Live Cell Fluorescence Microscopy – From Sample Preparation to Numbers and Plots

Jakub Zahumensky[1,*], Jan Malinsky[1]
[1]Department of Functional Organization of Biomembranes, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic
*For correspondence: jakub.zahumensky@iem.cas.cz

Abstract

Fluorescence microscopy images of biological samples contain a high amount of information. However, rigorous data analysis is required for a reliable assessment of the significance of changes in protein localization or fluorescence intensity changes. Most often, only the colocalization of two fluorescence signals is formally analyzed, where applicable. When additional analysis is made, both segmentation and measurements are performed manually. This is time-consuming, tedious and prone to experimenter error and bias. In our workflow, cells are immobilized on the microscope cover glass by a small agarose block, which enables the imaging of live cells in a state close to the cultivation conditions and enables the addition of chemicals during a time-lapse experiment. Cells in microscopy images are segmented using the Cellpose software and analyzed using custom-made Fiji (ImageJ) macros. Multiple parameters are measured automatically for each cell and the results table can be easily processed using GraphPad Prism or provided unix command line (bash) scripts. The use of the custom-written Fiji macros enables batch analysis across multiple large and independent data sets in a single run, in a non-biased manner.

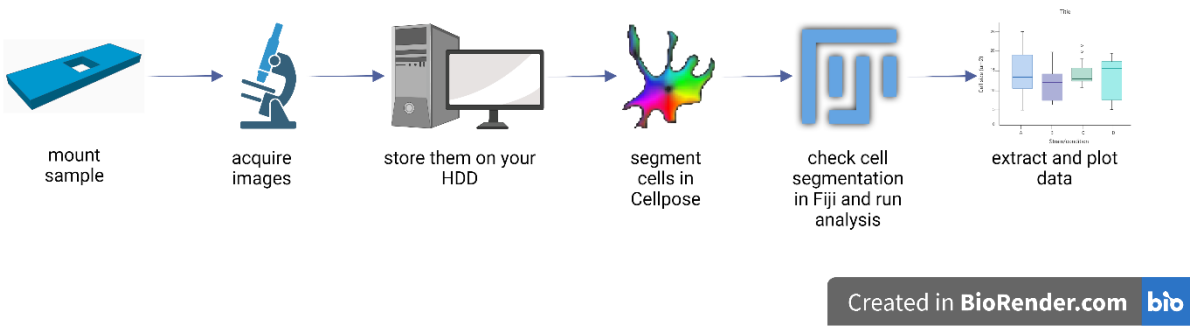
Key features

- Simple sample preparation that enables immobilization of live cell-walled cells in a monolayer, imaging at almost natural conditions and direct treatment during a time-lapse experiment.
- Analysis developed for budding yeast with GFP-tagged proteins that allows batch-analysis of large microscopy data sets, regardless of imaging platform, cell/organelle type and labeling.
- Multiple parameters are reported for each object, including size, fluorescence intensity (both in the plasma membrane and cytosol) and the number of microdomains (i.e., areas of high local intensity).
- Largely automated analysis, requiring only verification of segmentation from the user, with clear-cut rules, blinded on demand.

Keywords: Microscopy, Cells, Yeast, Data analysis, Cellpose, Fiji, ImageJ, Automated, Unbiased, Blinded

This protocol is used in: *Microbiol Spectr* (2022), DOI: 10.1128/spectrum.01961-22 (Zahumenský et al., 2022); *Microbiol Spectr* (2022), DOI: 10.1128/spectrum.02489-22 (Balazova et al., 2022); *J Cell Sci* (2023), DOI: 10.1242/jcs.260554 (Vesela et al., 2023)

Graphical overview



From fluorescence microscopy to numbers and plots – a generalized workflow

Introduction

Well-acquired microscopy images of biological samples contain high amounts of valuable information. However, the space available in scientific papers is generally insufficient to display multiple images for each biological replicate. Therefore, only a single representative image is usually shown, often of a single cell. Information on variation across biological replicates and cells can be communicated to the reader via formal rigorous statistical analysis. This way, it is readily clear how many times an experiment was performed, how many cells were analyzed in each replicate, and how significant the reported differences between strains/cell lines and/or conditions are. When performed, the formal analysis of microscopy images is often limited to quantification of colocalization, where applicable. When additional analysis is performed, the cells are segmented and analyzed manually (e.g., Gournas et al., 2017). However, any user-dependent steps limit the analysis and can introduce bias and errors.

