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Machine learning-assisted quantification of organelle abundance.

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Abstract

Organelle abundance is a key microscopic readout of organelle formation and, in many cases, function. Quantification of organelle abundance using confocal microscopy usually requires estimating of their area based on the fluorescence intensity of compartment-specific markers. This analysis usually depends on a user-defined intensity threshold to distinguish organelle regions from the surrounding cytoplasm, which introduces potential bias and variability. To address this issue, we present a machine learning-assisted algorithm that allows for the quantification of organelle density using the open-source Fiji platform and WEKA segmentation. Our method enables the automated quantification of organelle number, area, and density by learning from training data. This standardizes threshold selection and minimizes user intervention. We demonstrate the utility of this approach for both membrane and non-membrane organelles, such as peroxisomes and stress granules, in human cells and whole fish samples.

Key features

- The organelle abundance algorithm is an automated, open-source, Fiji-based tool that extracts the number and area of organelles and calculates organelle their abundance based on a single organelle marker.
- The macro measures the average intensity of all the segmented areas and quantifies their area.
- The algorithm is applicable to cellular compartments, including membrane-bound and membrane-less organelles.
- The training is performed on a sample dataset, enabling the algorithm to be applied to all images obtained with the same imaging parameters.

Keywords: organelle, peroxisome, stress granule, quantification, abundance, density, machine learning, Fiji, WEKA segmentation, PeroxiSPY

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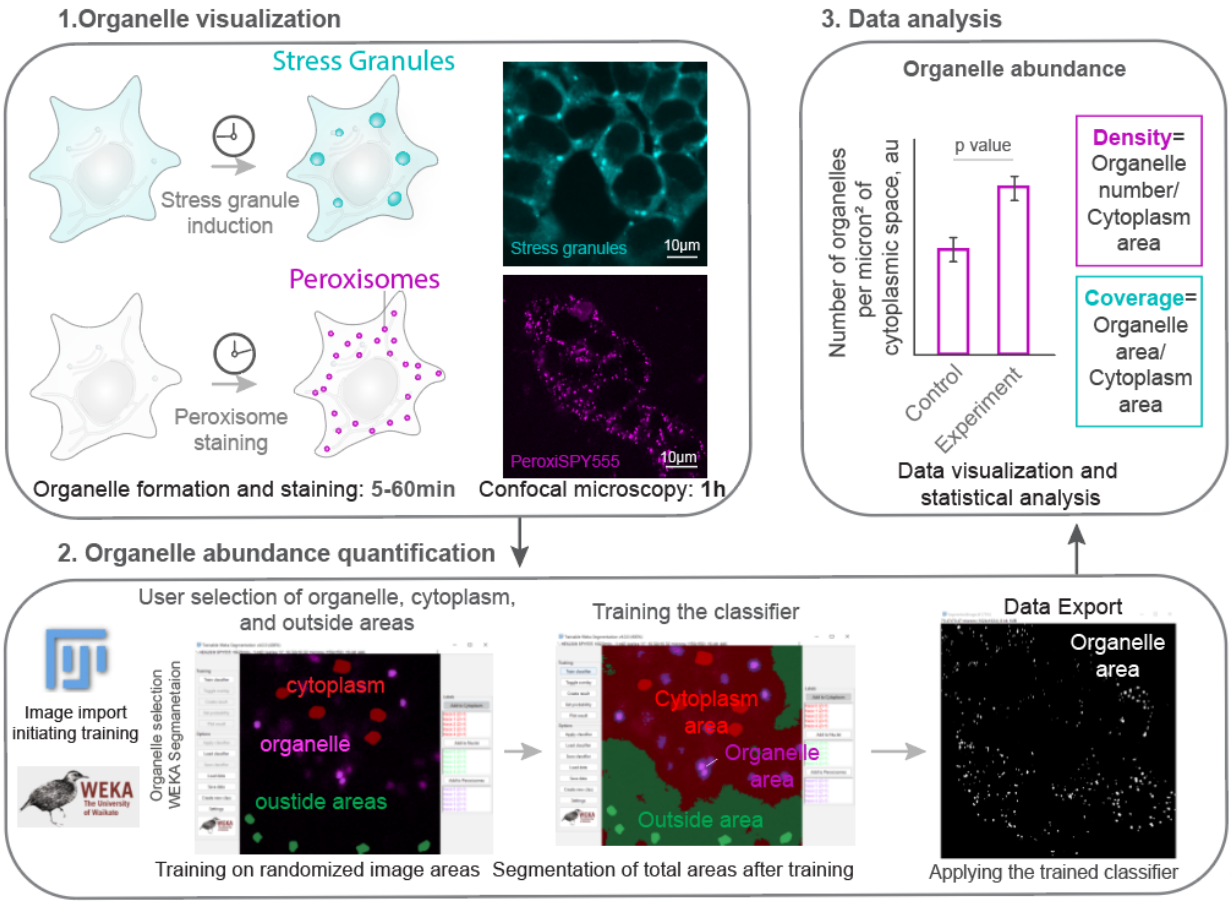
Graphical overview



Graphical overview of the protocol. Section 1 (Organelle visualization) corresponds with section A in the procedure. Section 2 (Organelle abundance quantification) corresponds with section B in the procedure. Section 3 (Data analysis) takes place after the procedure.

