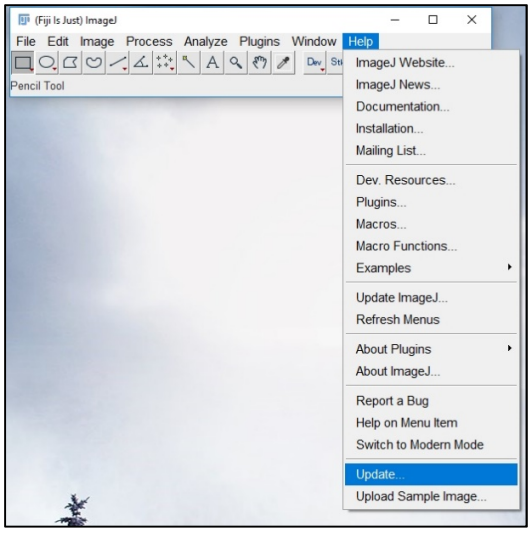
ALDQ Protocol, Instructions and Troubleshooting

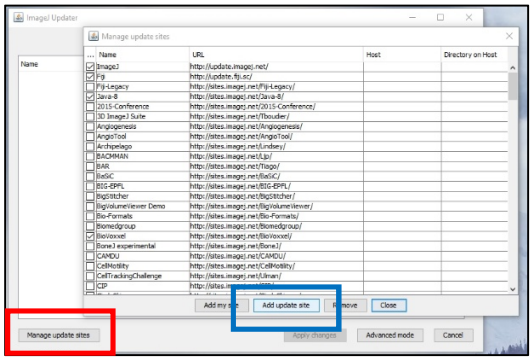
1. Installation of ALDQ

Download and install ImageJ/Fiji for your operating system. ImageJ/Fiji can be downloaded free of charge at the following link: https://imagej.net/Fiji/Downloads

After installation start ImageJ/Fiji and open the ImageJ/Fiji Updater:



ImageJ/FIJI menu => *Help* => *Update…*



ImageJ Updater => *Manage update sites* (red) => *Add update site*

Rename “New” to “ALDQ”, paste the URL:

“http://sites.imagej.net/Fuellekrug-Lab/”

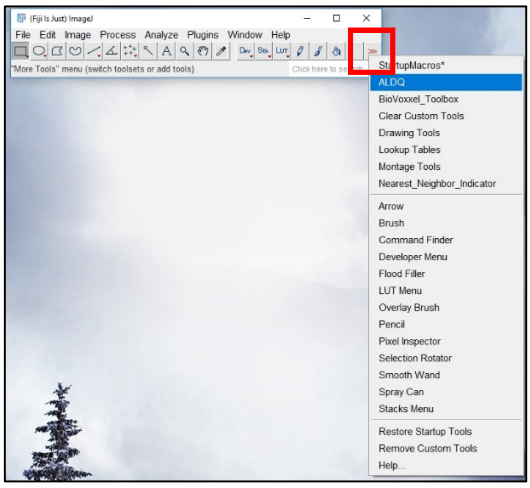
Restart ImageJ/Fiji

After restarting, go to

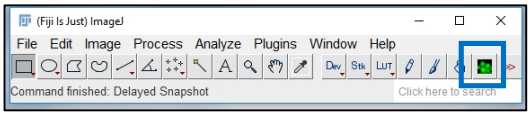
ImageJ/FIJI menu => Help => Update…

Update and restart ImageJ/Fiji. Note, two additional plugins are installed which are necessary for the script to run.

The script is now part of the user ImageJ/Fiji installation at \path\to\Fiji\Fiji.app\macros\toolsets.



After restarting, click on the two red arrows on the right-hand side (red square) and select “ALDQ”.

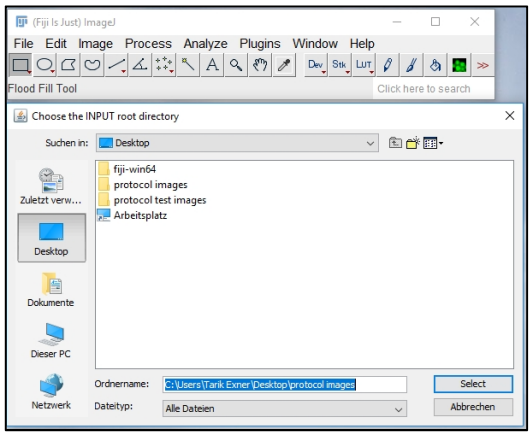


The ALDQ icon is shown as green lipid droplets in the Icon-Bar (blue square).