For the preparation of samples for live cell microscopy, concanavalin A or poly-L-lysine is often used to help the cells adhere to the glass surface via physical interactions. In our approach, (cell-walled) cells in thick suspensions are placed on microscopy cover glass and overlaid by a small block of 1% agarose prepared either in buffer or cultivation medium identical to that of the respective cell culture, ensuring a close-to-natural environment. The cover glass is taped to a custom-made microscopy sample holder (Fig. 1A). In this approach, the cells are immobilized on the cover glass just by mild mechanical forces, which does not induce a cellular stress response, as documented by normal actin structure in *C. elegans* (Chen and Pan, 2021) and by the absence of stress granules *S. cerevisiae* cells (Grousl et al., 2015; Vaškovičová et al., 2017). Furthermore, the cells spontaneously prefer a monolayer arrangement, i.e., they are mostly in the same focal plane. This makes microscopy imaging more efficient, saving both time and storage space. Since the agarose is permeable for small molecules, cells can be treated by directly adding chemicals onto the agarose block (in an inverted microscope) during time-lapse experiments (Grossmann et al., 2007; Grousl et al., 2015; Vaškovičová et al., 2017). Adding a cover glass on the other side of the sample holder (Fig. 2) prevents water evaporation and shrinking of the agarose block enabling long time-lapse experiments. The described arrangement thus represents an alternative to both surfaces coated with concanavalin A or poly-L-lysine, and expensive microfluidics systems.

The analysis protocol reported here integrates powerful automatic segmentation using Cellpose software (Stringer et al., 2021) with automatic cell-by-cell analysis. Large microscopy data sets ranging across unrelated experiments can be processed in batch mode, in a consistent and unbiased fashion. Besides the initial verification of proper segmentation (which can be blinded), the analysis does not require user input. It can, therefore, be run unassisted. Data from individual experiments can then be easily extracted and processed using either the provided Unix command line (bash)/R programming language scripts or using the *Multiple variables* data table in GraphPad Prism. Alternatively, the Results table can be processed in Microsoft Excel/LibreOffice Calc - manually, or by writing own macros.

Materials and reagents

Biological materials

The protocol was developed for *S. cerevisiae* and *C. albicans* cells and is described for these below. However, the analysis works with many cell types regardless of organism and/or tissue, and labeling (verified suppliers of yeast are Euroscarf or Dharmacon; choose depending on your location).

Reagents

1. Reagent grade non-fluorescent agarose (e.g., Sigma-Aldrich, catalog number: A9539)
2. KH_2PO_4 (e.g., Lach-Ner, catalog number: 30016-CP0; Sigma-Aldrich, catalog number: P0662)
3. K_2HPO_4 (e.g., Lach-Ner, catalog number: 30060-AP0; Sigma-Aldrich, catalog number: P3786)
4. distilled/ionex H_2O

Solutions

1. 50 mM potassium phosphate (KPi) buffer pH 6.3 (See Recipes)
2. 1% agarose in 50 mM KPi buffer pH 6.3 (See Recipes)

3. Optional: 1% conditioned agarose (See Recipes)

Recipes

Reagent	Final concentration	Quantity or Volume
KH_2PO_4 (1 M solution in H_2O)	38.75 mM	3.875 mL
K_2HPO_4 (1 M solution in H_2O)	11.25 mM	1.125 mL
H_2O	n/a	95 mL
Total	n/a	100 mL