Background

Quantifying organelle abundance by determining how many organelles are present in the cell and the area of the cytoplasm they cover provides a useful measure of organelle dynamics and offers insight into organelle function. For example, stress granules are membrane-less organelles that form from stalled translation pre-initiation complexes and other proteins in response to stress (Ivanov et al., 2019; Protter and Parker, 2016; Riggs et al., 2020). They function when they form in the cytoplasm. Similarly, peroxisomes are membrane-enclosed organelles that are essential for lipid and oxidative metabolism (Kumar et al., 2024; Wanders and Waterham, 2006; Wanders et al., 2018). They undergo division and increase in number when their metabolic activity is required. In both cases, quantifying organelle density in the cytoplasm correlates with organelle function in healthy cells.

Organelle abundance can be estimated from confocal imaging by visualizing resident proteins or established organelle markers (Metz et al., 2017). An organelle marker is any fluorescently labeled molecule that localizes to an organelle. Examples include overexpressing a fluorescent protein fused to an organelle localization signal, or an endogenously tagged protein. Examples of endogenously tagged proteins include the CRISPR/Cas9 genomic fusion of polyA-binding protein PABPC1 to Dendra2, which is used as a marker of stress granules (Amen and Kaganovich, 2021), and ATP-binding cassette sub-family D member 3 (ABCD3) fused to GFP as a marker of peroxisomes (Borisyuk et al., 2025)). Other examples include using a fluorescent probe that selectively targets the organelle, such as PeroxiSPY, which is used to label peroxisomes (Korotkova et al., 2024)), or using antibody staining of organelles post-fixation (Borisyuk et

al., 2025). The quantification requires segmentation, or defining the boundaries of compartments, which is often based on visual inspection. Manual segmentation is prone to error; for example, it is difficult to consistently outline highly variable, punctate, or overlapping patterns. To improve the reliability of quantification, thresholding methods set an intensity value independent of the user and improve consistency (Russell et al., 2009). Machine learning approaches further reduce errors of manual segmentation by providing consistent labeling based on user-defined parameters (Arganda-Carreras et al., 2017; Lam et al., 2025; Lefebvre et al., 2025).

Here we present a protocol for quantifying the abundance of membrane and membrane-less organelles based on a single marker using Fiji software (Schindelin et al., 2012). This protocol combines the WEKA Segmentation (Arganda-Carreras et al., 2017) and watershed algorithms into a user-friendly workflow. The quantification is based on trainable organelle recognition, which limits user-defined thresholding bias and improves the consistency of image processing (Isensee et al., 2021; Lefebvre et al., 2025; Newby et al., 2018). Recognition requires reliably outlining organelles for initial training, a task that can be done by experienced researchers. The trained classifier can then be applied to any number of images obtained with the same microscopy settings. Alternatively, a beginning researcher can train the classifier and include controls to verify whether the recognized area corresponds to an organelle area.

As a proof-of-concept, we validate that machine learning-assisted recognition of cellular compartments corresponds to an independent marker of cellular cytoplasmic and peroxisome areas, using a control marker for both the organelle and the cytoplasm. In many experimental setups, adding additional markers is not feasible, and manually extracting the cytoplasmic area from a single organelle marker is biased and time-consuming. Our results demonstrate that machine-learning assistance enables the consistent and reliable identification of these regions. Finally, we apply the macro to quantify the non-membrane compartment density of stress granules using a CRISPR/Cas9-tagged PABPC1 cell line (Amen and Kaganovich, 2020). The algorithm recognizes conditions in which there are no stress granule compartments and extracts areas based on a single marker.

This protocol can be modified further to estimate organelle coverage, the area of the cell occupied by the cytoplasm, or to extract abundance in 3D using stacks of 2D images obtained from tissues. This provides a useful tool for quantitative cell biology experiments.

Materials and reagents

Biological materials

Cell lines and organisms used in this study

1. Human embryonic kidney cells HEK293T (ATCC® CRL-3216™)
2. HEK293T PABPC1-DDR2 cell line (Amen and Kaganovich, 2021)3. Zebrafish *Danio rerio;*

Zebrafish husbandry and ethics:

All zebrafish were raised at the University of York in UK Home Office approved aquaria and maintained following standard protocol (Cunliffe, 2003). Tanks were maintained at 28 °C with a continuous re-circulating water supply and a daily light/dark cycle of 14/10 hours. The nacre WT background strain was used to perform injections.

Cell culture

HEK293T cells were maintained in high-glucose DMEM media supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, at 37 °C/5% CO_2. The media was replaced every 2nd day; the cells were seeded at a density of 50,000 cells per a well of a 4-well imaging plate. Cells were visualized at a 50-70% confluency, cells were split before they reached 90% confluency.

Stress granule formation

To visualize stress granules, replace the media in the glass-bottom plate for live-cell imaging with 200 µM sodium arsenite dissolved in the cell culture media and incubate for 1 h in the cell culture incubator at 37 °C/5% CO_2 (Amen and Kaganovich, 2020). To prepare 200 µM sodium arsenite, dissolve 2 µl of 100 mM sodium arsenite stock in water in 1 ml of media, use vortex mixing or pipetting.