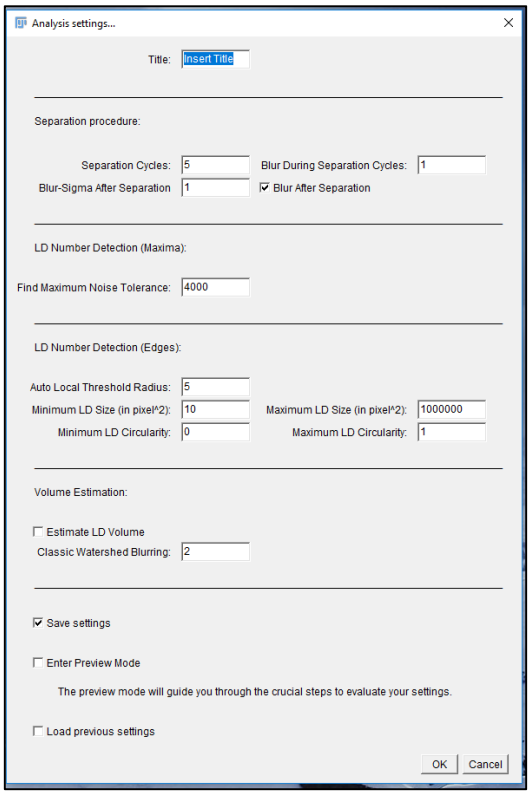
2. Use ALDQ

The raw images to analyze must be located in one generic folder and have the following requirements:

- 8-bit or 16-bit
- Single channel
- TIF/TIFF file format



The ALDQ analysis is started by clicking the ALDQ icon. The user can navigate to the input source directory and choose the folder with the raw images to process.

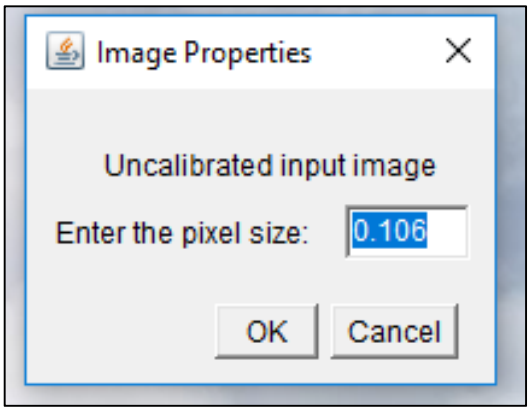


The dialog box “Analysis settings…” opens and the user can enter the input parameters necessary for the analysis.

For **recommendations**, refer to the Supplemental material of the original publication and to the tables “Guidelines for settings adjustment” and “Troubleshooting” below.

Enable the **preview mode** to optimize the default setting parameters (see section 4).

The user can load **previous settings** by enabling the checkbox “Load previous settings” at the bottom of the dialog box. By checking the box, the user can select the folder where the “Experimental_settings.txt” (see below) file is stored.



If the raw images are uncalibrated, the user can specify the pixel size in microns. In the example, 1 pixel measures 0.106 x 0.106 micrometer in xy dimensions.

Outline the cell borders with the polygon tool which is already pre-selected. Other tools can be selected by the user in the icon menu.

If the user doesn't select a region of interest the whole image is analyzed.

Tip: Use the right-click to close the polygon.

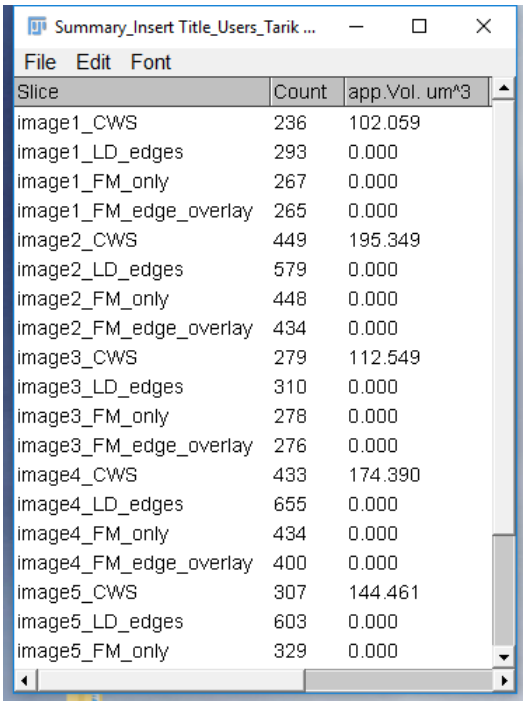
Click "OK".