Table 1. 50 mM KP_i buffer pH 6.3. *store at 4 °C for up to three months*

Reagent	Final concentration	Quantity or Volume
Agarose	1%	1 g
50 mM KP_i buffer pH 6.3	n/a	100 mL
Total	n/a	100 mL

Table 2. 1% agarose in 50 mM KP_i buffer pH 6.3 (for general microscopy experiments)

Heat the mixture in a microwave oven until all agarose dissolves. Let cool down to touch, then pour 9-10 mL into a 90 mm diameter Petri dish placed on a level surface. Wait until completely solidified (up to 30 min), then cover and seal the dish with Parafilm—store at 4 °C for up to a month. Before use, leave at room temperature for at least 20 minutes or put into an incubator at 30 °C for 5 minutes and cut with a scalpel blade into 1×1 cm squares. If you plan on adding chemicals directly to the agarose (e.g., during a time-lapse experiment), be precise with the cutting and measure the height of the agarose to calculate the volume of the agarose block.

Conditioned agarose (for specialized microscopy experiments, to be used immediately)

When harvesting (yeast) culture by centrifugation, remove 3 mL of the supernatant into a fresh 15 mL Falcon tube containing 0.03 g agarose. Close firmly, place in a tall glass beaker and heat carefully in a microwave oven on a low-intensity setting until all agarose dissolves (as the solution boils quickly, use multiple short intervals to prevent leaking of the solution out of the tube). Let cool down until you can hold the tube with bare hands, add chemicals (if desired) and pour into a 35 mm diameter Petri dish placed on a level surface (work quickly in this step, the agarose solidifies rapidly). Using conditioned agarose ensures that the environment in which the cells are grown is retained, minimizing stress exposure. This is especially important in situations when changes either in the composition of the media or its pH can result in significant changes in the localization, shape, or amount of the structure of interest, and also if the budding of yeast cells is to be monitored (Gournas et al., 2017; Grousl et al., 2015; Zahumenský et al., 2022)

Laboratory supplies

1. Petri dishes – 90 mm diameter (e.g., GAMA; catalog number: 400974)
2. Petri dishes – 35 mm diameter (optional) (e.g., Corning®, catalog number: CLS430165)
3. Erlenmeyer flasks (50-100 mL) for cultivation (e.g., PYREX®, catalog number: CLS49806L)
4. 15 and 50-mL Falcon tubes (e.g., Orange Scientific OrTubes, catalog numbers: 4440300 and 4440150)
5. 1.5 mL microcentrifuge tubes (e.g., Deltalab, catalog number: 200400P; Eppendorf Flex-Tube®, catalog number: 022364111)
6. 10 mL single-use pipettes (e.g., Orange Scientific, catalog number: PN10E1)
7. Compatible pipette tips (e.g., Gilson, catalog numbers: F161450, F161930, F161670; Eppendorf epT.I.P.S.®, catalog numbers: 022492004, 022492039, 022492055)
8. 20 or 50 mL single-use syringe (e.g., Chirana, catalog numbers: CH020L and CH050L)
9. Syringe filter with 0.2 µm pore size (e.g., Whatman, catalog number: 6896-2502)
10. Scalpel with no. 10 blade (e.g., Sigma, catalog number: S2896; Medin, catalog number: B397112910010)
11. Small spatula (home-made of old Western blot film works well, Fig. 1B)

12. High-transmission microscope cover glasses, 0.17 mm thick (e.g., Menzel Gläser 22×22 mm, catalog number: MZ-0025)
13. Lens cleaning papers (e.g., Kimtech Science, catalog number: 05511)
14. Scotch® Magic™ Tape
15. Permanent marker
16. Parafilm™ wrapping film