Peroxisome staining

Peroxisomes were stained with PeroxiSPY (Korotkova et al., 2024), as described in (Howman et al., 2025). To visualize

peroxisomes, replace the media in the glass-bottom plate for live-cell imaging with 1 µM PeroxiSPY dissolved in the cell culture media and incubate for 5-10 m in the cell culture incubator at 37 °C/5% CO_2.

Reagents

1. PeroxiSPY555 (Spirochrome, catalog or CAS number: SC207: Peroxi_SPY555). Store 1 mM stock in DMSO in -20 °C long-term, aliquotes can be stored at +4 °C.
2. Sodium arsenite (Thermo Fisher Scientific, catalog or CAS number: 7784-46-5)
3. Immersion liquid (Cargille, catalog or CAS number: 16482)4. Penicillin/Streptomycin for cell culture (Pan Biotech, catalog or CAS number: P06-07050)

Solutions

1. Dulbecco's Modified Eagle's Medium (DMEM) cell culture media with 10% fetal bovine serum (FBS) (Pan Biotech, P30-3031, P04-03590)
2. 100 mM sodium arsenite in water (use as a 500x stock). To dissolve in tissue culture medium add 2 µl of the stock to 1 ml of medium, mix by vortex mixing or pipetting before adding to the cells.
3. 1 µM PeroxiSPY in DMEM medium. To dissolve PeroxiSPY in media, add 1 µl of the 1 mM stock to an Eppendorf tube, then add 1 ml of media and mix by vortex mixing or pipetting before adding to the cells.

Laboratory supplies

1. 4-chamber glass bottom plates CellVis, catalog number: D35C4200N, cover glass (0.13-0.16 mm), used for confocal imaging
2. tissue culture 10 cm plates (Sigma) used for cell culture.

Note: *Plastic plate thickness will not allow 60x confocal imaging, however it is possible to image larger organelles through the plastic plates with lower magnification e.g. 20x objective, e.g. they can be used for nuclei segmentation.*

Equipment

1. Nikon A1r confocal microscope (Nikon, A1r), equipped with a CFI Plan Apo Lambda 60x oil NA 1.42 objective
2. Nikon W1 Spinning disk microscope (Nikon),), equiped with a CFI Plan Apochromat Lambda S 60XC Sil objective
3. CO_2 chamber and temperature control unit (OKO Lab)
4. CO_2 incubator for cell culture (Thermo Fisher Scientific.)
5. CFI Plan Apo Lambda 60x oil NA 1.42 objective (Nikon)
6. 561 nm laser and a 488 nm laser (Coherent)
5. Eppendorf tubes (Eppendorf)

Software and datasets

Type	Software/dataset/resource	Version	Date	License	Access (free or paid)
Data	Example dataset	N/A	01.12.2025	N/A	Free, Supplementary Dataset S1
Software	Fiji software (or ImageJ)	Java 21.0.7 (64-bit)	01.12.2025	(Schindelin et al., 2012; Schneider et al., 2012)	Free
Software	NIS Nikon software	4.1	N/A	N/A	Paid (microscope software)
Macro (organelle abundance algorithm)	Resource	1.0	01.12.2025	N/A	Free, Supplementary Code S1

Procedure

A. Image acquisition and wet-lab component of the protocol

Cell culture preparation for live-cell imaging, timing: 24 h

To prepare for confocal imaging we used standard cell culture protocols. Cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, at 37 °C/5% CO_2.

Caution: If your microscope detects the signal from phenol red in the media, use phenol red-free alternatives, e.g. P04-01158 (Pan Biotech).

1. One day before image acquisition split the cells into a glass-bottom imaging plate to achieve a 50-60% confluency the next day. We split HEK293T for peroxisome staining and HEK293T PABPC1-DDR2 for stress granule imaging at a density of 50.000 cells per well of the microscopy plate.

2. Live-cell image acquisition, Timing: 1 h

Imaging organelles requires high spatial resolution. For this protocol we used Nikon A1r confocal microscope equipped with a gallium arsenide phosphide cathodes (GaAsP) photomultiplier modules, a CO_2 chamber and a temperature control unit. The imaging was done using a bi-directional Galvano-scanning mode using CFI Plan Apo Lambda 60x oil NA 1.42 objective, using a 561 nm laser (Coherent, 50 mW) for PeroxiSPY555, and 488 nm laser (Coherent, 50 mW) for DDR2.

2.1. Turn on the microscope, required lasers, and the CO_2/temperature units. Ensure that the CO_2 and 37 °C temperature are stabilized for at least 15 minutes before the imaging.

2.2. Initiate the software and adjust the software parameters for acquisition. We used 0.5-2% laser power and HV (gain) of 100 and acquired a 1024*1024 px, zoom 3-4x images.

Note: *To ensure maximum resolution we recommend following Nyquist sampling (Shannon, 1949) – adjust the pixel size to be 2.3 times smaller than the smallest compartment that you are imaging. Increasing HV (gain) will increase the brightness but will also increase the noise. To ensure that data is usable, the same imaging parameters should be applied to all images within one experiment. Any confocal microscope can be used for this protocol.*

3. Staining and acquiring of the organelle images.

3.1. **Stress Granule imaging** requires inducing the formation of stress granules as previously described (Amen and Kaganovich, 2020). Pipette a 100 mM sodium arsenite solution as a 1/500 part of your total media volume in an empty Eppendorf tube, e.g. for 0.5 mL of media we pipette 1 µL of arsenite. Collect the media from the cells and mix with sodium arsenite by vortex mixing or pipetting. After mixing, add the mix to the plate and incubate for one hour in the cell culture incubator.