Wait for the script to finish the LD quantification. At the end of the process the next input image will be automatically loaded.

3. Data analysis and ALDQ output

3.1. Data analysis

Once all the raw images in the input source directory are analyzed, the script displays the "Summary Window". The "Summary Window" is additionally saved in the output root directory. We decided to leave the "Summary Window" open to continue directly with the statistic.



Slice	Count	app.Vol. um^3
image1_CWS	236	102.059
image1_LD_edges	293	0.000
image1_FM_only	267	0.000
image1_FM_edge_overlay	265	0.000
image2_CWS	449	195.349
image2_LD_edges	579	0.000
image2_FM_only	448	0.000
image2_FM_edge_overlay	434	0.000
image3_CWS	279	112.549
image3_LD_edges	310	0.000
image3_FM_only	278	0.000
image3_FM_edge_overlay	276	0.000
image4_CWS	433	174.390
image4_LD_edges	655	0.000
image4_FM_only	434	0.000
image4_FM_edge_overlay	400	0.000
image5_CWS	307	144.461
image5_LD_edges	603	0.000
image5_FM_only	329	0.000

Select all the rows and columns (CTRL+A) and transfer them to a software for statistical analysis.

For each image process you can find four rows in the summary window:

_CWS (only if the LD volume is estimated):

Count: LDs identified by image flooding (classic watershed; CWS)

App.Vol. um^3: Calculated approximated volume of LDs in the analyzed cell.

_LD_edges:

Count: LDs identified by edge detection.

_FM_only:

Count: LDs identified by the detection of central fluorescence maxima (FM)

_FM_edge_overlay:

Count: LDs identified by the overlay of a detectable edge and a central fluorescence maximum.

For the analysis, use the LD count "_FM_edge_overlay" and the calculated volume "_CWS".

The counts of "_LD_edge" and "_FM_only" are for control purposes. The values should be in the same range. Otherwise the detection settings might be too sensitive or too specific.

3.2. ALDQ output

Output root directory:

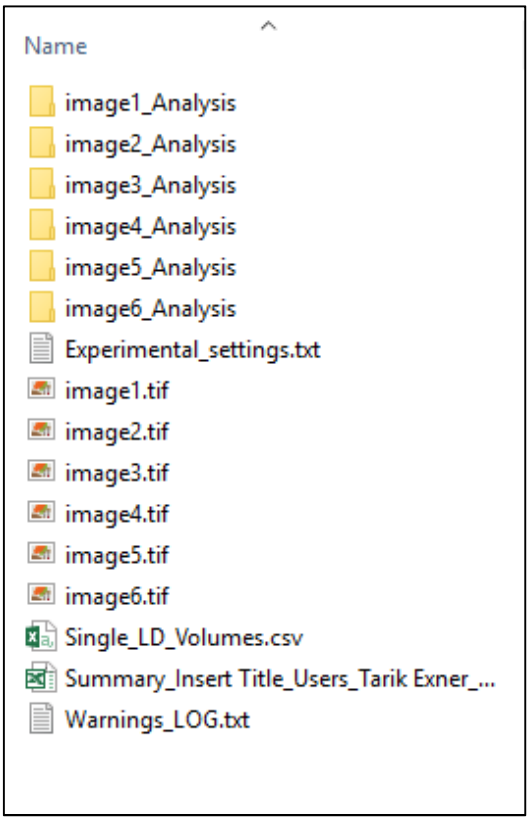


Image analysis folders

Experiment_settings.txt:

The text file summarizes the input user setting. This file can be loaded at the beginning of the analysis using the "Analysis settings…" window (see above)

Raw images

Single_LD_Volumes.csv:

Every analyzed lipid droplet from all images in that folder are stored in this .csv table with the respective area and the volume. This table allows to create a histogram plot for the single LD volumes.

