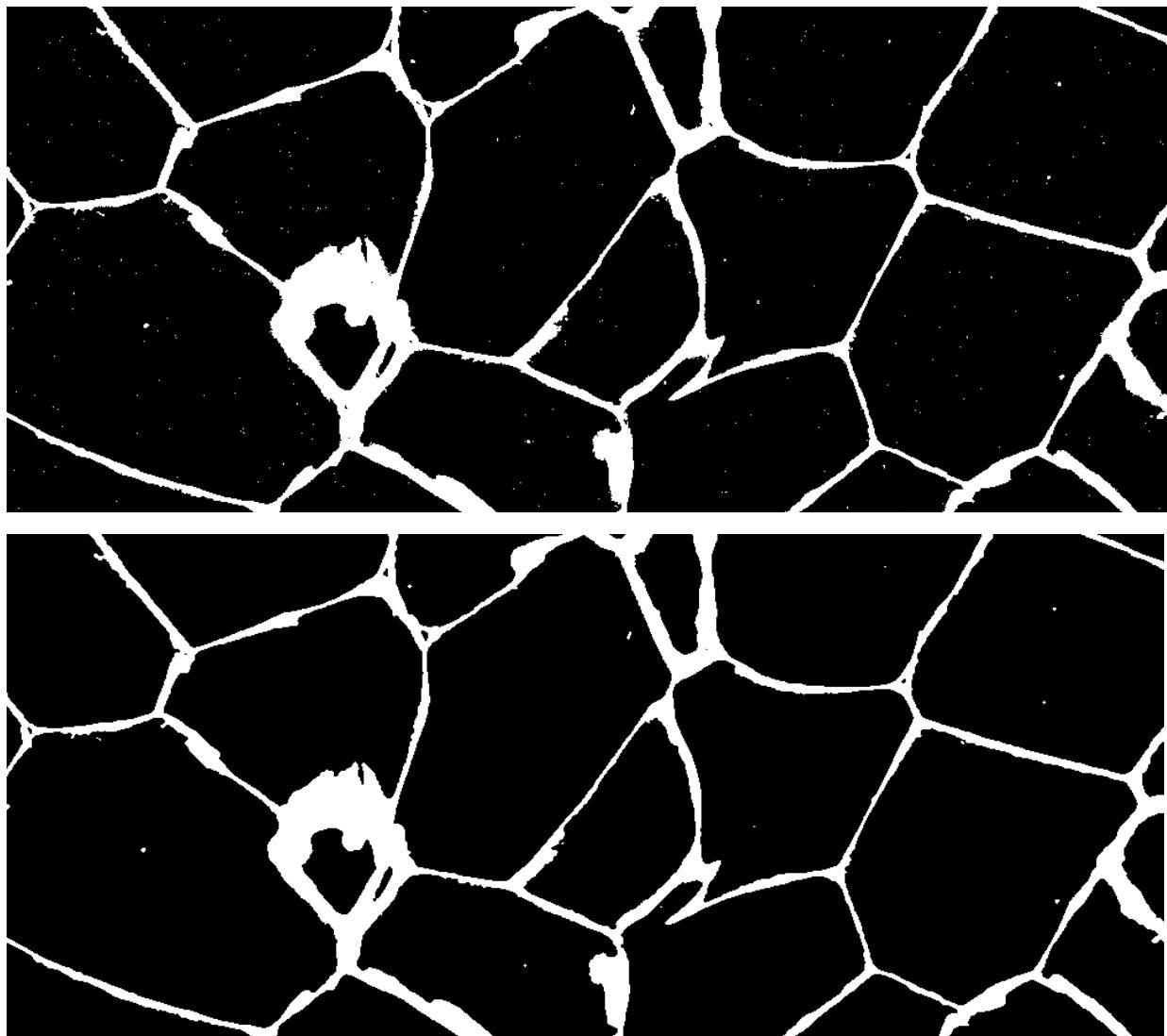
- Open the threshold tool (“Image > Adjust > Threshold...”) and select different thresholds. FIJI / ImageJ will automatically show you what they detect, e.g. with a red coloring.



- **Background definition:** Define whether you aim to detect bright objects on dark background or the opposite.
- **Exclude zero-pixels in threshold calculation - tolerated gap radius...:** When analyzing images that have been stitched (e.g. when imaged with a slide scanner), you can see that there are blank, black regions in the image. These regions falsify an automated threshold calculation, as they add a lot of zero pixels to the image's histogram. To circumvent that, AdipoQ Preparator includes a method to automatically detect these regions and ignore them during threshold calculation. This works as follows:
AdipoQ Preparator will detect all zero-pixels in the image and exclude them from the threshold calculation. However, zero-pixels may not only occur in such big regions where no image was recorded, but could also occur as individual pixels at boundaries of adipocytes (if the image intensity level is saturated). To avoid that such individual pixels from the sample are excluded in threshold calculation, AdipoQ Preparator performs operations to still include regions with zero-intensity pixels if they are individual and if they are surrounded by non-zero-intensity (= signal) pixels. The radius specified for this settings defines in which surrounding there must be non-zero-intensity pixels to include a zero-intensity pixel. Set this radius to a size that will be as wide as the thickness of the dark lines between the adipocytes.

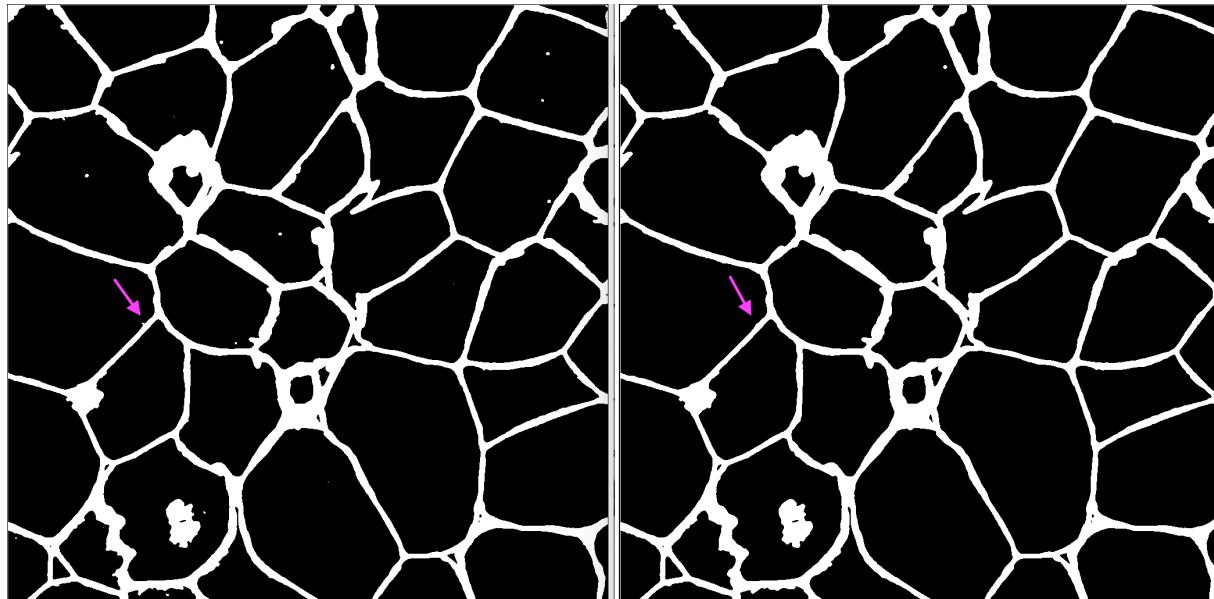


- **Despeckle image:** In the segmented image there can be a lot of small tiny dots (pixels) that emerge from detection noise. Such pixels would be detected as adipocyte boundaries although they are not. Despeckling the mask can remove those and allow a more precise detection of the adipocytes. Images below: before (top) and after (bottom) despeckling.

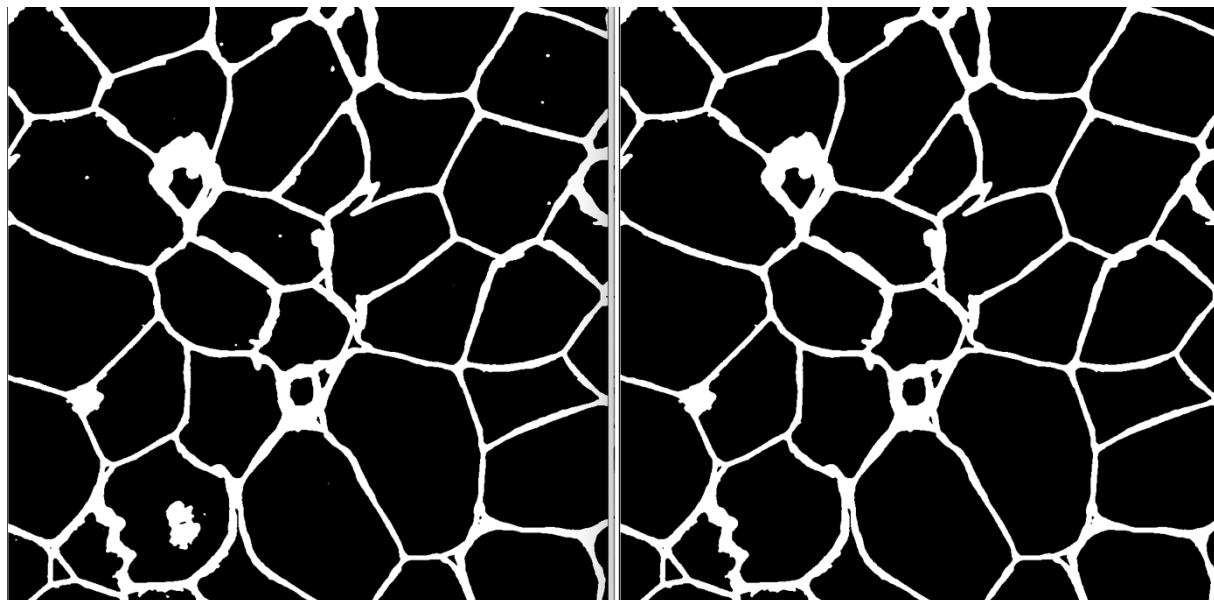


- **Detect regions and remove smaller regions:** Make sure to use this option, when analyzing HE-stained images - the reason for this is explained in the following. AdipoQ Preparator detects the boundaries of adipocytes in HE-staining images, not the adipocytes themselves. Thus, when there is blank space around the adipose tissue, this would be detected as a gigantic adipocyte. To avoid this, AdipoQ Preparator includes this function to detect a concatenated tissue region. Furthermore, this function allows to remove small tissue fragments surrounding the actual tissue of interest (which can occur in paraffin slices). The radius that can be specified defines the maximum radius of tissue fragments to be removed.

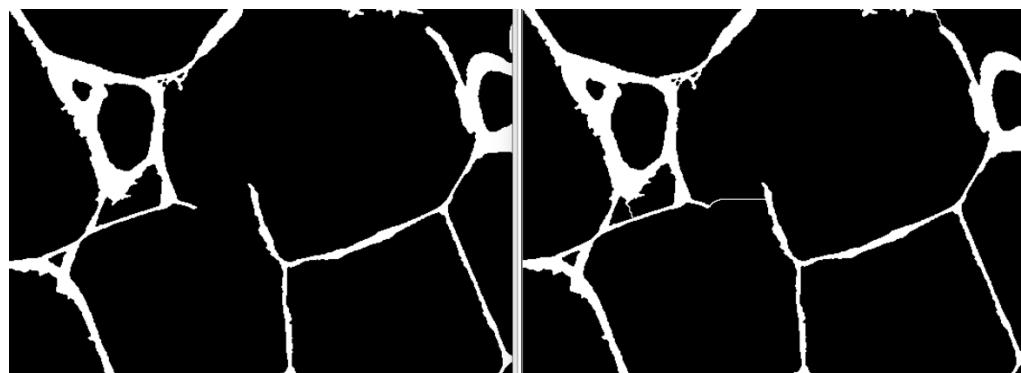
- **During detecting tissue regions, close gaps:** This option allows you to close “gaps” in the mask of individual adipocytes, which may image from noisy images or bad stainings. The defined radius defines the size of the maximal gap that is closed. Importantly, the gaps will also be closed when they are at the border of the adipocyte - such gaps would not be closed by the next mentioned preferences option “Fill holes”. Make sure that the radius is not too high, it should be at least 2x smaller than then lines between the adipocytes.
The image below shows a mask, where detected adipocytes are black, before (left) and (after) performing the close gaps option with a radius of 3 px. The function also removes noise at the adipocyte border (see magenta arrows).



- **Fill holes:** This option, when selected, fills holes in the adipocyte. The image below shows a mask, where detected adipocytes are black before (left) and after (right) filling holes.



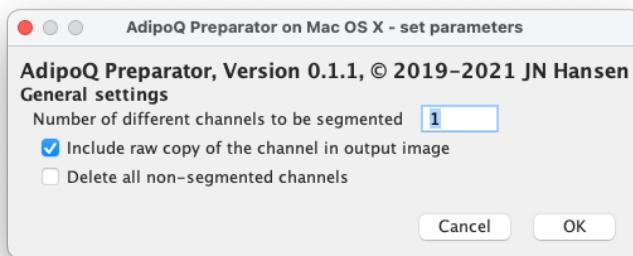
- **Apply Watershed algorithm:** This function can be useful if your images are of bad quality or if the lines encircling adipocytes are not very clear and continuous. Then, the Watershed algorithm may allow you to better detect adipocytes. Image below shows the mask (detected adipocytes = black) before (left) and after (right) applying the Watershed algorithm.



AdipoQ Preparator: Manually enter preferences - for “cultured cells”

Initially, a dialog pops up that allows you to select how many channels shall be segmented and whether the original channel and additional channels shall be kept in the image.

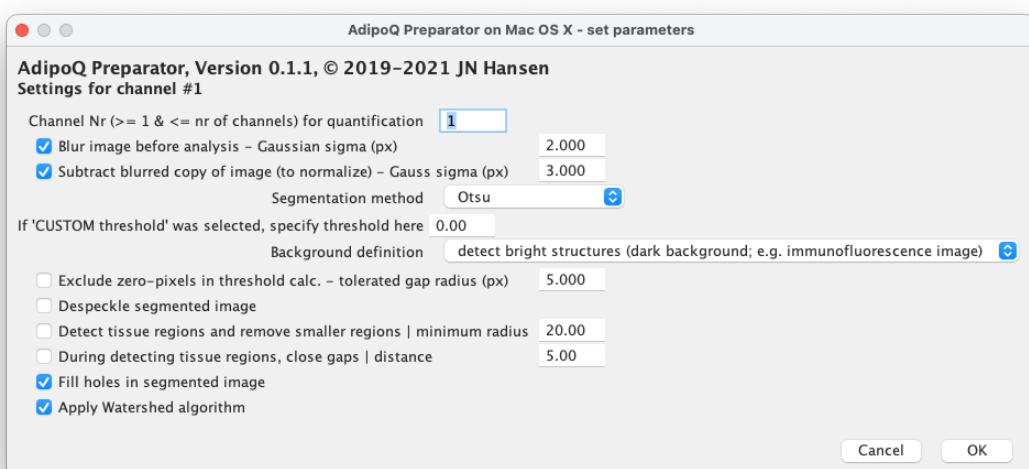
If you only plan to analyze droplets or nuclei, enter 1 in the field “Number of different channels to be segmented”. If you aim to analyze both, nuclei and droplets, you can enter 2 and you will be able to segment two channels in the next dialogs.