For **peroxisome imaging**, stain cells with PeroxiSPY for 10 m as previously described (Howman et al., 2025; Korotkova et al., 2024)To incubate the cells with 1 µM of PeroxiSPY555 for 10 m: pipette a 1 mM PeroxiSPY aliquot as a 1/1000 part of your total media volume in an empty Eppendorf tube, e.g. for 0.5 mL of media we pipette 0.5 µL of PeroxiSPY. Collect the media from the chosen microscope plate and mix with PeroxiSPY by

vortex mixing or pipetting. After mixing, add the mix to the plate and incubate for 5-10 m.

Caution: Do not leave the cells without media for over 20 s to avoid osmotic stress. To achieve uniform staining and organelle formation, all the media should be mixed with the molecules

3.2. Add immersion liquid to the objective (we use 60x oil objective) and position the plate. Adjust focus until the cells are visible and in-focus. Randomly choose imaging area and adjust parameters of acquisition (e,g, HV-100, Laser power = 1%, Zoom 3, 1024*1024 px, scan speed ½-1/4).

3.3. Acquire and save all images with accessible labels (we use .nd2 Nikon NIS Software), including cell line, staining, genetic modification, treatment, and time if applicable.

Pause point: *images can be stored and processed when needed.*

B. Image analysis – quantification of organelle density

1. Training the classifier for organelle abundance quantification. A classifier is an algorithm or model used to classify data (Bi et al., 2019). In the Fiji software this is stored as a ".model" file, and is used by the Trainable Weka Segmentation tool to classify sections of an image into organelles, cytoplasm, and area outside of the cytoplasm.

1.1. Open the macro file in Fiji (Supplementary code S1), either by dragging the file onto the Fiji window, or by clicking "File > Open" on the Fiji window.

1.2. Once the macro editor window appears, full screen the window and click "run" in the lower left corner.

1.3. Follow the prompt on the pop-up window. Type 1 in the dialog box to train a classifier from scratch, or type 0 to use a pre-trained classifier. If you type 0, skip to step 2.1

Note: *to open .nd2 images you will need to install the .nd2 reader in Fiji (https://imagej.net/ij/plugins/nd2-reader.html)*1.4. Using the pop-up window, select the folder containing your training image, ensuring this folder contains only the training image, and a folder in which to store the trained classifier and training data. Ensure that this second folder is empty.

1.5. Wait for the macro to run until a small window titled "Select Identifiable Features" appears. Additionally, 2 other important windows will appear, titled "Trainable Weka Segmentation" (Arganda-Carreras et al., 2017) and "Reference".

1.6. Using the "Trainable Weka Segmentation" window, and the "Reference" window for clarity, draw around up to 5 visible organelles, depending on how many are visible. Click the "Add to Organelles" button on the right of the window before drawing around each subsequent one.

Note: *peroxisomes are ~0.5µm round compartments in the cytoplasm that co-localize with peroxisome marker GFP-SKL in healthy cells (Figure 2A). When drawing in Weka segmentation, only select the area that is peroxisomes, avoid overdrawing.*

1.7. After selecting up to 5 visible organelles, select 5 sections of cytoplasm, each at least the size of the organelles selected in step 1.6. Click the "Add to Cytoplasm" button on the right of the window before drawing around each subsequent area.

1.8. Finally, select 5 sections outside of the cytoplasm, each at least the size of the organelles selected in step 1.6. Click the "Add to Nuclei" button on the right of the window before drawing around each subsequent area.

1.9. Click the "OK" button in the "Select Identifiable Features" window and wait until the training has been completed. To identify if the training has been completed, wait until the image displayed in the "Trainable Weka Segmentation" window changes to highlight the organelles (purple), cytoplasm (red), and nuclei/background (green).

1.10. A new window will appear, asking to continue or finish training. Type 1 into the dialog box to finish training, and type 0 to continue training. We recommend repeating the training steps (steps 1.6-1.10) 9 additional times, totaling 10 training loops. This number is based on data from Figure 2B, which suggests that after 10 training loops the change in identified area was minimal.

2. Using the Macro to analyze an image. The macro will use the trained classifier (1.1-1.10) and a watershed function to automatically determine the area and intensity of the visible organelles and cytoplasm, and the abundance of the visible organelles.

2.1. A pop-up window will appear asking whether you wish to analyze a new image using the previously trained classifier. Type 1 to continue with organelle analysis, or 0 to stop the macro. Stopping the macro is recommended if you wish to analyze more than one image. Typing 0 will result in the text "Training complete" being displayed.

2.2. In the next pop-up window titled "Select your Files", select the folder containing the image you wish to analyze. Ensure this folder contains only one image. Then select a folder to save the results, ensuring this folder is empty.

2.3. This step is only applicable if you are using a pre-trained classifier (if steps 1.4 – 1.10 were skipped). The same pop-up window described in step 2.3 will appear with an additional field. Select the folder which contains the pre-trained classifier. This folder should contain 2 files in the form of a classifier file (ending in .model), and a

data file.