Equipment

1. Microscopy sample holders (Fig. 1A; a Tinkercad file for 3D printing can be downloaded here)
2. Incubated orbital shaker (e.g., Lab Companion, model: SI-300R or N-biotek, model: NB205)
3. Centrifuge with a 1.5 mL microtube rotor (e.g., Labnet, model: Spectrafuge™ 24D)
4. Centrifuge with a 50 mL tube rotor (e.g., Hettich, model: Universal 320 R)
5. Micropipettes (e.g., Gilson, models: Pipetman P2, P200, P1000)
6. Pipette Controller (e.g., BrandTech, model: BRAND accu-jet®)
7. Laboratory scales (e.g., Kern, model series: 572)
8. Fluorescence microscope (e.g., Zeiss, model: 880 LSM; Olympus, model: FV1200 MPE or SpinSR10)
9. Microwave oven (e.g., ETA, model: Morelo)
10. Vortex (e.g., Labnet, model: VX-200 or Grant-bio, model: PV1)
11. Tube rack
12. A level surface
13. Personal computer/laptop

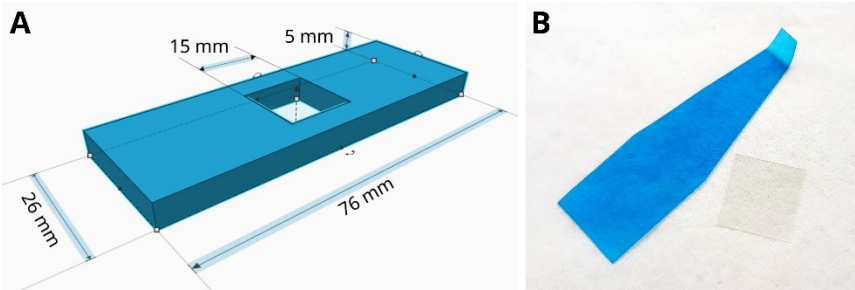


Figure 1. Equipment for microscopy sample preparation. *A) Image of the custom-made microscopy sample holder; Tinkercad file for 3D printing can be downloaded here. B) A spatula made of old Western blot film (a 22×22 mm microscopy cover glass for scale).*

Software and datasets

1. Cellpose 2.0 (Stringer et al., 2021) (; https://www.cellpose.org/; installation instructions at https://github.com/MouseLand/cellpose/blob/main/README.md)
   - The Cellpose Readme website includes instructions on the installation of Python (https://www.python.org/downloads/) and Anaconda (https://www.anaconda.com/download); both are required for the running of Cellpose
2. Fiji (ImageJ 1.53t or higher; https://imagej.net/software/fiji/downloads)
3. required plugins/libraries that are not part of the general Fiji download:
   - *SCF-MPI-CBG* and *BIG-EPFL* (to install, navigate through *Help → Update → Manage update sites*, then tick checkboxes for *BIG-EPFL* and *SCF MPI CBG*. Click *Apply and Close → Apply changes → OK*; restart Fiji)
   - *Watershed_* plugin (to install, download from http://bigwww.epfl.ch/sage/soft/watershed/ and put in the plugins folder of Fiji/ImageJ)
   - *Adjustable Watershed* plugin (to install, download *Adjustable_Watershed.class* from https://github.com/imagej/imagej.github.io/blob/main/media/adjustable-watershed and put it in the Plugins folder of Fiji/ImageJ)
4. recommended for data processing (specific steps for each approach are explained below in the Data Analysis section):
   - automatic: up-to-date version of R (including R Studio)

- automatic: Unix shell or Windows Subsystem for Linux (https://learn.microsoft.com/en-us/windows/wsl/install) and a user-friendly terminal (e.g., mintty 3.5.2: https://cygwin.com/install.html)
- semi-automatic: Prism 8 or higher (GraphPad)
- manual: LibreOffice Calc. or Microsoft Excel (can be automated using macros [not provided])

5. recommended for graphing: R, Prism 8 or higher (GraphPad), Sigmaplot 12 or higher (Systat)
6. All sample data and code have been deposited to GitHub: https://github.com/jakubzahumensky/microscopy_analysis