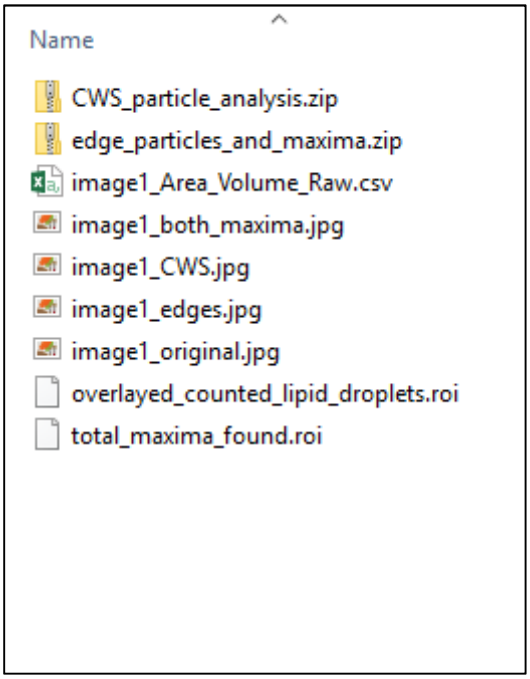
Summary.xls:

The "Summary Window" as described in 3.1. is saved here.

Warnings_LOG.txt:

Includes some major information on the process and possible warnings that were documented during the analysis.

Image specific output (Analysis folder):



CWS_particle_analysis.zip

Includes the coordinates of the LDs identified by Classic Watershed (CWS) as .roi files.

Edge_particles_and_maxima.zip

Includes the coordinates of the LDs identified by edge detection, the coordinates of the identified local intensity maxima and the cell border outline. Stored as .roi files.

_Area_Volume_Raw.csv

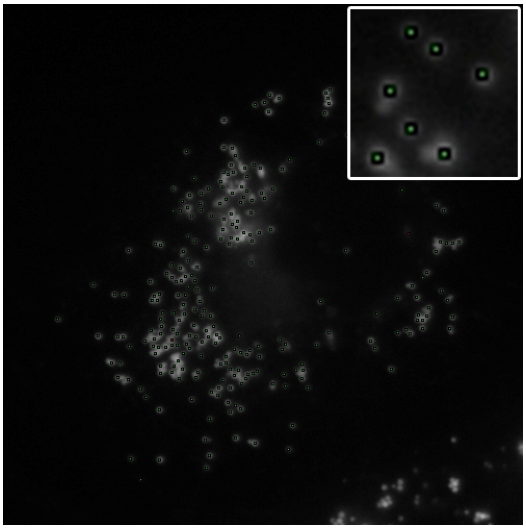
Includes the area and volume of the single LDs.

Overlayed_counted_lipid_droplets.roi.

Coordinates of the central fluorescence maxima that are located on edge-identified lipid droplets.

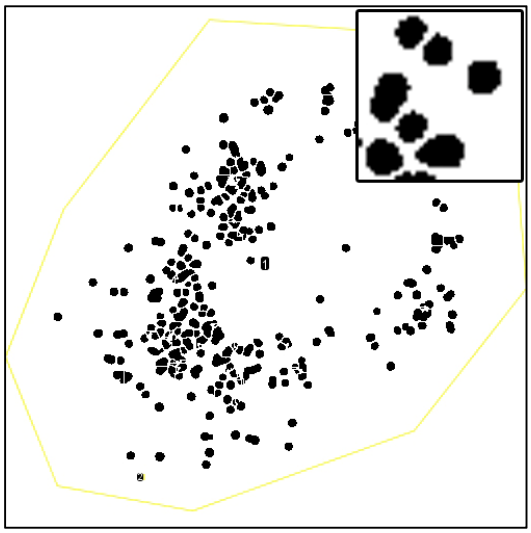
Total_maxima_found.roi

Coordinates of all fluorescence intensity maxima.



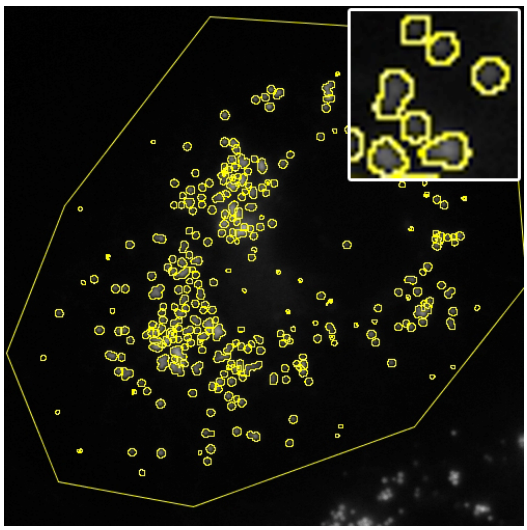
_both_maxima.jpg

Displays the raw image in overlay with LDs identified by LD edge and the central fluorescence maximum (green dot) and LDs identified by a central fluorescence maximum only (red dot).