2.4. Wait for the analysis section of the macro to run until a pop-up window asks whether you want to apply Fiji's watershed tool to the organelles. This will attempt to separate organelles that are too close, ensuring they are counted separately. This is useful in samples with high organelle density; higher density of organelles increases the likelihood of organelles overlapping and thus causing the macro to count fewer than are present in the image. Type 1 for yes, and 0 for no.

2.5. Wait until the text "Analysis complete" displays in the "log" window.

Figure 1. Organelle abundance quantification using FiJi macro

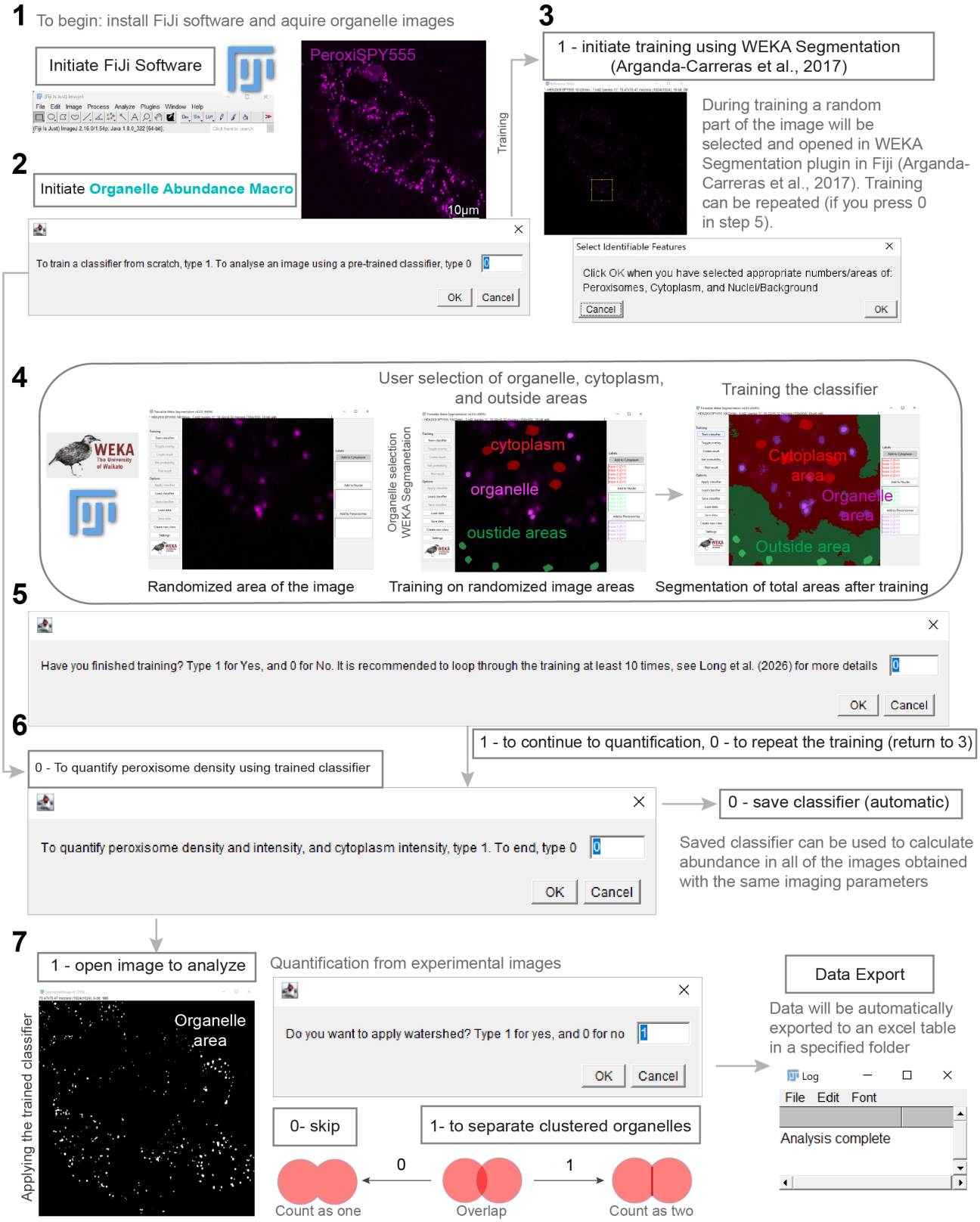


Figure 1. Organelle abundance quantification workflow (Code S1). Figure numbers correspond with procedure steps: 1 is procedure section A, 2 is 1.1-1.3 in procedure section B, 3 is 1.4 in procedure section B, 4 is 1.5-1.9 in procedure section B, 5 is 1.10 in procedure section B, 6 is 2.1-2.3 in procedure section B, and 7 is 2.4 in procedure section B.

Result interpretation

Expected output from this protocol is an excel table with the list of detected organelles (Supplementary table 1), their areas and corresponding average intensity per each area, with a calculated density of organelles in the cytoplasm in the form of number of organelles per square micron (abundance). Results can be plotted as a distribution of organelle areas (Figure 2A) or densities (Figure 3A). Coverage of the area can be extracted by dividing the sum or organelle areas by the sum of organelle and cytoplasmic areas.