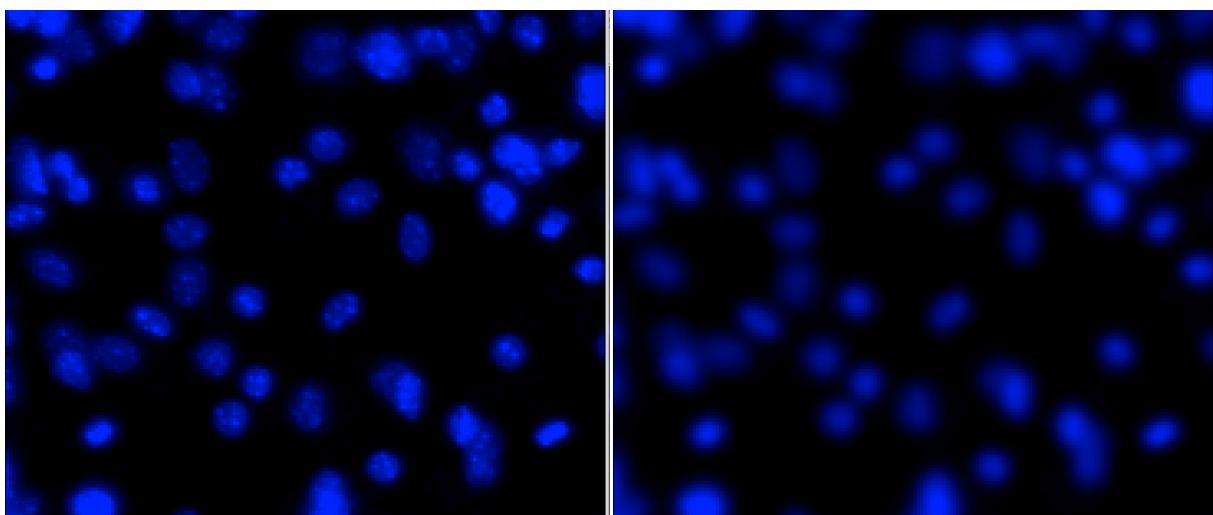
More detailed information:

- Include raw copy of the channel in output image:** If you select this option, the channel is duplicated before segmentation. In the output file you will find two versions of the channel, one segmented version and next to it the raw unsegmented version. This option is particularly helpful to check in the output image, whether the segmentation worked well and to eventually correct segmentation errors, as the output image will show an overlay of segmentation and raw channel.
- Delete all non-segmented channels:** In the output image, all channels except for the segmented one will be removed. This can reduce the size of output files.

When confirming the dialog, dialog of the following type will pop up - here you can enter the preferences for the first channel to be segmented (if you selected to segment more than one channel, additional dialogs for other channels to be segmented will pop up after this one - to see this pay attention to the headings (e.g. in the dialog below it is written “Settings for channel #1”. If you have selected to segment 2 channels, there will be another dialog popping up after this one labeled with “Settings for channel #2”).

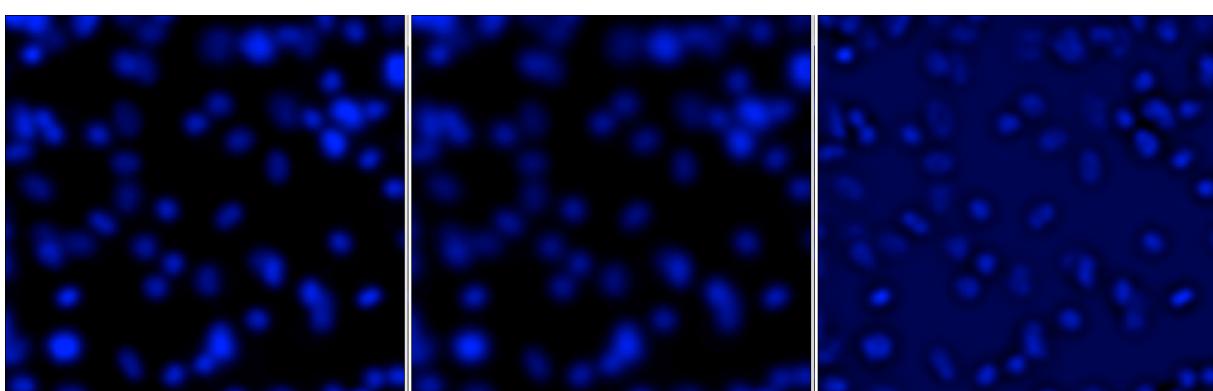


- **Channel Nr:** the number of the channel that needs to be processed (e.g. first channel = 1, second channel = 2, etc.). Select here the channel that describes nuclei or droplets best, e.g., the channel showing DAPI or a fluorescent lipid dye, respectively.
- AdipoQ Preparator allows two options to preprocess the image before threshold calculation. These options allow to reduce the noise in the image and normalize the image to the background. The combination of both methods is particularly useful for detecting droplets or nuclei, as they enhance gaps between adjacent lipid droplets.
 - **Blur image before analysis - Gaussian sigma (px):** 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 particular useful for segmenting a DAPI channel as otherwise these small points may be detected as objects instead of the whole DAPI nucleus.
 - Image below: raw image on left, blurred image on right (Gaussian sigma 2 px).



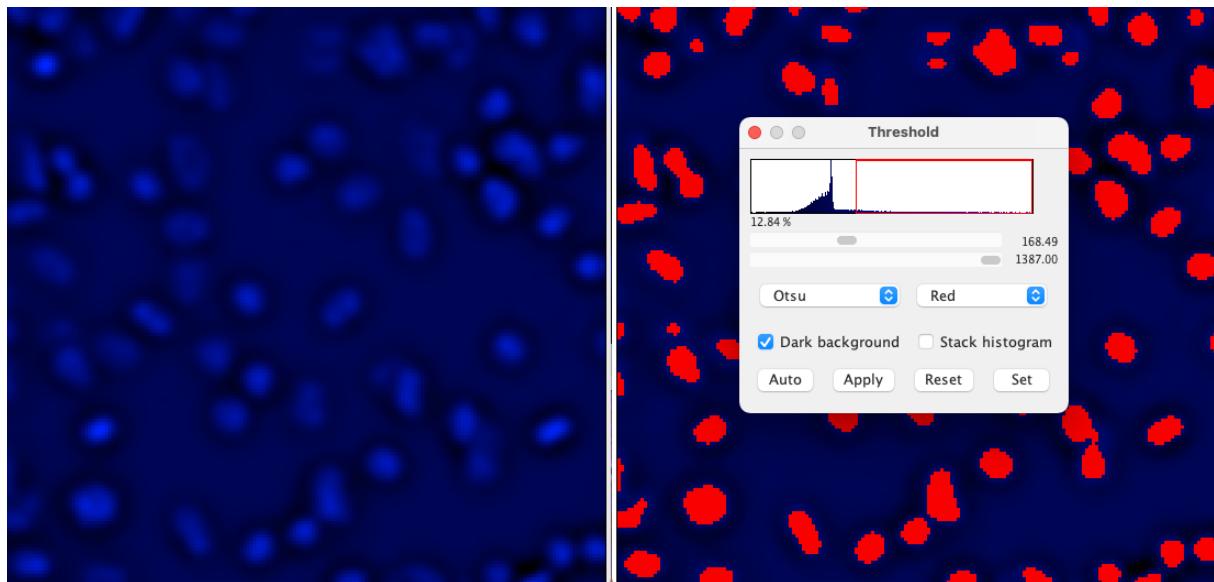
- **Subtract Blurred copy of image (to normalize)...:** A function that subtracts the local background and thereby, can normalize overall intensity differences in the image. This is also particularly helpful for images showing nuclei oder droplets as it allows to bring nuclei / droplets with different fluorescence level to the same overall intensity level. If you applied a blur already in the previous option ("Blur image before analysis"), then pick here a sigma that is definitely larger than the sigma selected in the previous option.

Image below: Left: image blurred with a sigma of 2 px, Center: image blurred with a sigma of 3 px, Right: Resulting image when subtracting Center image (sigma 3 px) from left image (sigma 2 px)



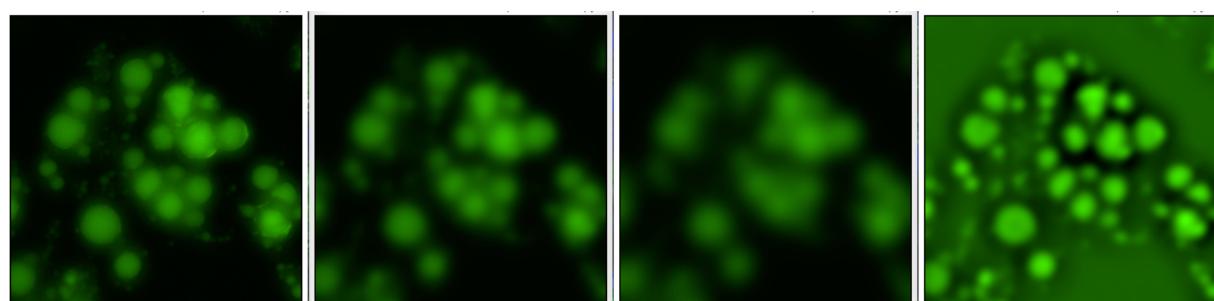
As can be seen in this image, the subtract methods yields an image containing small dark shades surrounding each nucleus. This is very useful for segmenting individual nuclei. For example, when applying now an intensity threshold algorithm (as introduced in the see next preferences), one would detect the following (red labeled) structures as nuclei.

Image below: Left: resulting difference image from the two differently blurred images. Right: difference image with a red mask overlayed, obtained by using the automatic intensity threshold method Otsu. Note: Small objects that may not represent nuclei can be later also removed in AdipoQ Analyzer by defining a minimum size.



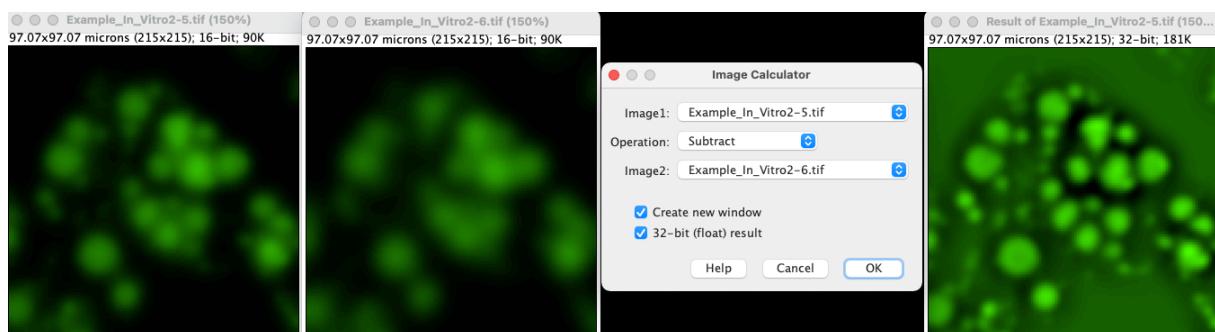
- Here is also an example for lipid droplet options showing that the combination of a blur and subtracting a blur with a larger sigma allows to normalize inter-droplet intensity and allows to better distinguish individual droplets:

Left: raw image; Center left: Image blurred with sigma 3 px; Center right: Image blurred with sigma 6 px; Right: resulting image when subtracting the image center right from the image center left:



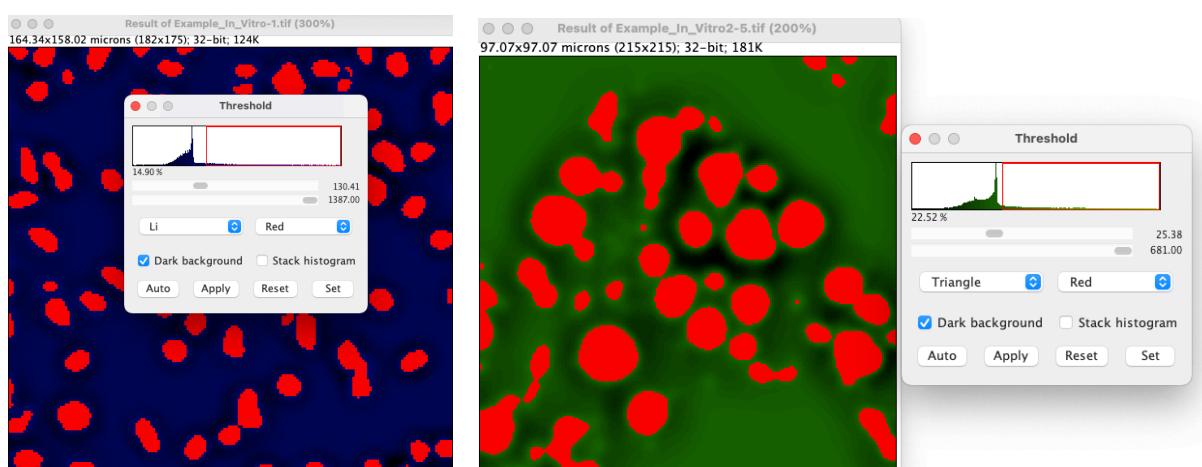
- **Segmentation method:** Here you can either select one of ImageJ's common intensity threshold algorithms (such as RenyiEntropy, Huang, Triangle, Li, Huang ...), or specify a CUSTOM threshold value that you then could enter in the number field below this settings. ImageJ's threshold algorithms separate objects from the background based on the histogram of the image. The algorithms differ in how they calculate the threshold. Thus, there are less harsh thresholds (that define rather more as background, e.g. MinError, Huang, Triangle, Li) and more harsh thresholds (that define rather less as foreground, e.g. Otsu, Renyi Entropy). If the segmentation does not work well, you should think about switching the threshold method. You can try the effect of different threshold methods in ImageJ as follows.

- Open an example image
- Preprocess the image as you would do automatically (see above).
 - Eventually, if you had selected this in AdipoQ Preparator, perform a Gaussian Blur by going to “Process > Filters > Gaussian Blur”,
 - Eventually, if you had selected this in AdipoQ Preparator, manually subtract a blurred copy of the image as follows:
 1. Duplicating the image (“Image > Duplicate”)
 2. Blurr it “Process > Filters > Gaussian Blur”
 3. Subtract it from the original image (“Process > Image calculator”, select original image (or already blurred image) as Image 1, Operation “Subtract”, duplicated blurred image as Image 2, and check 32-bit float result)



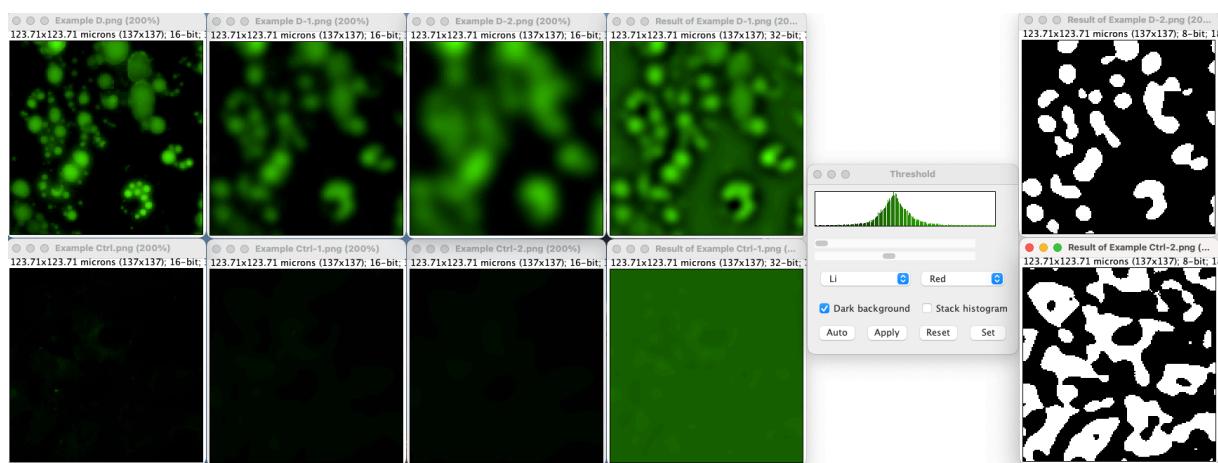
- Open the threshold tool (“Image > Adjust > Threshold...”) and select different thresholds. FIJI / ImageJ will automatically show you what they detect, e.g. with a red coloring.