Procedure

A. Microscopy sample preparation (yeast culture)

1. Cultivate cells under desired conditions.
2. Transfer the whole culture to a 50 ml Falcon tube (if not already used for cultivation) or remove 1 mL aliquot to a 1.5 mL microcentrifuge tube.
3. Centrifuge for 2 min at 1430 rcf at RT (room temperature).
4. Remove most of the supernatant by pipetting or decanting.
5. Vortex pellet in 3 one-second pulses (850 rpm).
   Note: Alternatively, keep the supernatant and gently disturb the pellet by pipetting.
   Note 2: If 1 ml of exponential culture is centrifuged in a 1.5 mL tube, repeat steps 2-5, pipetting the supernatant in step 4 both times.
6. Pipette 1 µL of the cell suspension onto a clean microscopy cover glass.
7. Cover the glass with a block of 1% agarose gel prepared with either buffer or cultivation media (Recipes 2 and 3). Use the small flat spatula (Fig. 1B) to manipulate the agarose block.
8. Place the cover glass into the sample holder, with the agarose block inside the chamber (Fig. 2).
9. Tape the cover glass to the holder with Scotch tape and mark with a permanent marker if you have more than one sample.
10. *Optional*: If you are performing a time-lapse experiment and/or use a microscope stage incubator to control temperature, flip the sample holder upside down onto a lens cleaning paper and tape another cover glass from the other side (this prevents the agarose block from drying out during longer experiments)
    Note: steps 6-10 are depicted in Fig. 2

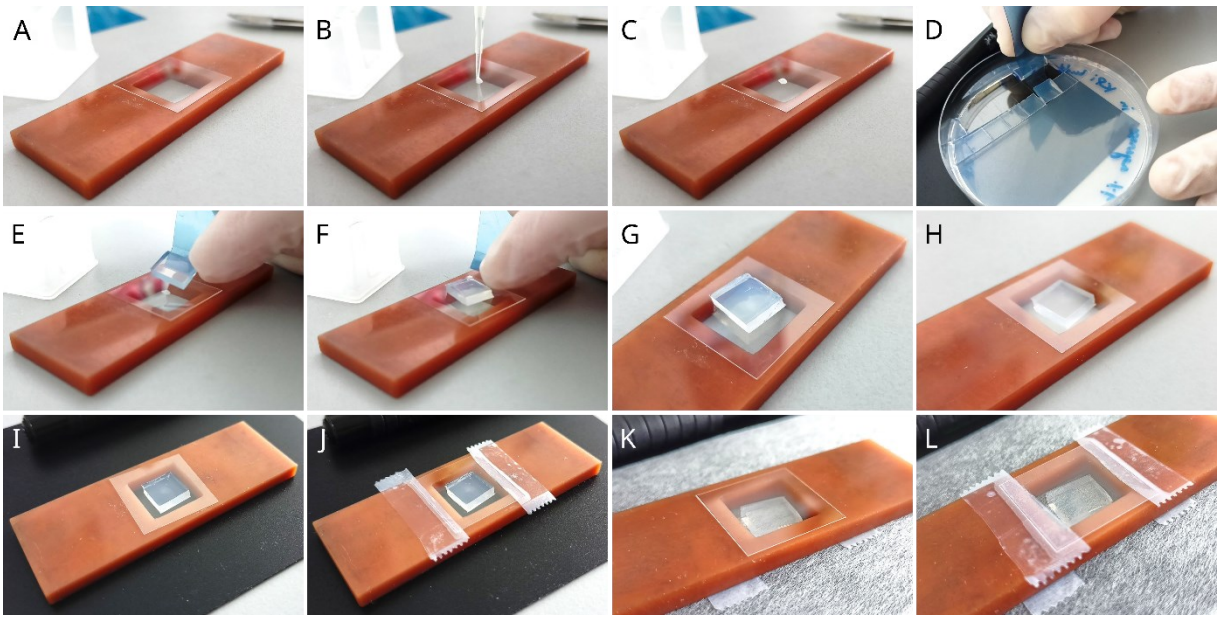


Figure 2. Microscopy specimen preparation. *A) place cover glass on a level surface, e.g., the sample holder. B-C) pipette a small amount of cell suspension on the cover glass. D) use the spatula to lift a small block of precut*

agarose (roughly 1x1 cm) from the Petr dish. E-G) cover the cell suspensions with the agarose block. H+I) Turn the agarose upside down so that the agarose block is inside the square hole in the sample holder. J) tape the cover glass to the holder. K-L) for time lapse experiments, turn the sample holder upside down and tape another cover glass to seal the chamber.