Image quality determines whether the data are usable and quantifiable. Image quality depends on the sample preparation and correct microscopy settings. For each organelle we recommend consulting with the literature on the expected size and number of the organelles in the cell line of choice. When imaging organelles for the first time, ensure that you have a verified organelle marker as a control (Figure 2A). To ensure maximum resolution, adjust the microscope settings to ensure that the pixel size is at least 2.3 times smaller than the smallest compartment that you are imaging. This can be done by increasing the image size or physical zoom. Avoid saturation of the organelle areas and ensure that the background noise is minimized by reducing the gain. The protocol estimates cytoplasmic areas based on the residual fluorescence, it is recommended to confirm whether the cytoplasmic area corresponds to actual cytoplasmic area using secondary markers (Figure 2A).

It is recommended to perform at least three biological replicates to obtain the data. The same classifier can be used to quantify the areas in all the images obtained with the same imaging parameters. Additionally, it is recommended to quantify organelle areas in at least 100 cells per condition. If the data follows normal distribution, determined by Shapiro-Wilks test, P values can be calculated by two-tailed Student's t test for two samples or one-way ANOVA for multiple samples. The equality of variances can be verified by the Brown–Forsythe or F test. Mann–Whitney (two groups) or Kruskal–Wallis (multiple groups) tests can be used for samples that do not follow a normal distribution. The sample sizes can be determined using power analysis. Results obtained from the images with different microscopy parameters cannot be reliably comparatively analysed (Figure 2C).

Validation of protocol

We confirmed that machine learning-assisted area segmentation corresponds to the physical cytoplasmic and organelle space using secondary markers (Figure 2A). Peroxisomal area quantified using organelle abundance macro is the same as a manually thresholded area defined by a GFP-SKL peroxisomal marker in the same cells (Figure 2A).

The segmentation relies on the intensity, therefore it is recommended to train the classifier for each set of imaging parameters, such as laser power or exposure. It takes only 5-10 training cycles, using the random area of the same image (Figure 1, Figure 2B), to reliably train the classifier. We showed that increased laser power corresponds to a gradual increase in areas recognized by the classifier (Figure 2C), thereby requiring classifier training for each independent dataset.

The classifier, once trained, can be applied to a necessary number of repeats of images obtained with the same microscope settings, at least 3 biological replicates were done for the experiments presented here. We applied a stress granule trained classifier to identify stress granule density in control and stress conditions (Figure 3A). The macro allowed extracting the absence of stress granules from all the control images.

This tool extracts organelle abundance data from 2D images, 3D space abundance can be extracted from a Z-stack of 2D images. Here we estimated peroxisome density in zebrafish *D. rerio* injected with PeroxiSPY peroxisome probes (Figure 3 B). Additionally, this protocol can be applied to other cellular organelles in different cell types, resulting in their reliable segmentation (Figure 3D, Lipid Droplets in neuronal cells).

Limitations

The protocol relies on the user to define the organelle to be classified, after that the procedure is not user biased. It is, therefore recommended that the classifier is trained by either an experiences researcher or the beginning researcher compares their classifier data to a secondary marker to ensure that the area corresponds to the organelle area of interest (see Figure 2A). Caution should be applied when estimating absolute coverage of organelles. As we show in Figure 2C, it depends on imaging parameters. Quantification of relative coverage, including negative and positive controls is recommended, e.g. organelle abundance between conditions imaged with the same microscopy parameters (e.g. Figure 3A).

To ensure independent validation we provide a dataset to train and apply the classifier (Supplementary dataset S1 that includes two images for training and detection), additionally we include the expected results table (Supplementary table S1).