Left image: example for DAPI (preprocessed as shown above), Right image: example for droplets (preprocesses as shown above).



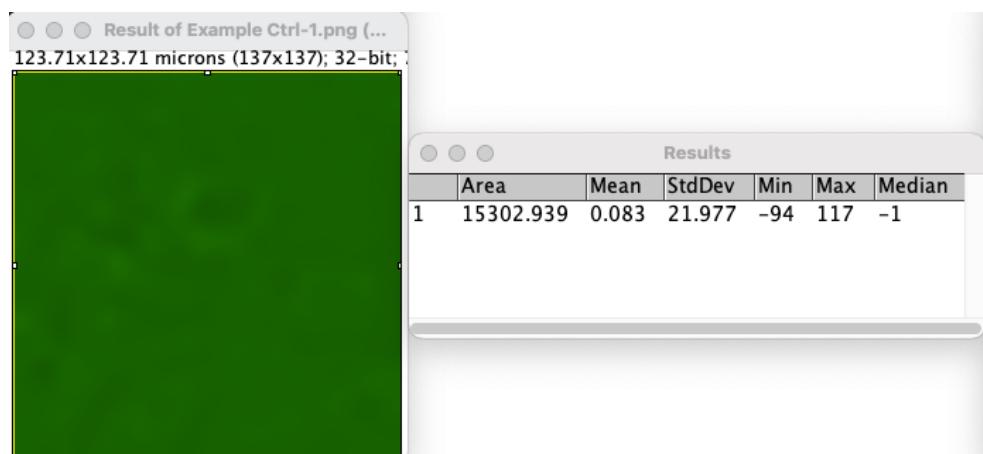
- **USEFUL HINT FOR ANALYZING LIPID DROPLET IMAGES:**

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. Have a look at the following example, which shows the whole analysis workflow as shown until threshold calculation for an image with lipid droplets and without lipid droplets. From left to right: (1) original image, (2) image blurred with sigma 3, (3) image blurred with sigma 6, (4) difference image where image blurred with sigma 6 is subtracted from image blurred with sigma 3, (5) Threshold dialog, (6) mask after segmentation with the auto-threshold Li. The upper row shows an image containing lipid droplets, the lower row shows an image containing no lipid droplets.

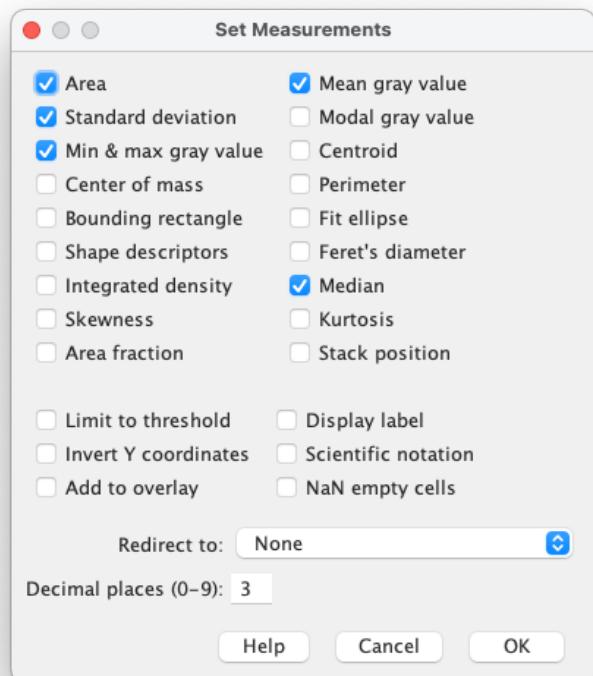


As can be seen, when the image does not contain any foreground structures (i.e., droplets), the background will be detected as foreground.

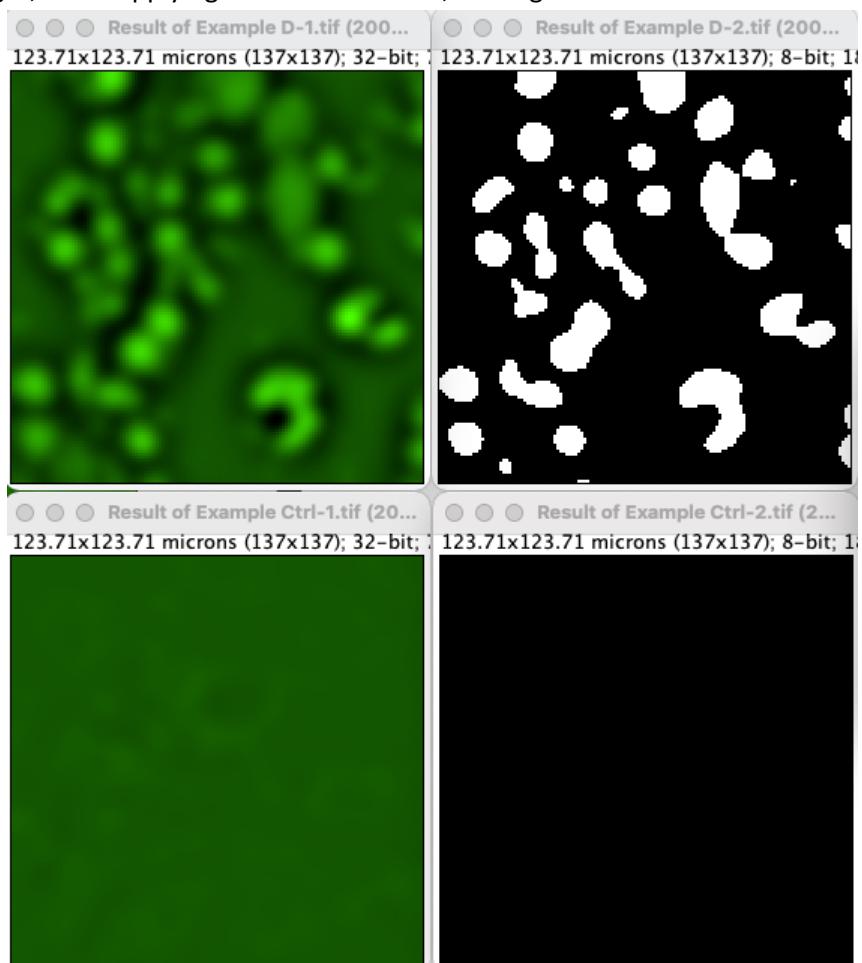
To circumvent this problem, we usually use a fixed threshold for the images from one experiment (that were stained at the same time). To determine the threshold, we use an image not showing any lipid droplets, perform the preprocessing as would be done by AdipoQ Preparator, and next, measure the intensities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



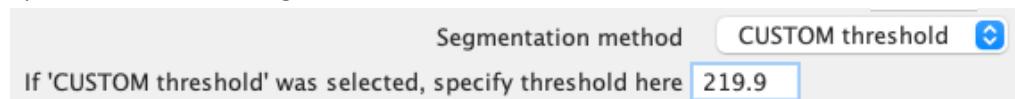
If the Results window does not show Mean and StdDev, click on the Results window, go to **RESULTS > SET MEASUREMENTS...** and make sure “Mean gray value” and “Standard deviation” is set.



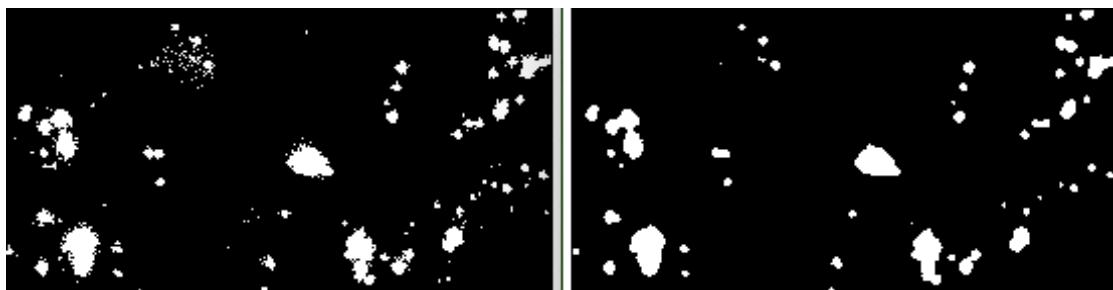
We then calculate the threshold as MEAN + 10*STDDEV. For the example shown above, this would be $0.083 + 10 * 21.977 = 219.853$. As can be seen in the following example images, when applying a threshold of 219, the segmentation works well.



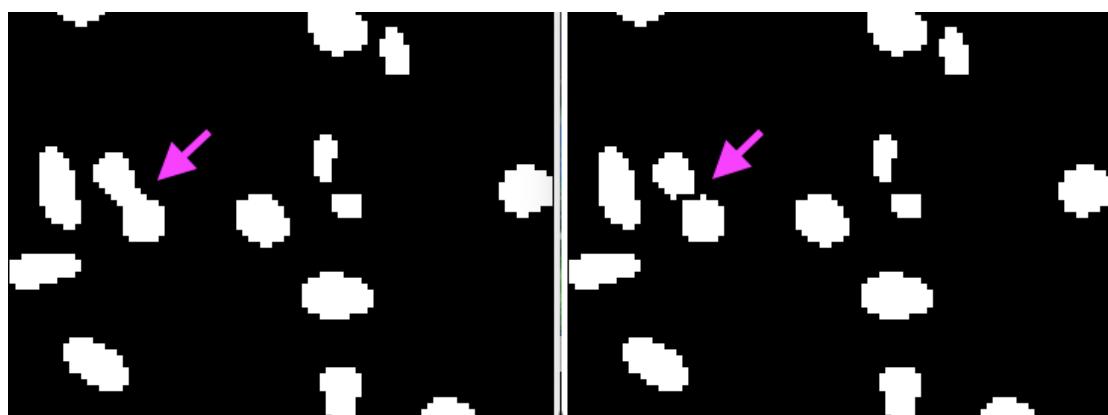
To select the according setting in AdipoQ Preparator, one would set the threshold options to the following:



- **Exclude zero-pixels in threshold calculation - tolerated gap radius...**: When analyzing images that have been stitched (e.g. when imaged with a slide scanner), you can see that there are blank, black regions in the image. These regions falsify an automated threshold calculation, as they add a lot of zero pixels to the image's histogram. To circumvent that, AdipoQ Preparator includes a method to automatically detect these regions and ignore them during threshold calculation. This option is usually only applicable for images from slide scanners (i.e., for slices and histological stainings) - for more information, please read the histology workflow.
- **Despeckle image**: Apply this method, if in the segmented image there are a lot of small tiny dots (pixels) that emerge from detection noise. Images below: before (left) and after (right) despeckling.



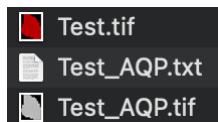
- **Detect regions and remove smaller regions**: This function should only be used for histological staining images (see chapter on histology above).
- **During detecting tissue regions, close gaps**: This function should only be used for histological staining images (see chapter on histology above).
- **Fill holes**: This option, when selected, fills holes in the mask. This is not recommended for these types of images, because it will fill also blank areas without droplets or nuclei in areas with a lot of droplets or nuclei.
- **Apply Watershed algorithm**: This function can be useful if your images are of bad quality or if the lines encircling adipocytes are not very clear and continuous. Then, the Watershed algorithm may allow you to better detect adipocytes. Image below shows the mask (detected adipocytes = black) before (left) and after (right) applying the Watershed algorithm.



AdipoQ Preparator: Output

AdipoQ Preparator saves the output image for a given input image as a single- or multi-channel tiff-image in the directory where the input image was located. The new file contains the original image's name and the additional ending “_AQP.tif”.

In addition, a metadata file with the ending “_AQP.txt” is saved.



The metadata file describes the input settings. Most importantly it also describes which channel in the output image corresponds to which channel in the input image:

```
Test_AQP.txt
Starting date: 2021-11-13      22:03:09
Image name: Test.tif
General settings:
    Channel duplicated to include a copy of the channel that is not processed.

Settings for channel #1:
    Channel Nr: 2

    Segmentation method: applying intensity threshold based on the Triangle threshold
algorithm.

    Background definition: detect dark objects on bright background.
    Excluded zero intensity pixels in threshold calculation – radius of tolerated gaps (px):
5.000000
    Despeckle mask
    Auto detect the region of interest – radius of excluded regions (px): 20.000000
    Fill holes in mask

    Used Triangle to determine the intensity threshold – threshold value: 188.000000

Channels in output image:
Channel 1: previous channel 1
Channel 2: previous channel 2 (segmented)
Channel 3: previous channel 2
Channel 4: previous channel 3

Datafile was generated on 2021-11-13 22:03:09 by 'AdipoQ Preparator', an ImageJ plug-in by Jan Niklas
Hansen (jan.hansen@uni-bonn.de, https://github.com/hansenjn/AdipoQ_Preparator).
The plug-in 'AdipoQ Preparator' is distributed in the hope that it will be useful, but WITHOUT ANY
WARRANTY; without even the implied warranty of MERCHANTABILITY or FITNESS FOR A PARTICULAR PURPOSE.
Plug-in version: V0.1.1
```

This information is however also stored in the .tif stack itself. Each slice image is labeled with the information from the raw image and whether it was segmented (labels of segmented images contain the prefix “segm: ”).

When you open an image in FIJI/ImageJ you find the slice image labels on the top of the image - example for the slice label of a segmented stack image:



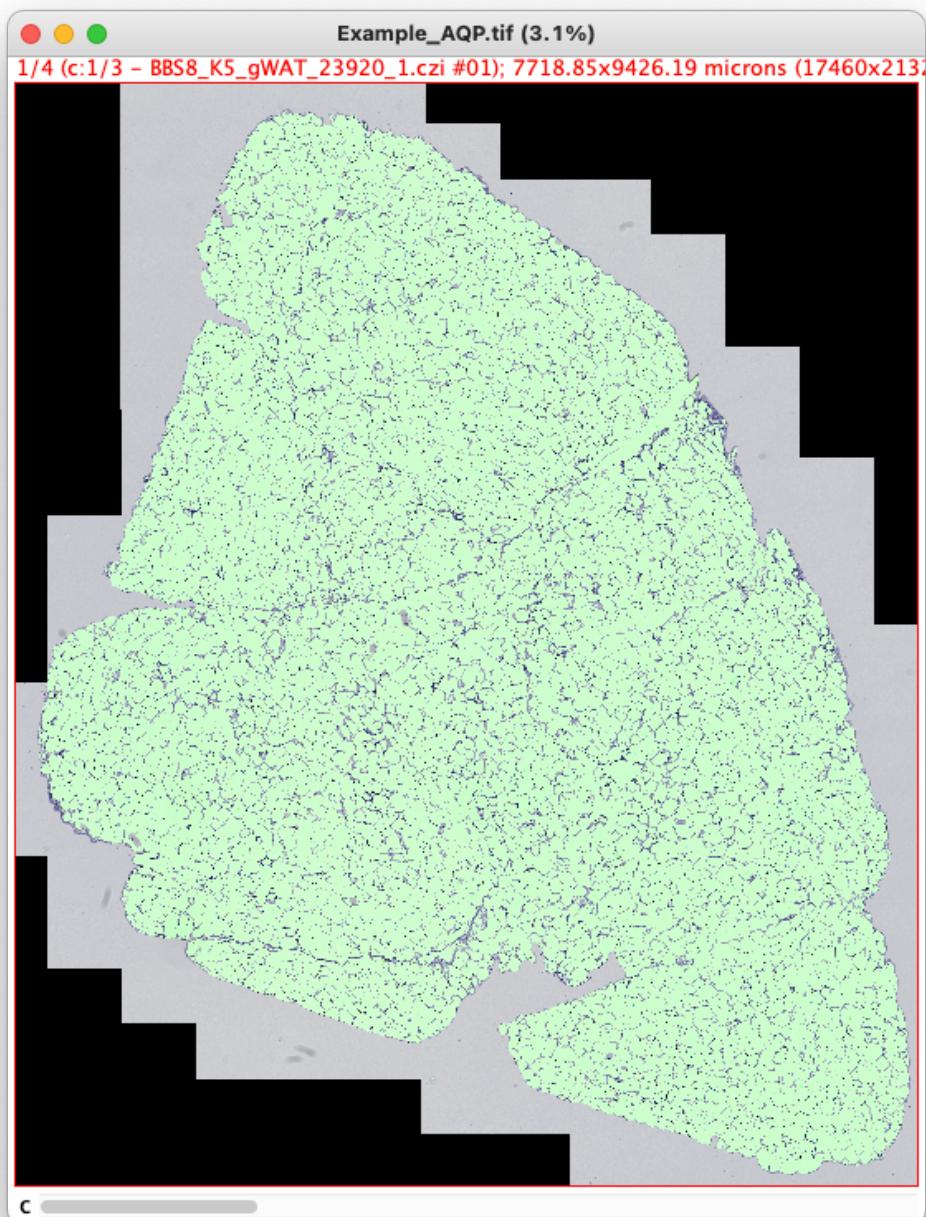
At the front, the channel in the output image is marked (“c:5/7”: channel number 5 of 7), and then in brackets the image label, where it is written that the shown image was segmented (“segm”) and the original channel / slice that the shown image was derived from (“c:3/4”).