B. Microscopy image acquisition

Critical notes: It is of paramount importance to observe a certain set of rules to make sure that the downstream data analysis works properly. We, therefore, give the following *7 microscopy commandments* (for a more detailed description see e.g., (Brown, 2007)):

1. Thou shall not overexpose your images.
2. Thou shall set offset properly (when using a photo-multiplier tube as the detector).
3. Thou shall not bleach your fluorescent tags.

   Note: Violations of any of 1-3 result in incorrect intensity readings

   Note: You can minimize bleaching by finding focus in one image area and imaging another area directly adjacent, using e.g., a tile scan option of your microscope or moving the stage in a defined manner using the stage controller in the microscope software.
4. Thou shall not make images with high noise.

   Note: While noise is always present in microscopy images, high noise levels can create issues for the segmentation of the cells and obscure sub-cellular structures
5. Thou shall check your images for bleed-through (crosstalk).

   Note: Bleed-through is caused by an overlap of emission spectra of two or more fluorescent markers. It can be easily mistaken for colocalization and hence lead to misinterpretation of data. Using sequential acquisition of the signal from individual channels greatly reduces the risk of bleed-through.
6. Thou shall not change imaging settings between imaging samples of the same series.

   Note: Changing the settings results in different intensities caused by the instrumentation, not the sample (strain, condition). As a result, a comparison of fluorescence intensities among the samples is impossible. Note that doubling the laser intensity generally does not result in doubling the fluorescence intensity.
7. Thou shall not directly compare intensities from samples obtained on different days.

   Note: Like 6, but more easily overlooked. In general, it is better to use intensity ratios of values originating from a single sample (e.g., ratio of mean intensities in the plasma membrane and cytosol), as these are based on an internal reference. Absolute fluorescence intensity readouts across samples should be compared only as a guide, and only from samples imaged in an immediate succession. Intensity readings are unreliable due to day-to-day fluctuations in the current, temperature, laser intensity, etc. In addition, to compare intensities across replicates, the intensities need to be first normalized – see General note 1.

Data analysis

The Fiji macros reported here facilitate automatic analysis of large amounts of segmented objects, such as cells, reporting a multitude of parameters for each, including size (area), fluorescence intensity (both integral and mean) and the number, fluorescence intensity and distribution of microdomains (areas with high local fluorescence intensity, also referred to as high-intensity foci) in the border areas of the objects. Shape descriptors are included (major, minor ellipse axis and eccentricity). The fluorescence intensity of the objects is reported separately for the whole objects, for their circumference and their inside. When whole cells are analyzed, this translates to intensity readings from the whole cell, the plasma membrane, and the intracellular space. In addition, various ratios are calculated and reported. The macros process all images of all biological replicates of all experiments found within the specified directory (folder) and all its sub-directories (i.e., they work recursively). For

the downstream processing of results to work properly, data from various biological replicates need to be stored in separate folders and named consistently (see Fig. 3). The data are reported in a single Results table where each line contains information on a single cell (analyzed object), including which experiment and biological replicate it belongs to.

All reported macros can be run from Fiji by clicking *Plugins → Macros → Run...* and opening the macro from the shown dialog window, or by drag-and-dropping the respective macro onto the Fiji window and pressing *Run* in the macro edit window. The macros are numbered in the order in which they are to be used. Each macro run starts by displaying a dialog window, where multiple parameters need to be set by the user. Path to the folder to be processed can be copy-pasted or selected using the "Browse" button.