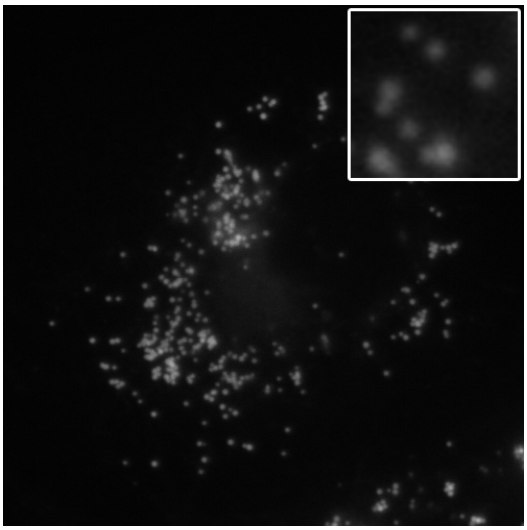
_CWS.jpg

Displays LDs identified by Classic Watershed (CWS). The area of those particles is used for LD volume determination.



_LD_edges.jpg

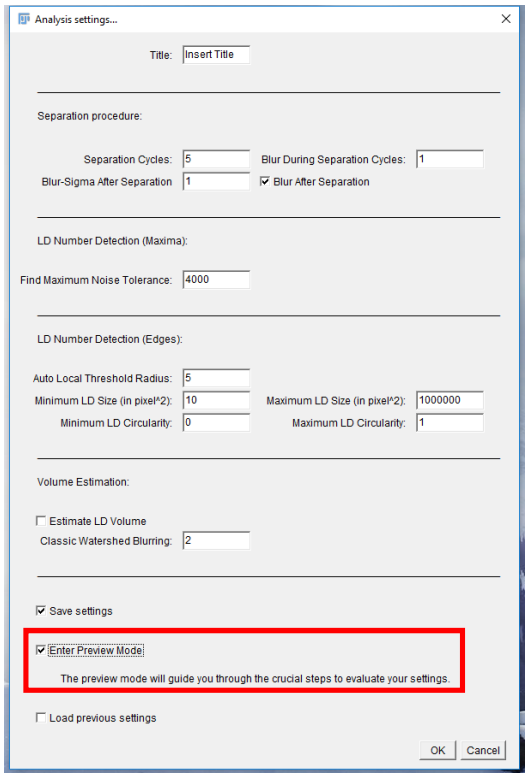
Displays the identified LD edges as an overlay on the raw input image (yellow line).



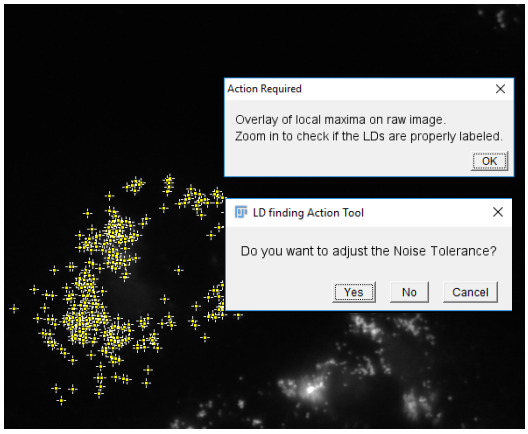
_original.jpg

Raw input image for comparison.