Scrutinizing and eventually correcting segmentation

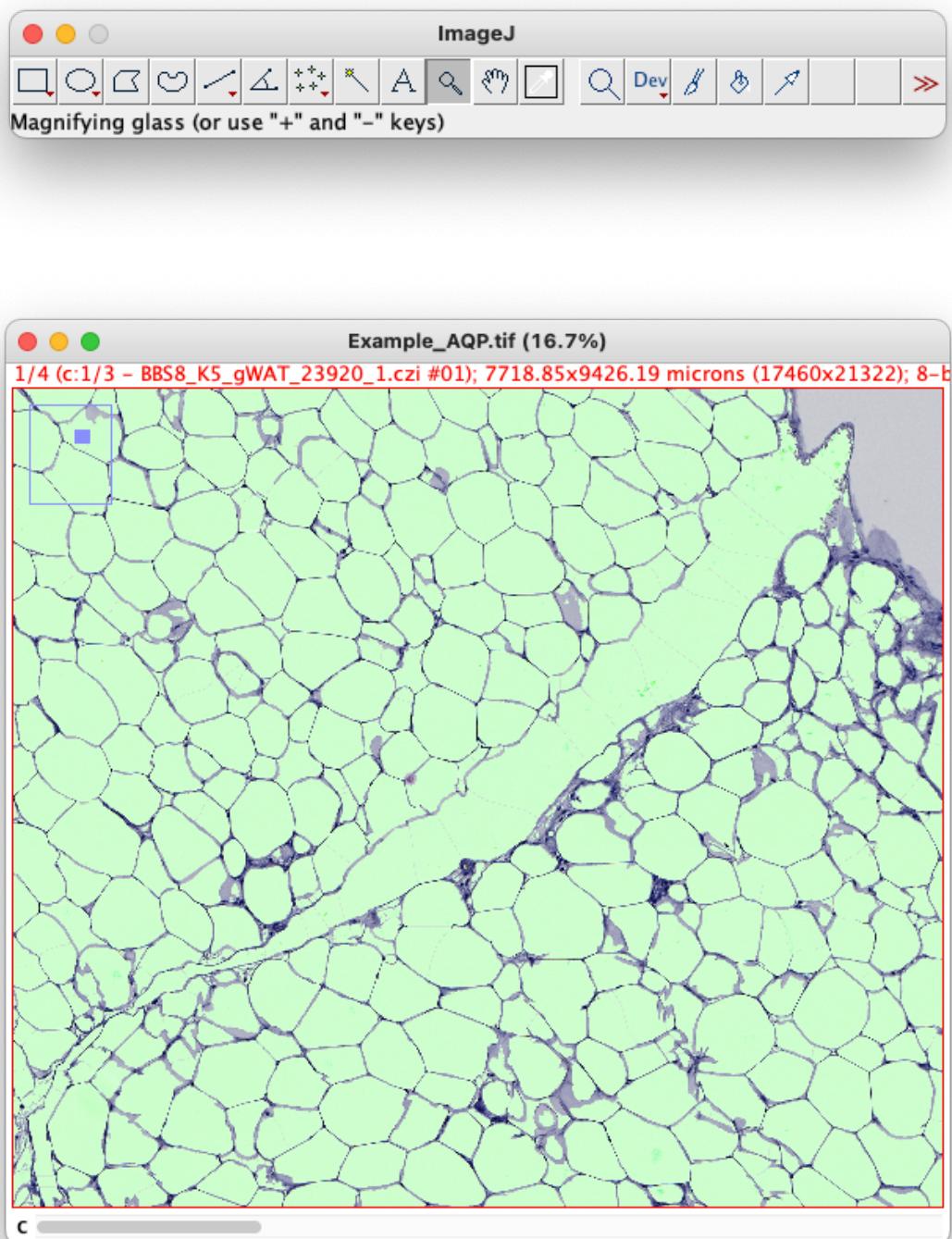
Usually, in histological staining images there are so many adipocytes analyzed that few incorrectly detected adipocytes or other structures do not matter. However, you can also be very precise and correct - at least big - errors. The same applies to fluorescence images, where also usually many droplets or nuclei are detected so that small errors do not matter. Nonetheless, in this chapter, we show how you can manually correct the segmentation. We show it exemplarily for a histological staining but the same workflow applies to fluorescence images.

(Note: It is highly recommended that you use a setting in AdipoQ Preparator that does not delete all other channels in the image, as then you can well see whether the adipocytes have been picked up accurately (see image below).

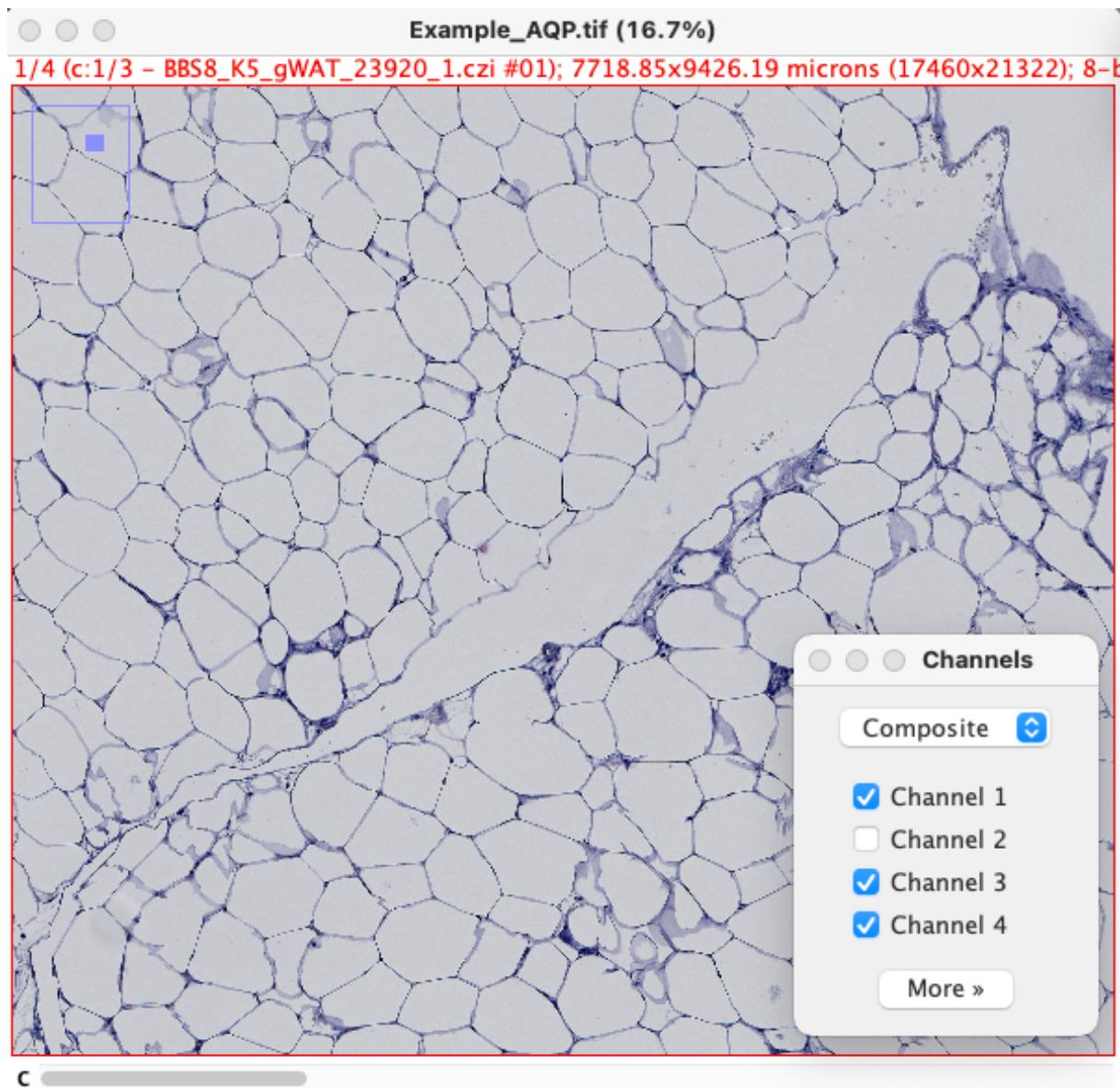
Open the output AQP.tif file in ImageJ / FIJI by drag and drop into the status bar.



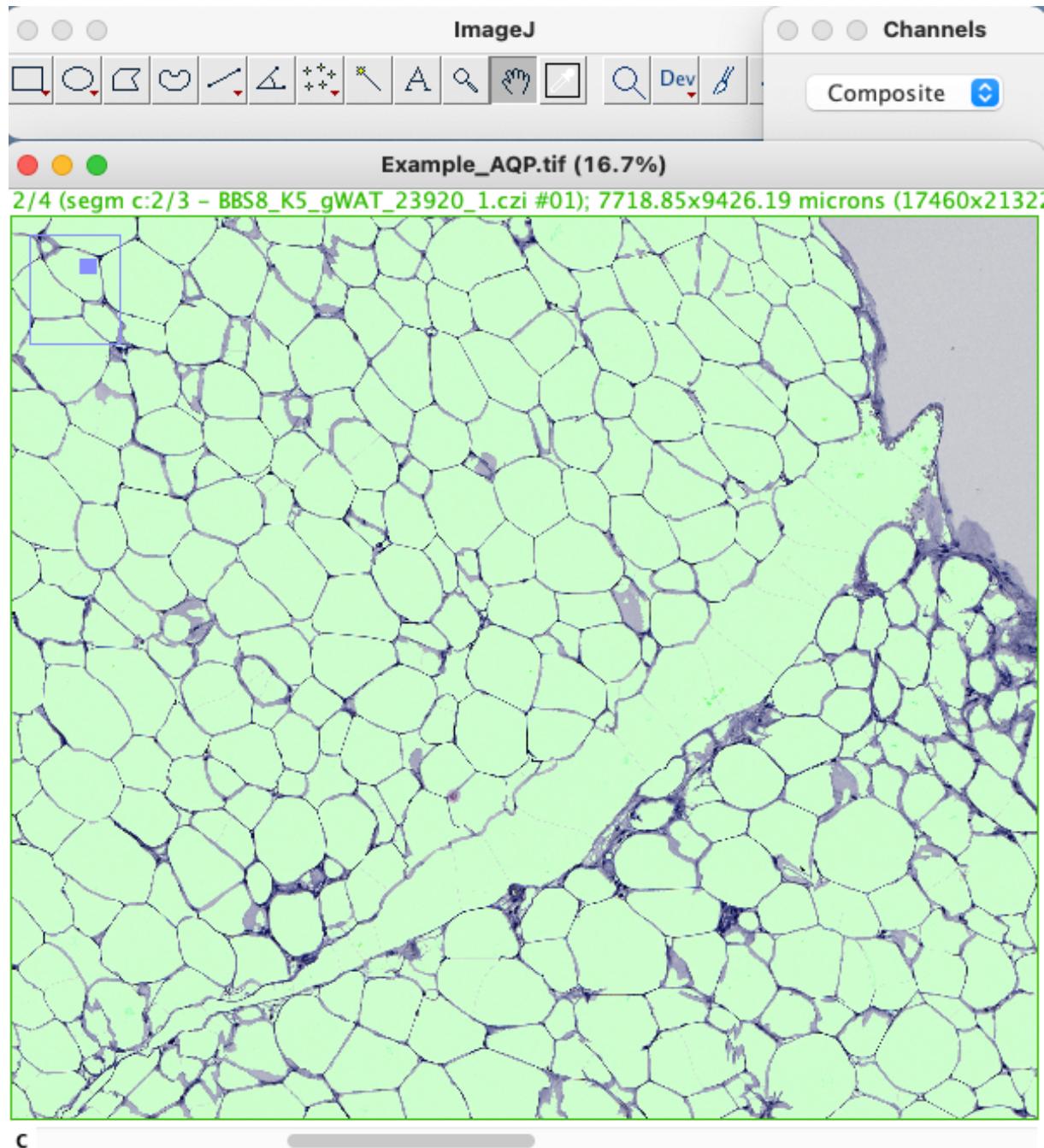
Use the magnification glass tool to zoom into different image parts - you may eventually discover some structures detected as adipocyte (green in image below):



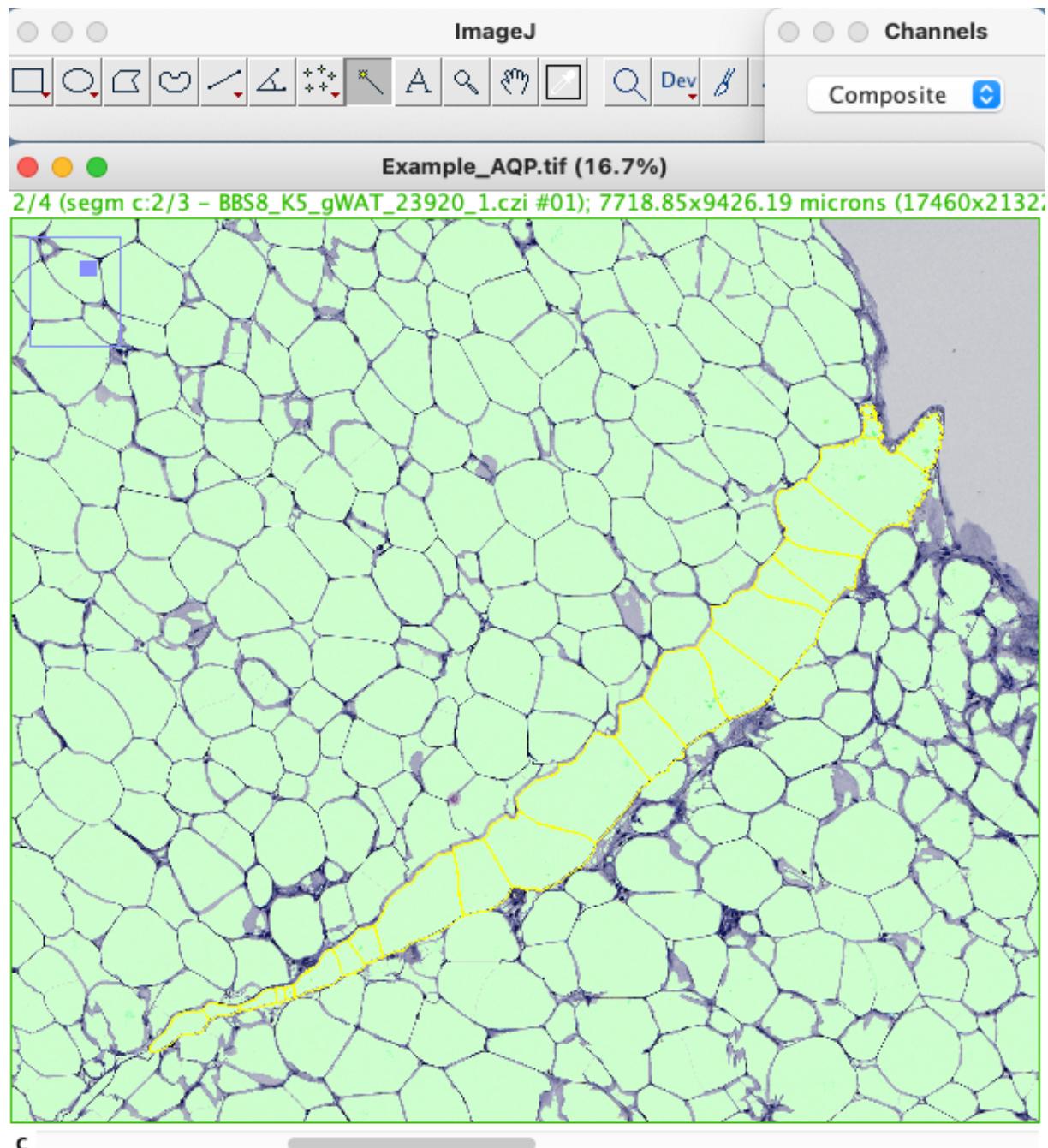
Open the channels tool ([IMAGE > COLOR > CHANNELS TOOL](#)) - this allows you to enable / disable individual channels or see whether the detected structures are adipocytes or something else (e.g. blood vessels or tissue disruptions).