All dialog windows displayed by the macros include a "Help" button, which shows information on individual parameters. Once the running macro is finished, a message (*"Finito!"*) is shown to the user, informing them that the macro has finished successfully. If there are some irregularities during a macro run, the message also gives a warning and additional information (e.g., that not all images have defined regions of interest, with their list written in one of the log windows).

Data organization

For the analysis to work, a certain data organization structure is required (Fig. 3). If you plan on processing data from multiple experiments at the same time, it is advantageous to use experimental (accession) codes for your experiments. These also facilitate cross-referencing between the digital data and hard-copy lab notebook (as well as between related experiments). An example experiment code: "JZ-FM-αβγ", where JZ are initials of the experimenter (here "Jakub Zahumensky", FM denotes "*fluorescence microscopy*" and αβγ is a three-digit number. All data belonging to a specific experiment should be stored in a single folder whose name starts with the experiment code. Within this folder, each biological replicate should have a separate folder where data are stored. We recommend using the date in the format YYMMDD as it keeps the folders ordered chronologically. In each replicate folder, separate folders for transversal (images of cell cross-sections) and tangential images (depicting cell surfaces) are to be created, each for the respective images. In these, a folder called "data" is for storing the actual data to be analyzed. All additional folders that are required are created by the provided macros. If images require pre-processing, such as drift correction, the use of an extra "data-raw" (or similar) folder is recommended. The actual filenames should include the following information: cell type (yeast strain/cell line etc.), fluorescent tag(s) listed in the order in which channels are saved in multichannel images, cultivation medium (if multiple options exist) and time, treatment and frame number (multiple frames of each strain and condition are required). Information on what part of cells is imaged (transversal/tangential section, z-stack) should be included as well. Data from time-lapse experiments need to be first corrected for drift (and bleaching, if desirable) and separated into individual images (which can be facilitated by the supplied "*0. correct and project.ijm*" macro). As the Results table is comma-separated, the information in filenames needs to be comma-separated, ideally without spaces (example in Fig. 3; any spaces are substituted with underscores in the Results table). The experiment codes, biological replicate folder names and filenames need to be used consistently (including using the exact same notation for the same treatment each time), as these are reported in the Results table and enable automatic data filtering and processing (using unix bash/R/Graphpad Prism), as well as manual processing (Excel/Calc). Experiments destined for analysis can be temporarily moved to a single folder (called e.g., "analysis") and analyzed in a single run.

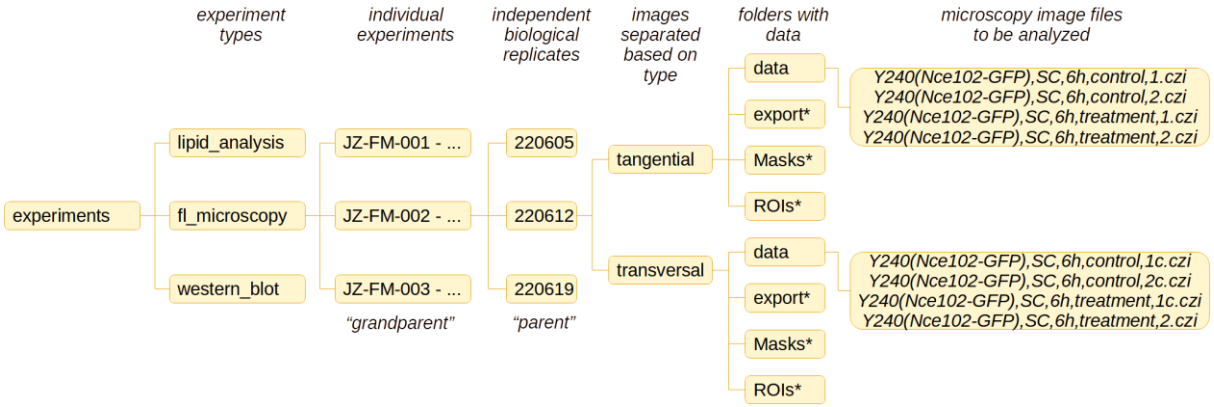


Figure 3. Suggested data organization structure. *Note that the folders marked with an asterisk are created automatically by the provided macros.*