Figure 2. Protocol validation

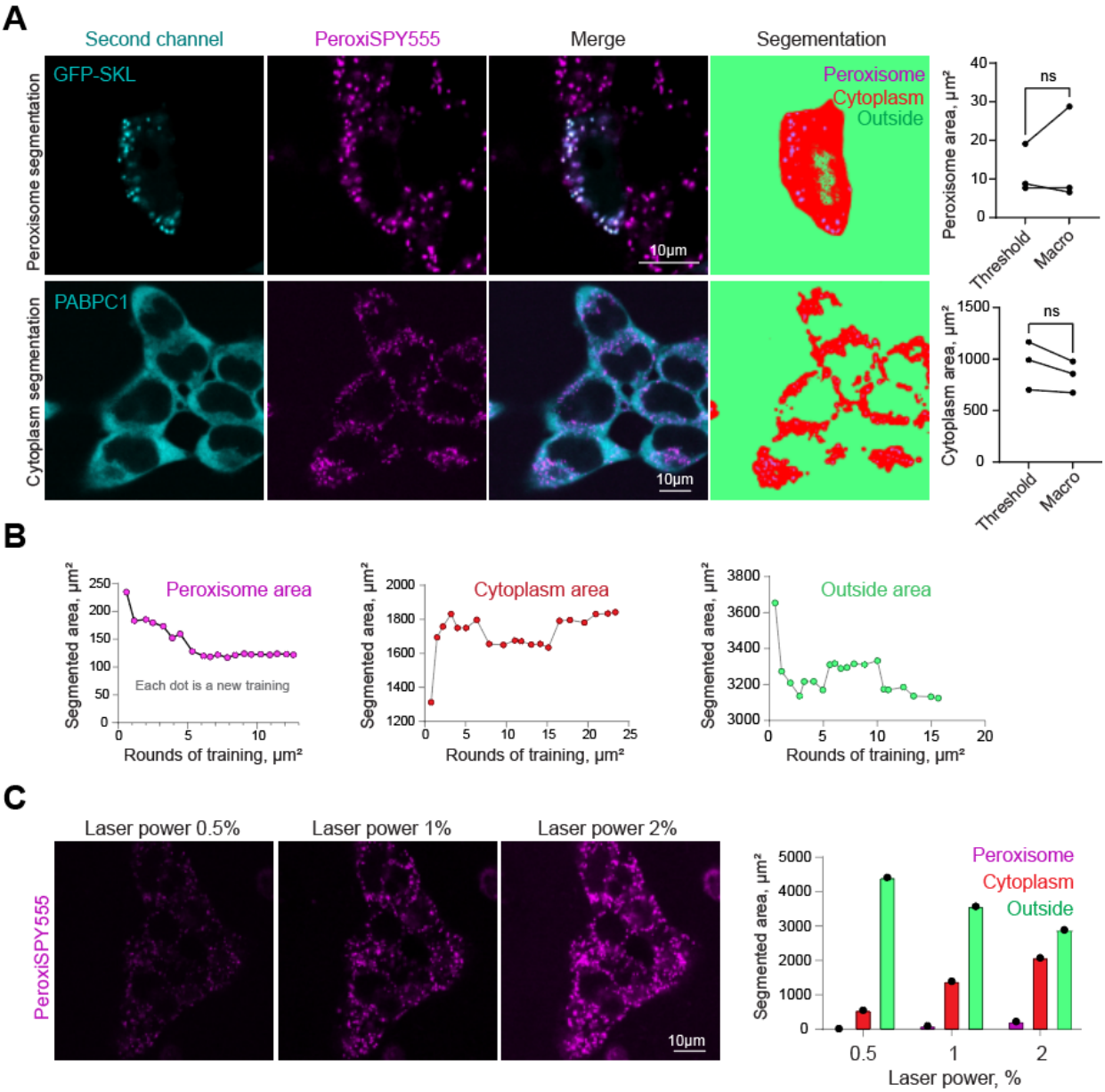


Figure 2. Protocol validation A. Confocal microscopy of peroxisomes in HEK293T cells (upper row) expressing peroxisome marker GFP-SKL, stained with PeroxiSPY (1 μM for 10 m) or (bottom row) expressing CRISPR/Cas9 PABPC1-DDR2 and stained with PeroxiSPY(1 μM for 10 m). Segmentation panels (column 4) show areas recognized by the classifier. Quantification shows the area size detected with the manual threshold method and the area segmented by classifier, N=3, mean± SEM ns – non-significant. **B.** Determination of segmented areas and number of sequential training loops required for appropriate classifier training. Each dot represents additional training area including the data from the previous training. **C.** Confocal microscopy of peroxisomes in HEK293T cells stained with PeroxiSPY (1 μM for 10 m) imaged with 0.5, 1, or 2% laser power. Quantification shows the area size detected with the classifier trained on laser power 1% image.