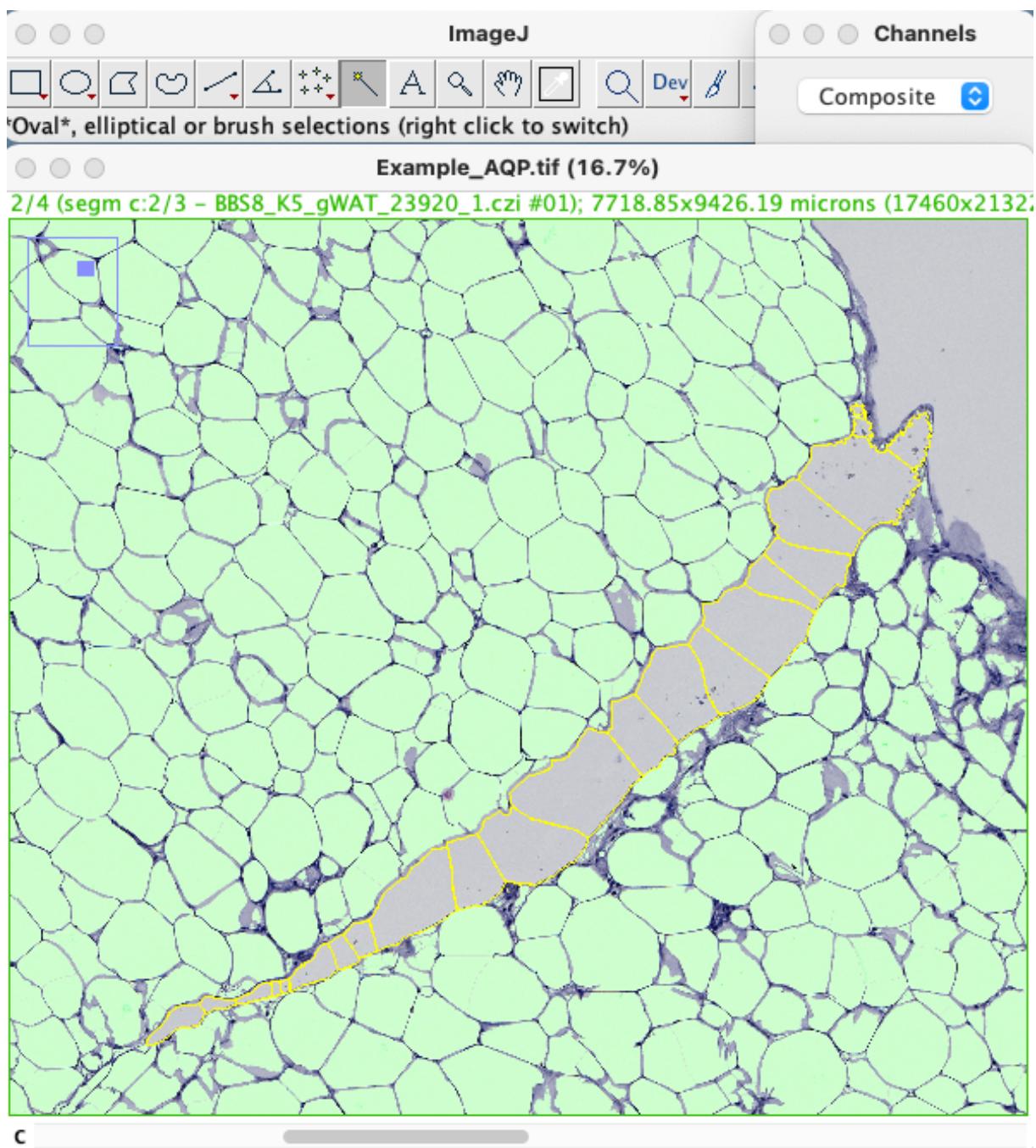
To remove structures from the mask, do the following: Switch the Channel bar to the segmented channel (in this case channel 2, you can also see that this is the segmented channel by the status note about the image on top “2/4 (segm c:2/3...”).



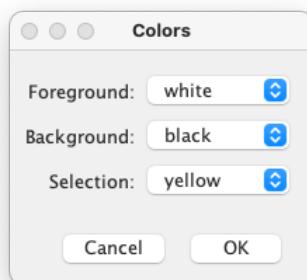
Next, use the Wand tool and click on the segments you want to remove. When you hold the SHIFT key you can select additional segments by clicking on them. When you press the ALT key ("option" key on Mac computers) you can unselect individual elements.



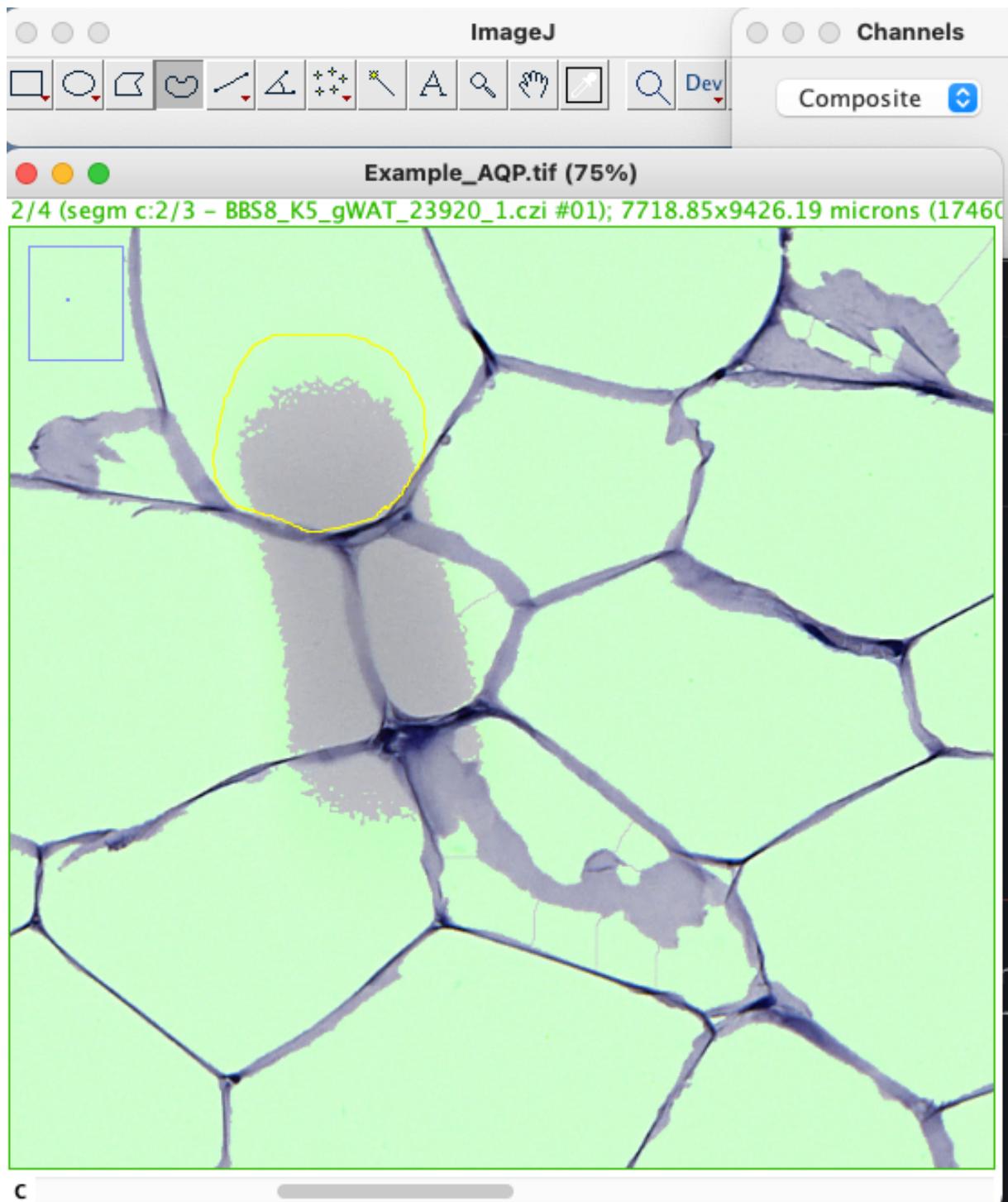
Now, press the backspace key, which will remove those elements from the mask.



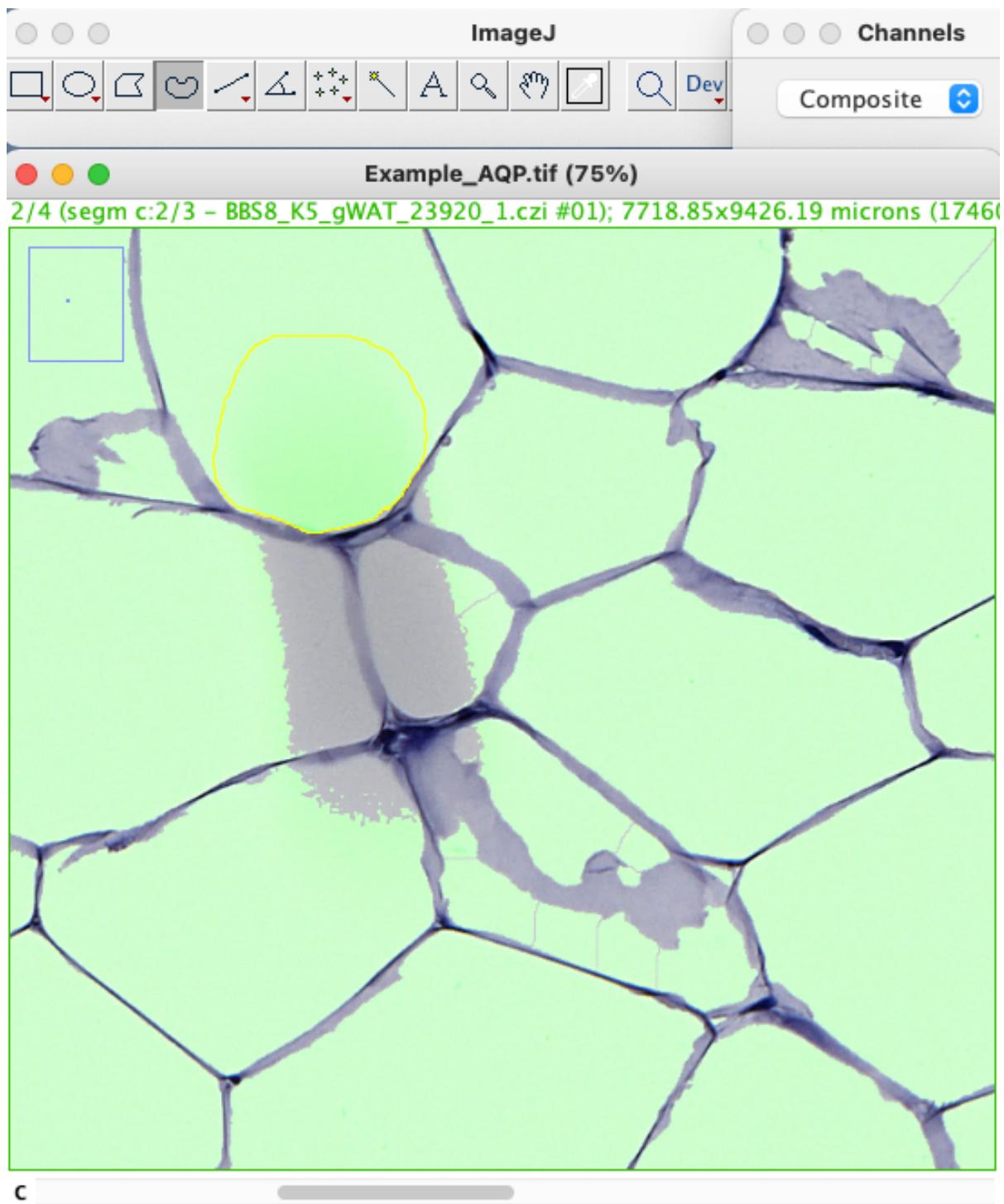
If this does not work, go to Edit > Options > Colors and make sure the dialog is set as follows:



If you want to add parts to the mask, select them with the lasso tool /or with other ROIS (e.g. polygon, ellipse, ...). You can add to the selection when pressing SHIFT and cut parts away from the selection when pressing ALT. (Sidenote: to remove all selections on the image just click somewhere outside the drawn ROI in the image and it will disappear.)



To add the part to the mask, press the key "f" on the keyboard.

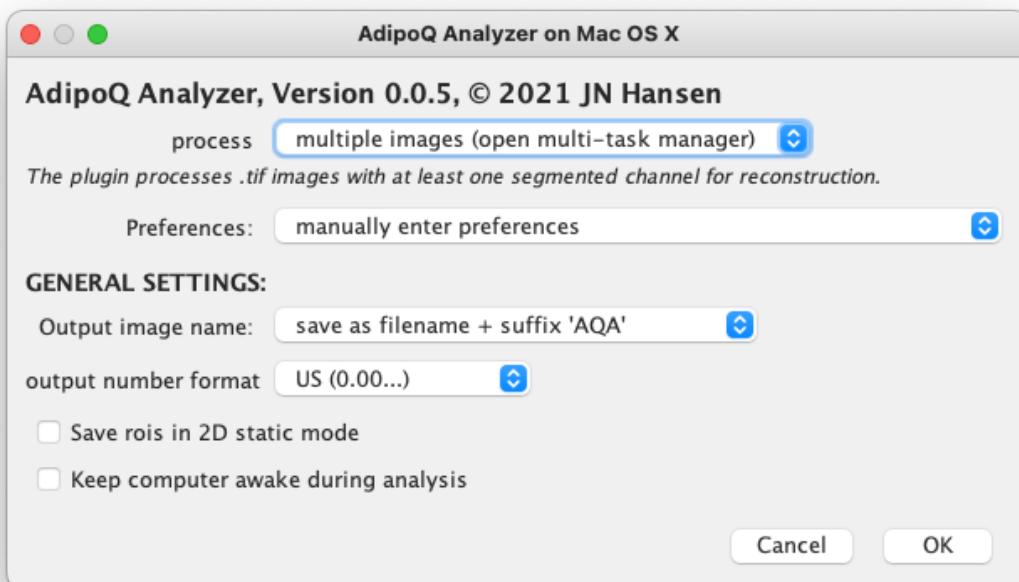


When you are done with editing, save the image (e.g. under a new name to make sure that you remember that it was manually added). Now you can feed it into AdipoQ Analyzer.