4. Preview Mode

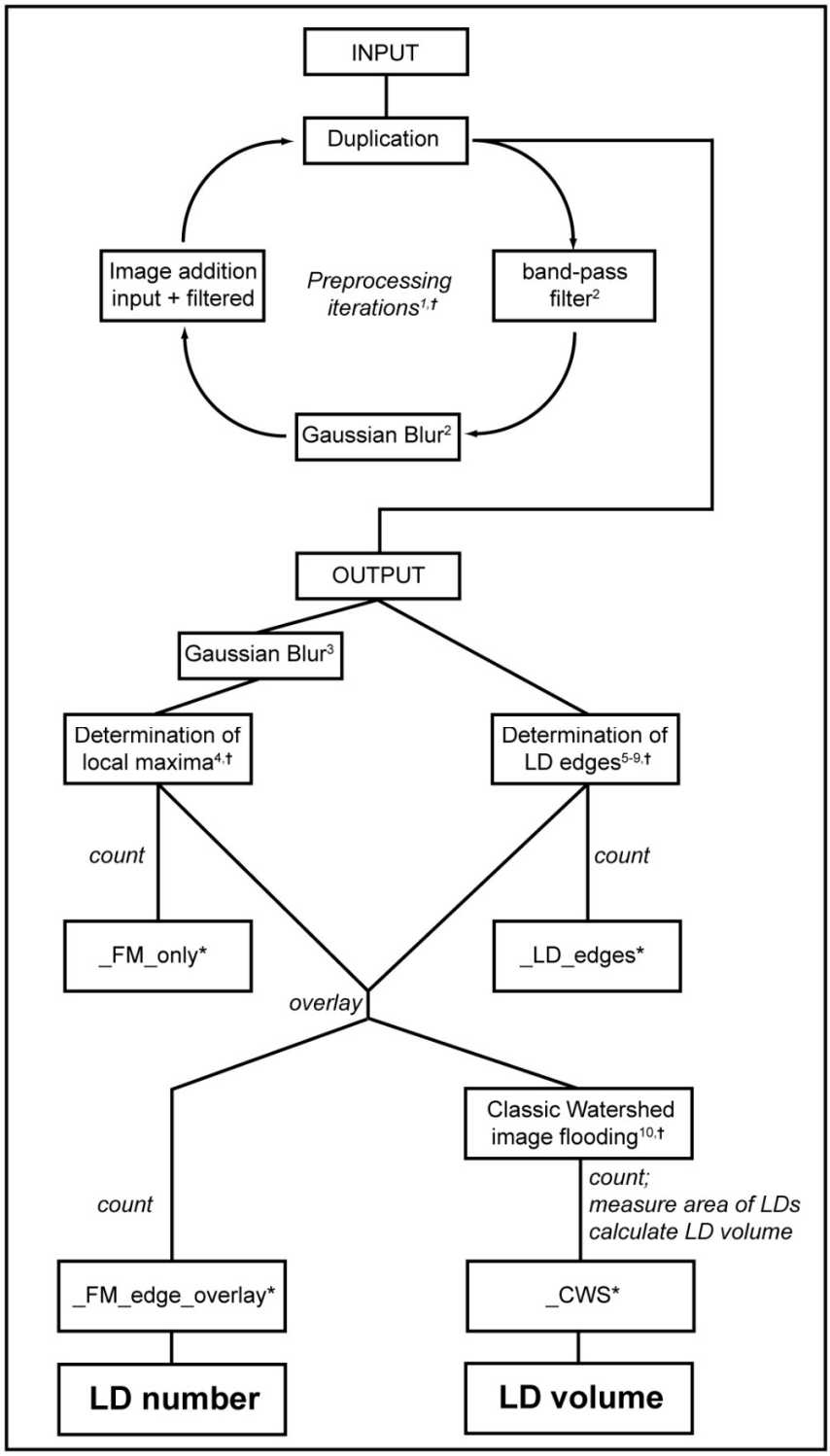


To enable the preview mode, check the "Enter Preview Mode" option in the "Analysis settings…" dialog window (red rectangle).



The preview mode is designed to stop the script at pre-defined critical steps and displays the processed image. The user can zoom in and check if the LDs are properly segmented or identified by their central fluorescence maximum, detected by their edge or segmented by image flooding. If the LD identification lacks sensitivity or specificity the settings can be adjusted during the analysis to gain the best detection. The settings are then saved in the respective analysis folder. To adjust the settings, use the recommended settings provided in Table 1 and Table 2 (see below).

5. Analysis flow of ALDQ



For legend see next page

Analysis workflow of ALDQ (continued)

Flowchart of the steps carried out by the ALDQ algorithm. The summarizing output parameters are labeled with * (compare section 3.1). The changeable analysis settings are assigned to the respective analysis steps (superscript numbers). Analysis steps after which the preview mode stops the script and displays the images are marked by †.