Figure 2. Protocol application

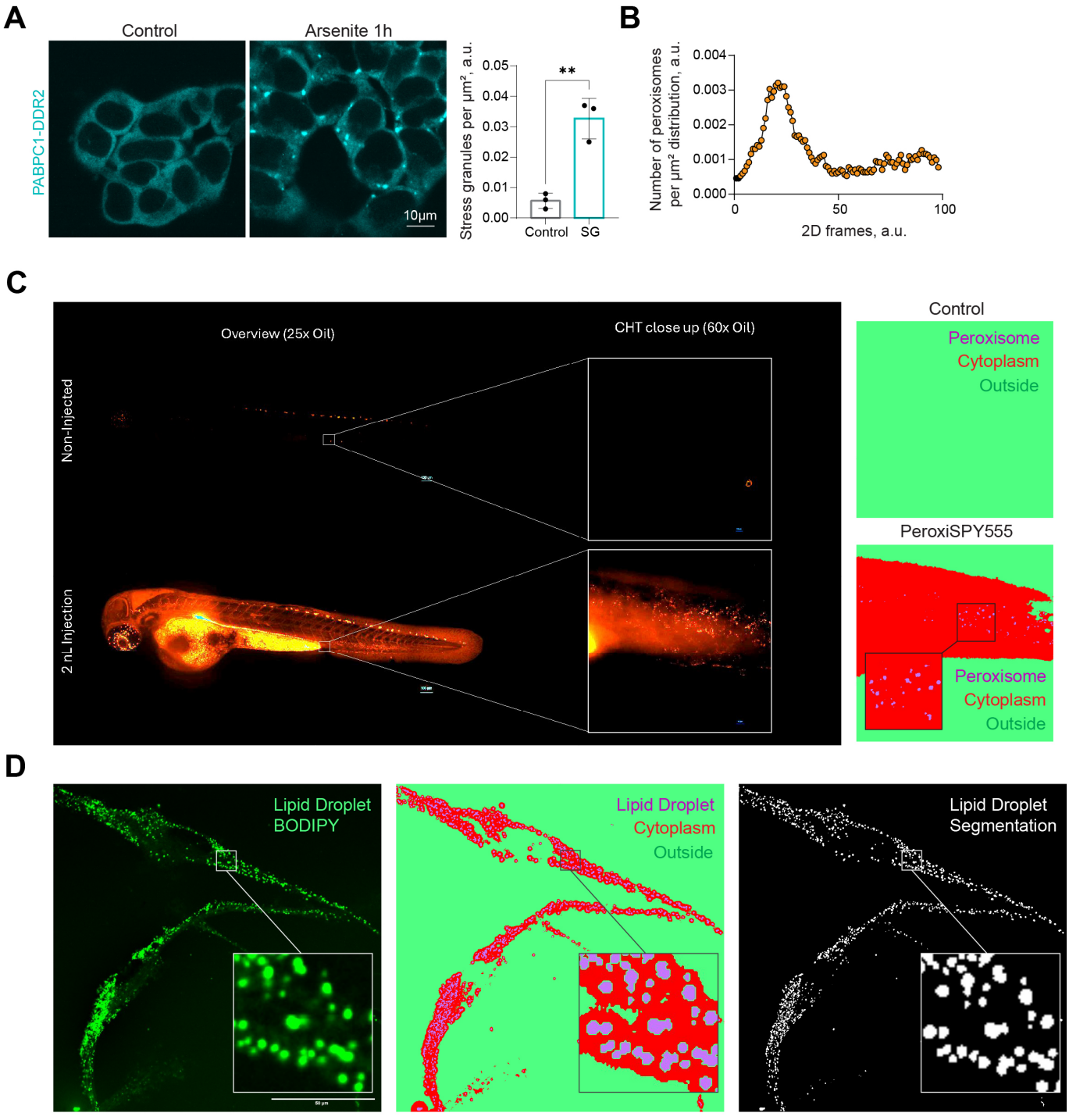


Figure 3. Protocol application A. Confocal microscopy of stress granules in HEK293T PABPC1-DDR2 cells in control and 200 µM arsenite (60 m) conditions. Quantification shows the cytoplasmic density of stress granules, N=3, mean± SEM. ** - p<0.01. **B-C**. Confocal images of zebrafish embryos at 3-day post fertilization injected with 2 nl of PeroxySpy555 at 1 mM in the blood circulation by the duct of Cuvier. Images were acquired 2 h after injection, using a W1 Nikon Spinning disk microscope. Images were segmented using macro and peroxisomal areas were detected and plotted on the histogram (B). (D) Macro-based segmentation of lipid droplets in human iPSC-derived microglia. Representative fluorescence image of human iPSC-derived microglia at day 21 of differentiation (D21) stained with BODIPY 493/503 to label lipid droplets. Corresponding WEKA segmentation and binary segmentation mask generated

using the custom macro; white indicates segmented lipid droplets and black denotes cytoplasm/background. Scale bars, 50 µm.

General notes and troubleshooting

General notes

1. The classifiers used in this protocol are trained on the data using macro (code S1) and can be used to estimate organelle abundance. The users can also follow the training algorithm to train their own classifier to be applied in different conditions, or for a different cellular compartment.
2. The protocol has been tested in several microscope settings (e.g. laser power changes), using several organelle markers. Its applicability to a different organelle needs to be confirmed with a secondary marker as we have done in figure 2.
3. It is recommended to train the classifier for 5-10 loops on each dataset (using a representative image) with new microscopy parameters.

Troubleshooting

Problem 1: Unable to detect organelle areas
Possible cause: Image quality is low
Solution: Optimize the imaging acquisition parameters. For example, HV (gain) will increase brightness but will also increase noise. Increase laser power and decrease gain to improve the signal-to-noise ratio.
Possible cause: photobleaching
Solution: Some fluorescent molecules are photostable (e.g. PeroxiSPY), while others can bleach with laser exposure (e.g. DDR2). Reduce light exposure when handling samples by wrapping the plate in aluminum foil or working in the dark room. When imaging, do not image the same area repeatedly. Find the area on a low laser power setting and then switch to the higher laser power setting before acquiring the final image. Use mounting media with an antifade protection if using fixed samples.

Problem 2: Organelle areas are too big
Possible cause: User defined areas are not specific to organelle during the selection process
Solution: Select areas strictly within the organelle
Possible cause: oversaturation
Solution: When setting microscopy parameters avoid saturation by reducing the gain and the laser power until only few or no pixels remain saturated.

Problem 3: Organelle cluster prevents quantification of the numbers
Possible cause: Watershed is not applied during the procedure (point 2.4, type 1 – to apply)
Solution: Apply the watershed that separates clusters into separate compartments

Problem 4: Not enough organelles are visible while training
Possible cause: The Randomly selected training area is too small
Solution: Navigate to lines 4 and 5 in the macro while it is open in Fiji and not running. Edit lines 4 and 5 in the macro so “w” and “h” variables are larger. For example, lines 4 and 5 could be replaced with:
Line 4: “w = 250;”
Line 5: “h = 250;”

Supplementary information

The following supporting information can be downloaded here (link available when this protocol is published online):
1. Dataset S1. Example images to train and quantify the density of peroxisomes
2. Code S1. Macro to quantify organelle abundance
3. Table S1. Example output results table.
4. Video S1. Video explanation of the workflow.

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Competing interests

The authors declare no conflicts of interest.

Ethical considerations

This protocol does not use human or animal subjects.

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