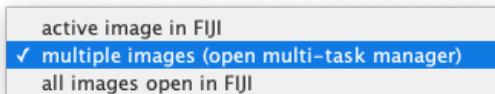
Quantification with AdipoQ Analyzer

To quantify the images segmented with AdipoQ Preparator (no matter whether they were from cultured cells or tissues), launch AdipoQ Analyzer via [PLUGINS > ADIPOQ > ADIPOQ ANALYZER](#).

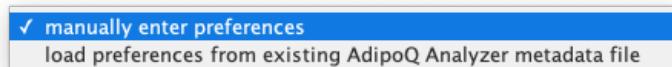
A first dialog pops up that shows general settings:



- Process: Under the process option you can again select how you want to subject input images to the plugin (for more information, see the notes for AdipoQ Preparator, which contains the same file management system).

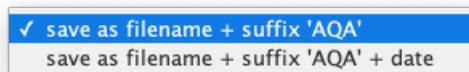


- Preferences:
 - If load preferences from existing AdipoQ Analyzer metadata file is selected, a dialog will open that requests you to select a AQA.txt file to import settings – no settings need to be manually entered. AQA.txt files are output by AdipoQ Analyzer and contain the settings from the analysis (see below).
 - If manually enter preferences is selected, further dialogs are displayed to enter the preferences.



AdipoQ Analyzer: General settings

The Output image name option allows to add the date and time of processing for each output file. This is particularly useful when you reanalyze files, because commonly AdipoQ Analyzer overrides the output files when you run it again. If you however select to add the date and time to the file name, output file names will be unique and you will not override a previous run.



The Output number format option defines in which format numbers will appear in the metadata text file - this is usually not relevant for AdipoQ Preparator runs as you never read in these numbers. It is more important when you run AdipoQ Analyzer (see below).

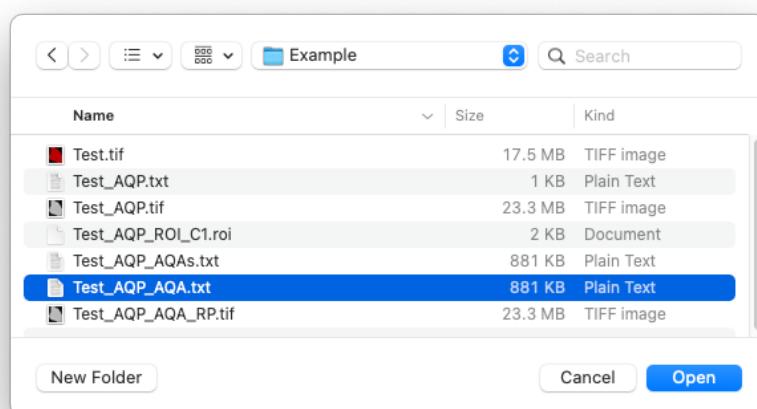


Save rois in 2D static mode: If this option is selected, AdipoQ Analyzer will also output a .zip file that contains a set of ROIs - one ROI for each detected object. This zip file can be imported into ImageJ by drag and drop into the status bar of the ImageJ window. Then the ROIs will be loaded to the ROI Manager, from where they can be displayed in the image of your choice (For example, you could also open the ..._AQP.tif file and then map the ROIs there to see which objects have not been detected / which have been detected).

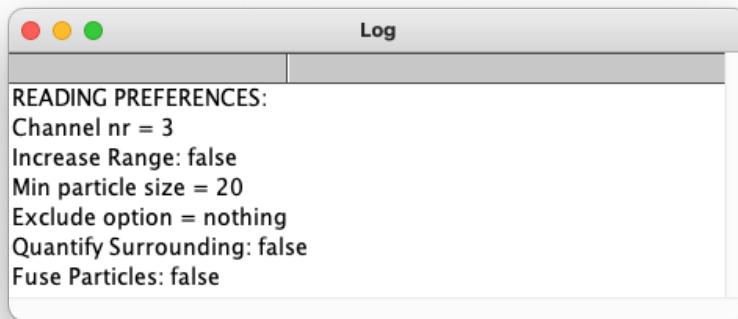
The option Keep computer awake during processing allows to, when selected, prevent your computer from going to sleep mode during processing, which could pause the analysis. It is useful, when you run a large batch and have no control on a computer to prevent going to sleep (e.g., on Mac Books this can be difficult and thus select this option definitely if you are running the program on a Mac book).

AdipoQ Analyzer: Automatically load preferences

When you select to automatically load preferences, a dialog pops up and you are requested to open a file. Navigate to the folder where the pre-existing AdipoQ Preparator run was stored and select the AQP.txt file from it to load the settings from that run.



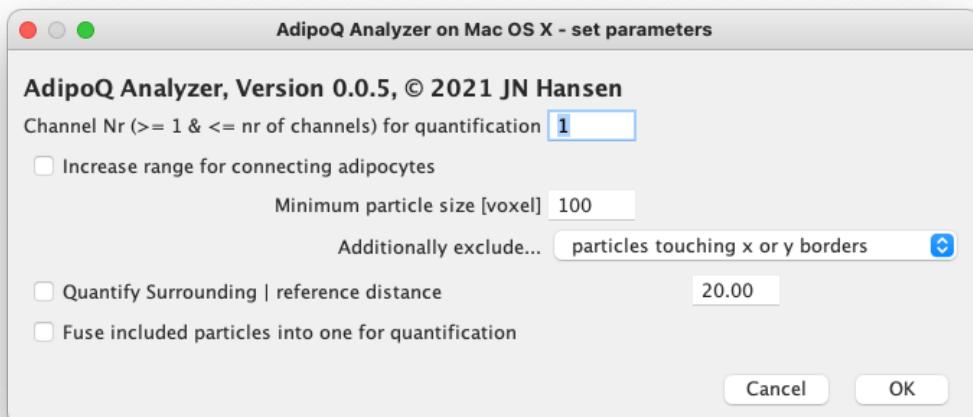
In the Log windows that pops up next, you can check the loaded preferences.



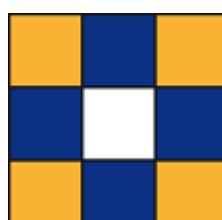
Next, either AdipoQ Preparator directly launches or you will be directed to the dialog to load the input files.

AdipoQ Analyzer: Manually enter preferences

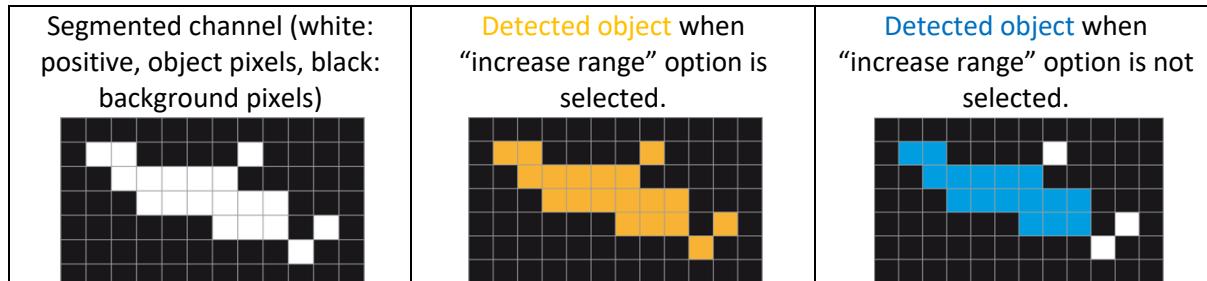
When you select to manually enter preferences, a dialog pops up that allows you entering those settings:



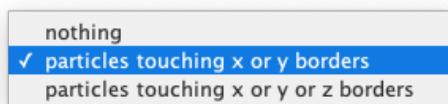
- Increase range for connecting adipocytes: This option determines which mode is used to connect adjacent voxels to form an object.
 - When the box is checked, AdipoQ Analyzer will connect a pixel in the image to all direct (blue voxels in image below) and diagonally adjacent voxels (orange voxels in image below) to form an object. If the box is not checked, diagonally adjacent voxels (orange in image below) will not be connected but only directly adjacent voxels (blue).



- The “increase range” function is helpful when the image features a bad signal-to-noise-ratio or when the objects are incompletely labeled and contain small gaps in labeling. However, the “increase range” function also features the disadvantage that noise voxels can be added to objects and that adjacent objects may be fused, if there is no wide gap inbetween.



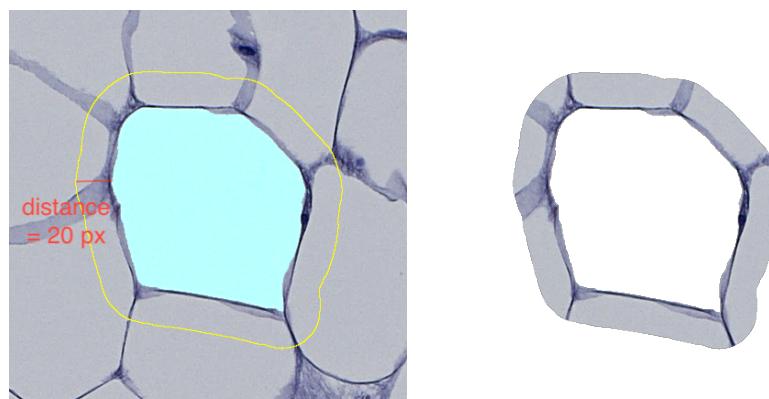
- Minimum particle size [voxel]: This defines the minimum size of objects that will be detected and quantified in the unit pixel. It allows you to filter out noisy small pixels. Note, that you could also still later apply a different size threshold in a post-hoc analysis (see R scripts).
- Additionally exclude...: This option allows you to remove incomplete objects at the image borders. AdipoQ Analyzer allows to exclude objects that do not lie completely within the imaged field of view. Objects contacting the image borders in x, y, and z are automatically removed if the following option is set as follows:



Note: If you have a 2D image, and you will select the option for x or y or z, all particles will be removed as they contact the z border. Also, a 3D mode is not yet fully implemented in AdipoQ Analyzer, the setting for x or y or z is preliminary and not of use for now.

- Quantify surrounding: When this option is selected, AdipoQ Analyzer will also quantify intensities in the surrounding of each detected object and output as surrounding parameters (see parameter list). The distance specified is the maximum distance of a pixel to the outline of the object to be considered into the surrounding.

Image below shows an example: **LEFT:** For the adipocyte detected (cyan) the pixels considered will be those within a distance of 20 px (as 20 px was set) to the adipocyte. The outer most pixels considered in the surrounding are marked with the yellow line. **RIGHT:** An image showing the pixels that will be quantified in parameters describing the surrounding.



- Fuse included particles into one for quantification: This option is experimental. It can be used if you do not want to distinguish individual objects in the image but only want to quantify the total area / fluorescence of particles above the size threshold without caring about individual particles.

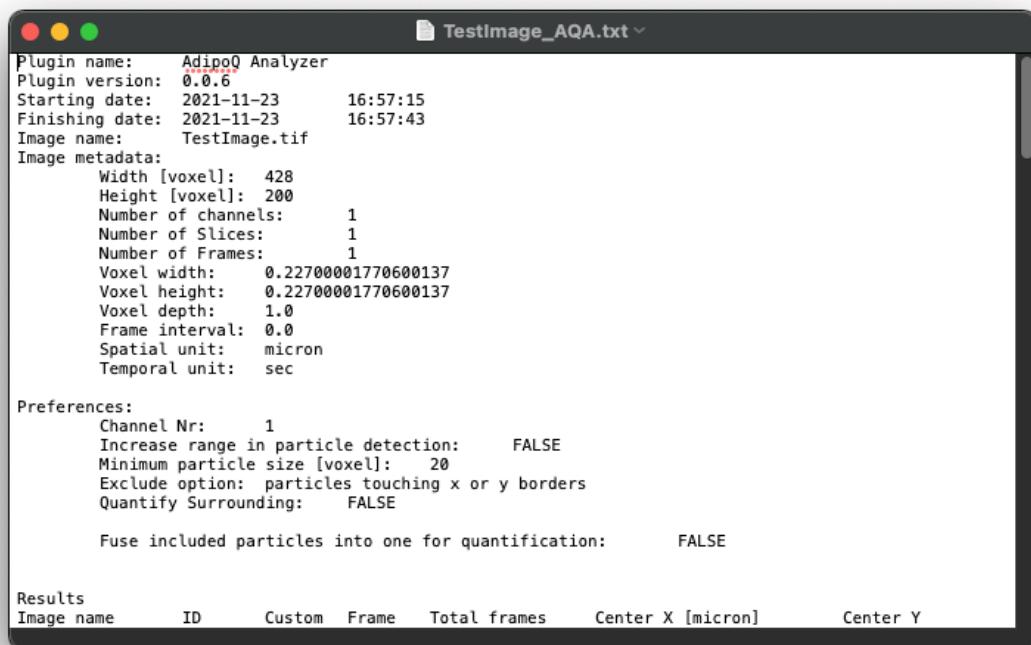
AdipoQ Analyzer: Output file

All output files for a given input image are saved in the directory of the input image and contain the name of the analyzed input image plus the suffix “_AQA” and, eventually, extra-suffixes depending on the type of output file (e.g., if the date was added).

The following files are output:

Name	Art
Test.tif	TIFF-Bild
Test_AQP.txt	Reiner Text
Test_AQP.tif	TIFF-Bild
Test_AQP_ROI_C1.roi	Dokument
Test_AQP_AQAs.txt	Reiner Text
Test_AQP_AQA.txt	Reiner Text
Test_AQP_AQA_RP.tif	TIFF-Bild

- Filename suffix _AQA.txt: A tab-delimited text file containing the analysis settings and results



```

Plugin name: AdipoQ Analyzer
Plugin version: 0.0.6
Starting date: 2021-11-23      16:57:15
Finishing date: 2021-11-23     16:57:43
Image name: TestImage.tif
Image metadata:
    Width [voxel]: 428
    Height [voxel]: 200
    Number of channels: 1
    Number of Slices: 1
    Number of Frames: 1
    Voxel width: 0.22700001770600137
    Voxel height: 0.22700001770600137
    Voxel depth: 1.0
    Frame interval: 0.0
    Spatial unit: micron
    Temporal unit: sec

Preferences:
    Channel Nr: 1
    Increase range in particle detection: FALSE
    Minimum particle size [voxel]: 20
    Exclude option: particles touching x or y borders
    Quantify Surrounding: FALSE

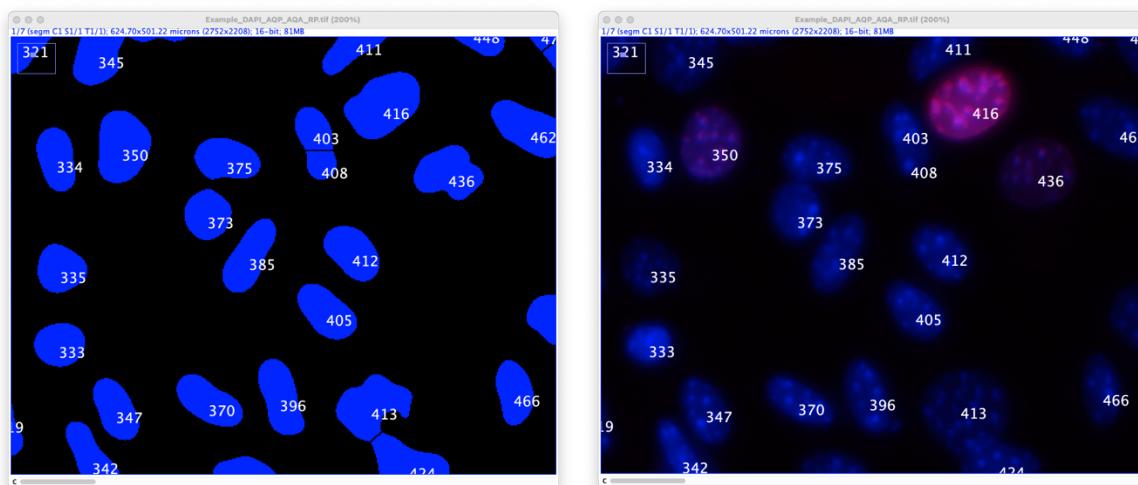
    Fuse included particles into one for quantification: FALSE

Results
Image name      ID      Custom   Frame   Total frames   Center X [micron]   Center Y

```

- Filename suffix _AQA_RP.tif: A copy of the input image after filtering out particles that are too small. This image also contains labels of the detected objects (labels correspond to the column ID in the output text files, so one can find out which object yielded which result). Note, that this file is also very useful to inspect the results and check whether all objects are picked up correctly, as one can see the detected masks and the IDs.