* output of the summary window; † displayed in the preview mode; [1] Preprocessing iterations; [2] Blur During Preprocessing Iterations; [3] Blur After Preprocessing; [4] Find Maximum Noise tolerance; [5] Auto Local Threshold Radius; [6] Minimum Particle Size; [7] Maximum Particle Size; [8] Minimum Particle Circularity; [9] Maximum Particle Circularity; [10] Classic Watershed Blurring

Table 1: Guidelines for settings adjustment

	Purpose	recommended starting point	recommended change rate	Effect of increasing (+) and decreasing (-)
Preprocessing Iterations	LD enhancement, background suppression	3*	steps of 1*	(+) more LD enhancement and background suppression, higher probability of over-enhancement (-) less enhancement of LDs and background suppression, decreased sensitivity of local maxima detection
Blur During Preprocessing Iterations	efficiency of the band-pass filter	1	steps of 1	(+) Less over-enhancement of larger lipid droplets, less efficient separation of small LDs (-) More over-enhancement of larger lipid droplets, more efficient separation of small LDs
Blur After Preprocessing (value)	attenuation of unspecific local maxima	1	steps of 1	(+) less sensitive local maxima detection (-) more sensitive local maxima detection
Find Maximum Noise Tolerance	Sensitivity of local maxima detection	4000*	steps of 500*	(+) less sensitive local maxima detection (-) more sensitive local maxima detection
Auto Local Threshold radius (edge detection)	Sensitivity of edge detection	10	steps of 10	(+) more sensitive edge detection (-) less sensitive edge detection
Minimum LD Size	Exclusion of small particles (area [px])	15	steps of 10	(+) larger edge defined particles get excluded from the analysis (-) smaller edge defined particles get included in the analysis
Maximum LD Size	Exclusion of large particles (area [px])	1.000.000		(-) larger edge defined particles get excluded from the analysis
Minimum LD Circularity	Exclusion of non-circular objects	0.4	steps of 0.05	(+) non-circular objects are more excluded from the analysis (-) non-circular objects are more included in the analysis
Maximum LD Circularity	Exclusion of circular objects	1		(-) circular objects are excluded from the analysis
Classic Watershed Blurring	Image smoothing before flooding	2	steps of 0.5	(+) more smoothing, higher probability of under-segmentation (-) less smoothing, higher probability of over-segmentation

The different settings from Suppl. Table 1-5 of the main manuscript and section 5 of the protocol are assigned a purpose, a recommended starting point and a recommended change rate for 16-bit images. The effect of adjusting the settings is explained for increasing ((+)) and decreasing ((-)) the respective value. Highlighted in gray are settings that needed frequent changes dependent on the LD morphology in different cell lines (see Discussion in the manuscript). (*) values are referring to 16-bit images and need to be scaled accordingly for other image depths. px: pixel

Table 2: Troubleshooting

LD number measurement	
Lipid droplets are not recognized by local maxima (no marker on LDs*)	(1) Decrease “Find Maximum Noise Tolerance” (2) Increase the number of “Preprocessing Iterations”
Background is falsely identified as lipid droplets (green marker on background structures*)	(1) Increase “Find Maximum Noise Tolerance” (2) Decrease the number of “Preprocessing Iterations” (3) Increase “Blur After Preprocessing” (4) Increase “Minimum Particle Circularity”
Lipid droplets are recognized by local maxima but not assigned a lipid droplet (red marker on LDs*)	(1) Increase “Auto Local Threshold radius”
More than one green or red marker on one lipid droplet *	(1) decrease number of “Preprocessing Iterations” (overenhancement) (2) Increase “Blur During Preprocessing Iterations”
LD volume estimation	
LDs are oversegmented **	(1) Decrease number of “Preprocessing Iterations” (2) Increase “Classic Watershed Blurring”
LDs are not separated **	(1) Increase number of “Preprocessing Iterations” (2) Decrease “Classic Watershed Blurring”
LDs are missing **	(1) Increase “Auto Local Threshold radius”
Particles do not correspond to lipid droplets **	(1) Decrease “Auto Local Threshold radius”

Common problems and possible solutions to optimise LD detection. The algorithm outputs images comprising the detected local maxima (*), edges and largest cross-sections (**), among other data (section 3). These images are supposed to be used to adjust the settings accordingly as displayed here. This table can also be used for changes during the preview mode.