Image below: _AQA_RP.tif file from an example image where DAPI nuclei were detected. Left: only the mask shown; Right: no mask but original DAPI (blue) and additional fluorescence channel (magenta) displayed. The image allows to see which nuclei received which ID to find them in the results text files. To display only selected channels, open the channels tool (Image > Color > Channels tool) and unselect channels that shall not be displayed.



- Filename suffix _AQAs.txt: A tab-delimited text file containing only the results lines from the file with suffix _AQA.txt, including the table caption. This file can be readily read in for a programming-based post-hoc analysis (Python, R, MATLAB) or into table calculation software for a more manual post-hoc analysis (e.g. Microsoft Excel)

TestImage_AQAs.txt						
Image name	ID	Custom	Frame	Total frames	Center X [micron]	Outline
Center Y [micron]		Center Z [micron]	Voxels	Area [micron^2]	C1: Integrated Intensity	C1: Max Intensity
2D-Asphericity Index					C1: Average Intensity	C1: SD of Intensities
Median Intensity					C1: Min Intensity	C1: Max Intensity
TestImage.tif	1		1	1	3.483276	2.559621
1.494341 26.332002			6.076508	0.000000	0.000000	0.000000
TestImage.tif	2		1	1	81.330863	3.721179
1.442812 25.424002			5.970821	0.000000	0.000000	0.000000
TestImage.tif	3		1	1	83.799960	4.481931
2.215747 39.044003			7.399277	0.000000	0.000000	0.000000
TestImage.tif	4		1	1	6.504044	5.369044
1.185167 20.884002			5.411516	0.000000	0.000000	0.000000
TestImage.tif	5		1	1	2.643883	5.492510
2.627979 46.308004			8.058239	0.000000	0.000000	0.000000
TestImage.tif	6		1	1	85.841615	6.280334
2.627979 46.308004			8.058239	0.000000	0.000000	0.000000
TestImage.tif	7		1	1	94.638893	7.476633
8.141583 143.464011			14.183506	0.000000	0.000000	0.000000
0.000000						
TestImage.tif	8		1	1	13.166001	6.810001
1.803515 31.780002			6.675581	0.000000	0.000000	0.000000
T	T	T	T	T	T	T

- NOTE: Both, the _AQAs.txt and the AQA.txt files, can be readily pasted into an Excel sheet to inspect the results. Just open them in the text editor and copy paste them into an Excel sheet.

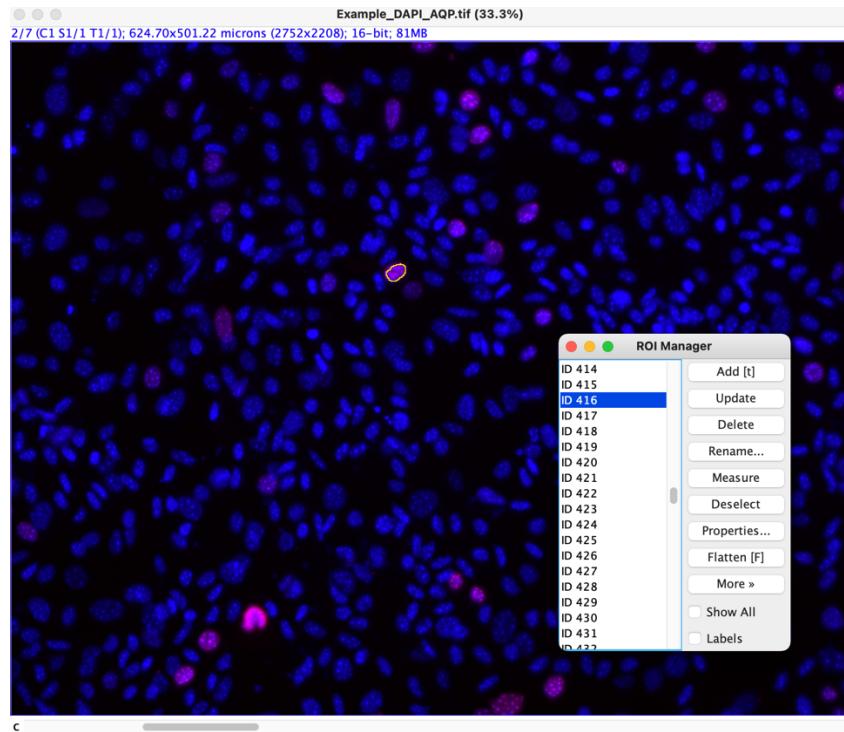
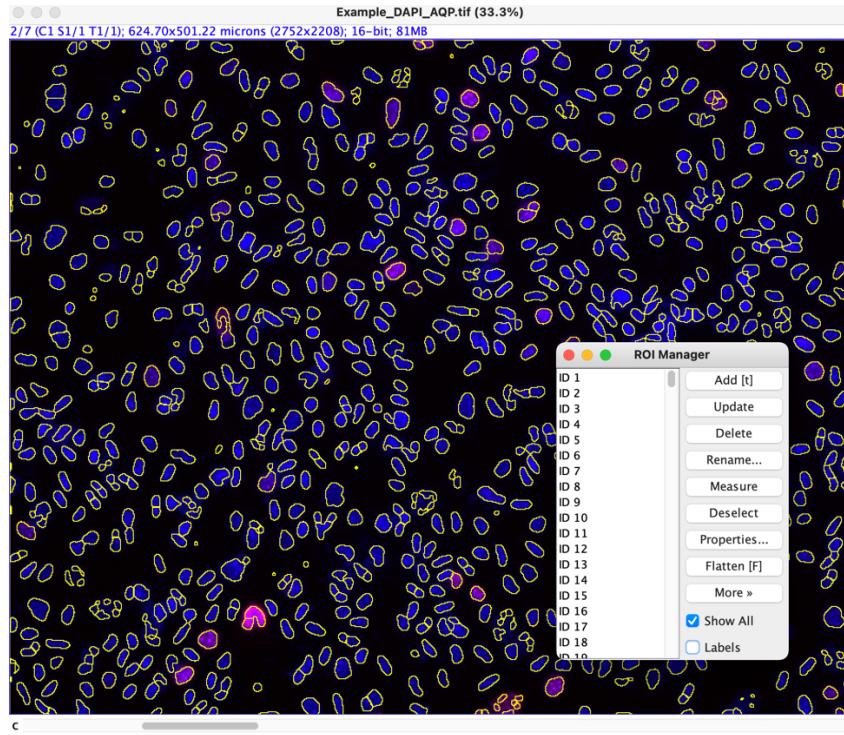
Book1

	A	B	C	D	E	F	G	H	I	J	K	L
1	Plugin name:	AdipoQ Analyzer										
2	Plugin version:	0.0.5										
3	Starting date:	23.11.21 03:30:08										
4	Finishing date:	23.11.21 03:36:35										
5	Image name:	New-02_Sh_s235_AQP.tif										
6	Image metadata:											
7	Width [voxel]:	2752										
8	Height [voxel]:	2208										
9	Number of channels:	7										
10	Number of Slices:	1										
11	Number of Frames:	1										
12	Voxel width:	0.227000018										
13	Voxel height:	0.227000018										
14	Voxel depth:	2										
15	Frame interval:	0										
16	Spatial unit:	micron										
17	Temporal unit:	sec										
18	Preferences:											
20	Channel Nr:	1										
21	Increase range in particle detection:	FALSE										
22	Minimum particle size [voxel]:	20										
23	Exclude option:	nothing										
24	Quantify crown-like structures:	FALSE										
25	Fuse included particles into one for qu:	FALSE										
27	Results											
30	Image name	ID	Custom	Frame	Total frames	Center X [mic]	Center Y [mic]	Center Z [mic]	Voxels	Area [micron]	Outline [mic]	2D-Aspheric C1:
31	New-02_Sh_s235_AQP.tif	1		1	1	2.143754	80.705587	0	561	28.907774	17.479001	0.917074
32	New-02_Sh_s235_AQP.tif	2		1	1	0.65209	117.141646	0	212	10.92415	11.577001	0.988092
33	New-02_Sh_s235_AQP.tif	3		1	1	0.583928	124.48263	0	152	7.832409	9.761001	0.983879
34	New-02_Sh_s235_AQP.tif	4		1	1	3.820046	145.73679	0	979	50.446899	27.240002	1.081895
35	New-02_Sh_s235_AQP.tif	5		1	1	3.74094	195.72773	0	1344	69.254987	28.375002	0.961846
36	New-02_Sh_s235_AQP.tif	6		1	1	1.355121	270.32792	0	429	22.105944	14.074001	0.844419
37	New-02_Sh_s235_AQP.tif	7		1	1	0.326149	316.95465	0	87	4.483024	8.172001	1.088775
38	New-02_Sh_s235_AQP.tif	8		1	1	1.271752	329.87044	0	576	29.680709	19.522002	1.01084
39	New-02_Sh_s235_AQP.tif	9		1	1	0.384913	349.19906	0	115	5.925836	8.853001	1.025914

Book1

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Image name	ID	Custom	Frame	Total frames	Center X [mic]	Center Y [mic]	Center Z [mic]	Voxels	Area [micron]	Outline [mic]	2D-Aspheric C1:	Aver
2	New-02_Sh_	1		1	1	2.143754	80.705587	0	561	28.907774	17.479001	0.917074	65
3	New-02_Sh_	2		1	1	0.65209	117.141646	0	212	10.92415	11.577001	0.988092	65
4	New-02_Sh_	3		1	1	0.583928	124.482628	0	152	7.832409	9.761001	0.983879	65
5	New-02_Sh_	4		1	1	3.820046	145.736794	0	979	50.446899	27.240002	1.081895	65
6	New-02_Sh_	5		1	1	3.74094	195.727725	0	1344	69.254987	28.375002	0.961846	65
7	New-02_Sh_	6		1	1	1.355121	270.327919	0	429	22.105944	14.074001	0.844419	65
8	New-02_Sh_	7		1	1	0.326149	316.954645	0	87	4.483024	8.172001	1.088775	65
9	New-02_Sh_	8		1	1	1.271752	329.870435	0	576	29.680709	19.522002	1.01084	65
10	New-02_Sh_	9		1	1	0.384913	349.199062	0	115	5.925836	8.853001	1.025914	65
11	New-02_Sh_	10		1	1	3.333919	384.597319	0	693	35.709603	25.424002	1.20018	65
12	New-02_Sh_	11		1	1	2.093938	434.588007	0	869	44.778708	21.792002	0.918663	65

- Filename suffix _AQAr.zip: This file is only output if Save rois in 2D static mode is selected in the AdipoQ Analyzer preferences. The file contains a ROI for each detected structure and may be useful to perform further custom quantifications in ImageJ. The file can be imported into ImageJ by drag and drop into the status bar. Open the original image before dropping the .zip file, then you can see the ROIs overlaid on the original image:



AdipoQ Analyzer: Description of output parameters

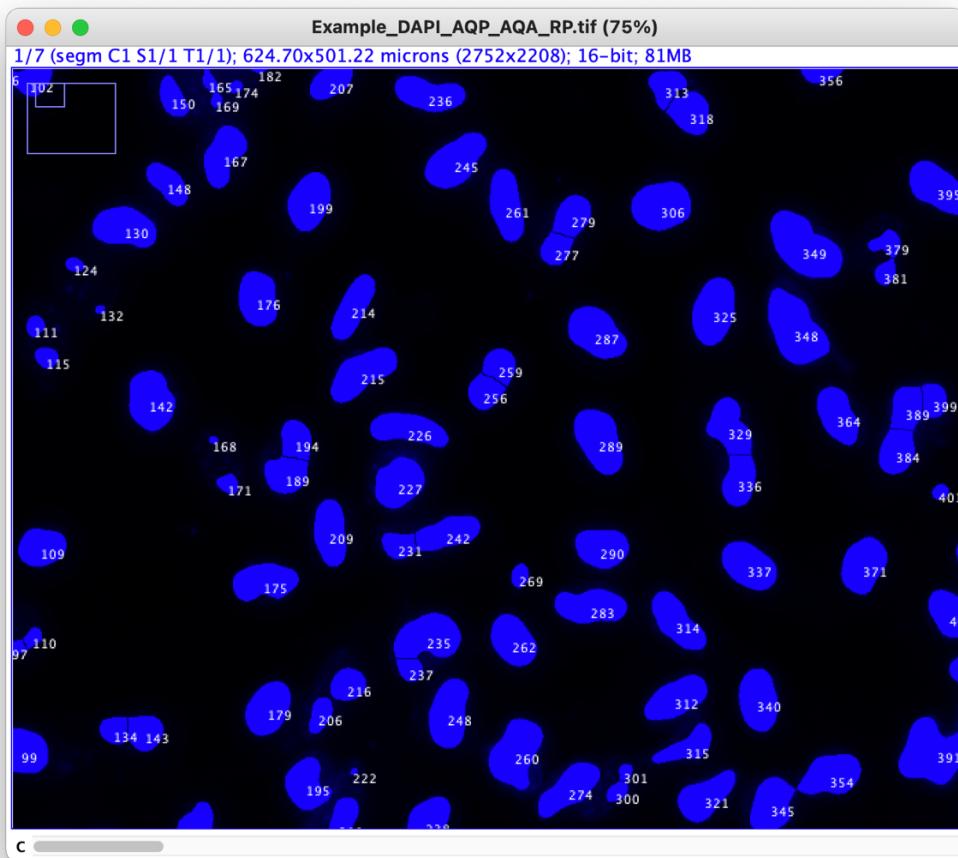
Please note that many output parameters are very technical and not relevant for the standard user. A detailed description of all parameters follows here. Each parameter is output in a separate column in the AQAs.txt file or in the *Results* section of the AQA.txt file.

Image name

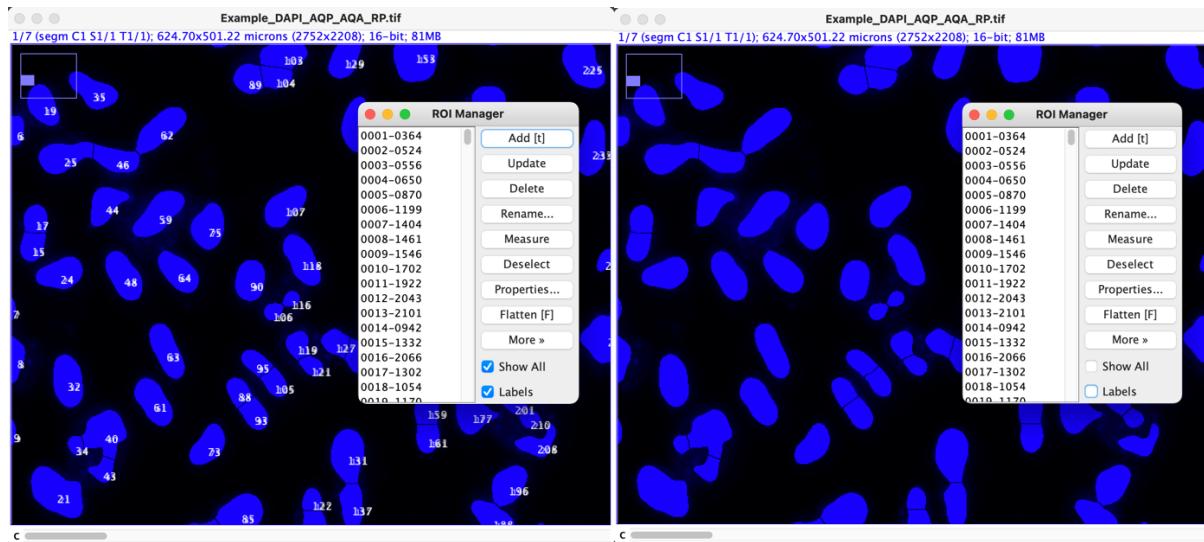
- The first column displays the name of the image where the adipocyte was detected. This is useful to find out from which image the object (i.e., adipocyte, droplet, or nucleus) came, e.g., in case you merge results from multiple images.

ID

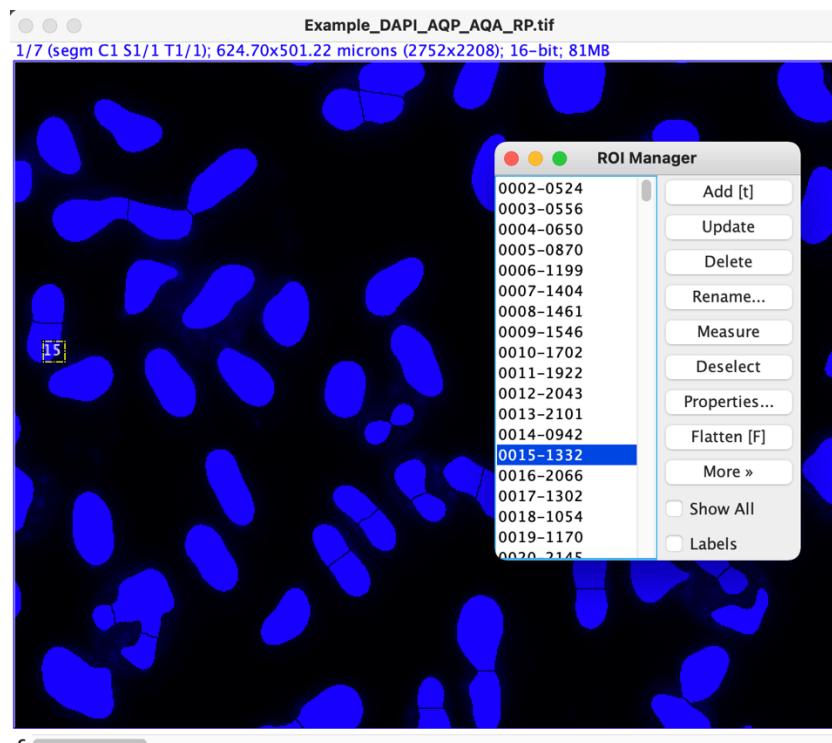
- The ID is unique per image: for each image, each detected object receives an ID. This ID allows you to find the object in the image when opening the AQA_RP.tif file, where objects are labeled with an ID.



- Side note: Sometimes, the image may contain thousands of objects and thus, it will not be easy to find the object in the AQA_RP.tif image. There is however one way in ImageJ to display only individual numbers in the AQA_RP.tif file. The numbers shown there are all stored in the so-called “Overlay” of the image. You can retrieve the numbers from there as individual ROIs via the command Image > Overlay > To ROI Manager.
- Now all numbers are moved to the ROI Manager which pops up.
- Unselect show ROIs to remove the display of the ROIs



- Now you can select an individual ROI in the Manager and it will be shown in the image solely, which allows you to simply find the Object with the specific ID. Note the ROIs in the Manager always start with the ID number.



Center X, Center Y, Center Z (μm)

- The center of the object in image coordinates, given in micron.
- Indicates the position in the image.

Voxels

- The number of pixels belonging to the object

Area (μm^2)

- The area covered by the object

Outline (μm)

- The outline of the object
- Calculated by summing up the outlines of all pixels belonging to the object
- The surface of a pixel is determined as the sum of
 - #sides without neighbor in x and y direction * pixel width
- Note: If the object contains a hole also the outline of the hole will be added to this parameter.

2D-Asphericity Index

- Is calculated by comparing the outline of the object to the outline of a sphere containing the same area as the object.
- The smaller the more circular the shape (1 = perfectly circular)
- The higher the more non-circular and complex the shape

Average, Integrated, Median, SD, Min, and Max Intensity

- Provided for any channel in the image - the channel nr is given at the beginning of the parameter label (e.g. “C2: Average Intensity” indicates that the parameter refers to the pixel intensities in channel 2):

C1: Average Intensity	C1: Integrated Intensity	C1: Median Intensity	C1: SD of Intensities	C1: Min Intensity	C1: Max Intensity	C2: Average Intensity	C2: Integrated Intensity	C2: Median Intensity	C2: SD of Intensities	C2: Min Intensity	C2: Max Intensity	C3: Average Intensity	C3: Integrated Intensity	C3: Median Intensity	C3: SD of Intensities
65535	36765135	65535	0	65535	65535	7505.46702	4210567	7281	3950.87852	1889	14296	1471.27451	825385	1463	58.754
65535	13893420	65535	0	65535	65535	3260.13679	691149	3248	741.365619	1755	4736	1505.02359	319065	1497	54.282
65535	9961320	65535	0	65535	65535	2668.41447	405599	2675.5	784.680636	1156	4591	1640.5	249356	1629	68.18

- These parameters describe the pixel intensity values of the pixels belonging to the object.
 - Average Intensity = average of all values
 - Integrated Intensity = Sum of all values
 - Median Intensity = Median of all values
 - SD Intensity = Standard deviation of all values
 - Min Intensity = Minimum intensity of all values
 - Max Intensity = Maximum intensity of all values
- Note that the pixels at holes in the object are not included, as their pixels do not belong to the object.

*Surroundings Parameters**Surr Voxels*

- The number of pixels that is considered as belonging to the surroundings of the object.
- This parameter is only output if the Quantify-Surrounding option in AdipoQ Analyzer is selected:

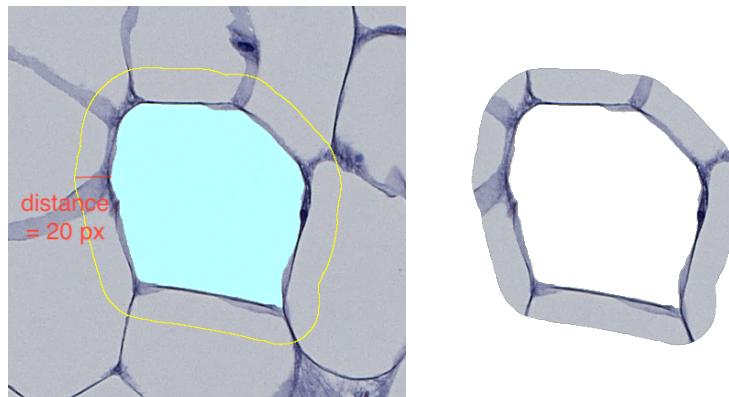
Quantify Surrounding | reference distance

20.00

- Pixels from the image are considered to belong to the surroundings of the object, if they do not belong to the object while their Euclidian distance to at least one pixel belonging to the

object is less than a user defined value. This distance value is pre-defined by the user in AdipoQ Analyzer (See also the explanations for AdipoQ Analyzer).

Example image below: For the adipocyte labeled in cyan, the pixels that are closer than or maximum 20 px apart (20 px is the value set by the user) from at least one pixel belonging to the adipocyte would be considered (left image). The right image shows only the pixels that are considered (all non-considered pixels are white).



Surr Average, Surr Integrated, Surr Median, Surr SD, Surr Min, and Surr Max Intensity

- Provided for any channel in the image - the channel nr is given at the beginning of the parameter label (e.g. “C2: Surr Average Intensity” indicates that the parameter refers to the pixel intensities in channel 2).
- These parameters are only output if the Quantify-Surrounding option in AdipoQ Analyzer is selected:

<input checked="" type="checkbox"/> Quantify Surrounding reference distance	20.00
---	-------

- These parameters describe the pixel intensity values of the pixels surrounding the object within a user-defined distance (see explanations for the parameter Surr Voxels). Based on the intensity values of these selected pixels the different parameters are quantified:
 - Surr Average Intensity = Average of all values
 - Surr Integrated Intensity = Sum of all values
 - Surr Median Intensity = Median of all values
 - Surr SD Intensity = Standard deviation of all values
 - Surr Min Intensity = Minimum intensity of all values
 - Surr Max Intensity = Maximum intensity of all values
- Note that the pixels at holes in the object are also included in this parameter, as their pixels do not belong to the object and are in close proximity to the object.
- To get an impression on what the parameters mean, please have a look at the next section showing an Example.

Surr Average Intensity Min 5%, Surr Average Intensity Min 25%, Surr Average Intensity Max 5%, Surr Average Intensity Max 25%

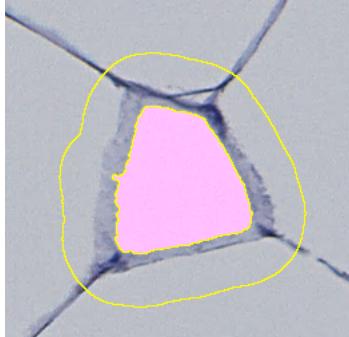
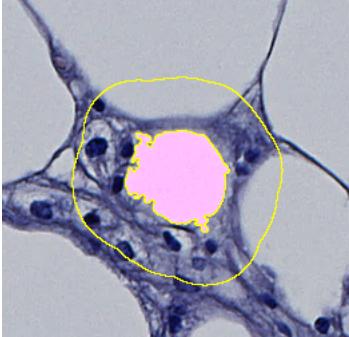
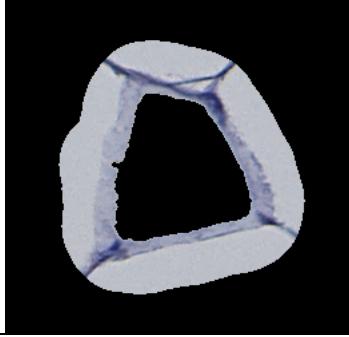
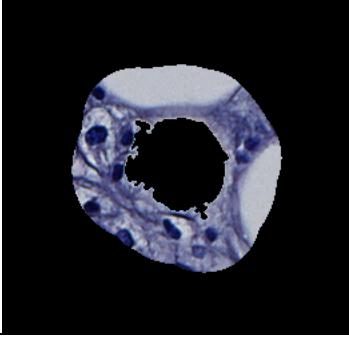
- Provided for any channel in the image - the channel nr is given at the beginning of the parameter label (e.g., “C2: Surr Average Intensity Min 5%” indicates that the parameter refers to the pixel intensities in channel 2).
- These parameters are only output if the Quantify-Surrounding option in AdipoQ Analyzer is selected:

<input checked="" type="checkbox"/> Quantify Surrounding reference distance	20.00
---	-------

- These parameters describe the pixel intensity values of a certain subset of pixels surrounding the object within a user-defined distance (see explanations for the parameter Surr Voxels). From this pool of surrounding pixels only a certain fraction is extracted and their intensities are averaged to determine the parameter:
 - Surr Average Intensity Min 5% = Only the 5% of all surroundings pixels with the lowest intensity values among all surroundings pixels are quantified. The average of their intensity values is output.
 - Surr Average Intensity Min 25% = Only the 25% of all surroundings pixels with the lowest intensity values among all surroundings pixels are quantified. The average of their intensity values is output.
 - Surr Average Intensity Max 5% = Only the 5% of all surroundings pixels with the highest intensity values among all surroundings pixels are quantified. The average of their intensity values is output.
 - Surr Average Intensity Max 25% = Only the 25% of all surroundings pixels with the highest intensity values among all surroundings pixels are quantified. The average of their intensity values is output.
- To get an impression on what the parameters mean, please have a look at the next section showing an Example.

Example for the surroundings parameters

To get an impression on how the parameters for the surroundings describe the surroundings of an adipocyte in a stained tissue (e.g., Hematoxylin-Eosin staining), compare the following two adipocytes:

	Mostly adipocytes in surroundings	Many structures other than adipocytes in the surroundings
Image with yellow lines marking the surroundings for the object labeled in magenta.		
Pixels in surrounding (20 px)		
Surr Voxels	56878	47642
C3: Surr Average Intensity	185.36	150.76 (= lower)
C3: Surr Integrated Intensity	10543071	7182292 (= lower but do not use (see below))
C3: Surr Median Intensity	191	187 (= lower)
C3: Surr SD of Intensities	19.84331	52.30 (= higher)
C3: Surr Min Intensity	23	0 (= lower)
C3: Surr Max Intensity	223	234 (= similar)
C3: Surr Average Intensity Min 5%	111.87	31.15 (= lower)
C3: Surr Average Intensity Min 25%	164.59	71.59 (= lower)
C3: Surr Average Intensity Max 5%	198.29	199.52 (= similar)
C3: Surr Average Intensity Max 25%	194.77	193.85 (= similar)

Evaluation and hints:

- Note that all these hints are mostly referring to using this parameter for quantifying an HE staining. When you have immunofluorescence images, then these parameters may also be useful but depending on your research question their may be different important aspects you want to look at and thus different parameters more relevant than highlighted here. Thus, just use this example here to understand the parameters, but carefully rethink the parameters when translating them to a different paradigm.

- As can be seen from the example, in particular, the average, median, SD, min 5% and min 25% values differ between the two adipocytes.
- Be cautious with Min and Integrated intensity because
 - Min Intensity just relies on a sole pixel value and thus is generally very shaky because largely affected by detection noise.
 - The integrated intensity depends on how many pixels belong to surroundings > if an adipocyte is larger, the parameter value will be directly larger. However, this parameter may be useful if you have a fluorescence channel. For example, if you would have a fluorescence channel that labeled specific dot structures that are all of same size (e.g., specific nuclei), then the integrated intensity could quantitate how many dotty structures are near. However, this value will be only accurate, if you had segmented the channel to be quantified and pixels not belonging to the surroundings object of interested would be 0.
- The Surr Average Intensity Max 5% and Max 25% parameters are stable, as they reflect the intensity of surrounding adipocytes. They might be usable as a reference value for the image background.
- The Surr Average Intensity Min 5% and Min 25% are good indicators for there are surroundings structures. The advantage of averaging only the lowest intensity pixels is that the parameters also detect surrounding structures when they are only on one side of the adipocyte and ignores the intensities from other surrounding adipocytes, which will be high. Still, they are better than the Min value, as they do not rely on one pixel value only but on multiple pixel values. However, note that this parameter has limitations. It will not well distinguish differences between adipocytes with many surroundings structures as there the min 5% or min 25% will always be similar but the other intensities in the surrounding beyond those selected low pixel values make the difference. Thus, this parameter is good to indicate whether there is some surrounding structure but not for comparing the extent of surrounding structures. Accordingly, the parameter could be used in a post-hoc analysis to filter for “interesting” adipocytes, whose surroundings could then be compared using other surroundings parameters (e.g., SD or Average).
- Note that if you use fluorescence to label certain surroundings structures of interest, then the strongest signals will be represented as highest intensity values (whereas in an HE staining, this is the opposite, as in such images surrounding structures are of lower intensity values). Thus, for fluorescence images, the min 5% and min 25% parameters may be the background estimates whereas the Max 5% and Max 25% parameters may be the interesting parameters to filter for surroundings structures.
- The SD is the only parameter that rises with more surrounding structures. This is because it predominantly describes the heterogeneity in the surrounding. Very heterogeneous surroundings (containing adipocytes, nuclei, and other structures) will get a high SD.

Explore data sets in a post-hoc analysis

To explore data sets analyzed with AdipoQ programmatically, have a look at the R scripts that we provide in the AdipoQ GitHub repository:

<https://github.com/hansenjn/AdipoQ/tree/main/R%20Scripts>.