Preparation for analysis (image export and object segmentation)

To segment objects (typically cells and/or nuclei) in microscopy images, we use Cellpose 2.0 (Stringer et al., 2021) (see Software). Other segmentation options are available in Fiji (ImageJ), or Cell Profiler (Stirling et al., 2021), but these are not covered in this protocol. Cellpose 2.0 can be used either through its GUI (graphical user interface) or via the command line (Anaconda; see Software). The GUI is useful for finding the segmentation parameters for the given cell type and experiment. The command line can then be used to run the segmentation in batch mode.

Export

To prepare the images for segmentation, they need to be first exported into "tif" format, as Cellpose does not work with raw microscopy images. We highly recommend combining this step with the alignment of channels, z-stacks, and time-lapses (if not already performed). Fiji plugins "StackReg" (with the "translation" option) or "3D drift correction" often give good results. To export all images across biological replicates (and/or experiments), run the "*1. export.ijm*" macro. This macro, like all the others, works recursively, i.e., images in all subfolders called "data" will be exported. During this process, the respective "export" folders are created automatically. For example, the data structure depicted in Fig. 3: running the macro from the JZ-M-002 folder processes all three biological replicates and stores the exported images in *export* folders within the respective BR folder. For the export, there is an option to adjust the contrast of the images, which might be beneficial for the following segmentation in Cellpose (note that if the contrast is adjusted, the images should only be used for segmentation, not for the analysis itself). The macro allows the user to specify which channels are to be exported. The segmentation masks and in turn ROIs are then used to quantify both channels. Therefore, it is advisable to export the channel with the brighter fluorescence signal and clearly visible plasma membrane.

The analysis (described below) processes single frames at a time. Therefore, for time-lapse experiments, the images need to be split into individual frames. To make the segmentation process more efficient, we recommend the following:

1. correct the timelapse or z-stack for drift (*StackReg* or *3D-DriftCorrection* plugin)
2. correct for photobleaching (*Bleach correct* plugin)
3. *Z-project* -> MAX (this results in the best possible contrast)
4. use the final image for cell segmentation in Cellpose and its verification in Fiji
5. during the "*ROI_check*" step (segmentation verification; described below), discard ROIs for cells that have moved/rotated relative to the other cells during the experiment
6. make copies of the final ROISet, as each frame needs its own (use for example the Double Commander (https://doublecommander.com/) multi-rename tool for automatic renaming)

Note: steps 1-3 can be easily performed with the provided "*0. correct_and_project.ijm*" macro (also includes the option to save Mean/SUM/MAX intensity projection); for this to work, images need to be stored in a "data-raw" folder.

Segmentation

To perform segmentation, follow instructions on the official Cellpose README website (section "*Run cellpose locally*"; includes a demo) to open the GUI. Open one of your exported images, and input the (average) diameter of your cells in the *Segmentation* tab. In the *model zoo* tab, select one of the models (cyto2 works well for yeast cells), which starts the segmentation. Once finished, change the flow_threshold (maximum allowed error of the flows for each mask), cellprob_threshold (higher values lead to smaller segmentation masks) and stitch_threshold (for segmentation of z-stacks) to find the parameters that give the best results (detailed description of parameters can be found in the Cellpose documentation). For the analysis macro (below) to work properly, the size of the Masks/ROIs for transversal images should be set in a way that their edges are placed in the middle of the plasma membrane. For tangential images, Masks should be just as big as the visible parts of the cells (Fig. 4).

Once you have obtained the segmentation parameters (write them down), close the GUI and use the command line to segment images in batch mode (detailed information can be found in the Cellpose documentation:). Example Anaconda command:

```
python -m cellpose --dir D:\Cellpose\export\ --pretrained_model cyto2 --
chan 0 --diameter 80 --mask_threshold 3.0 --flow_threshold 0.2 --use_gpu --
no_npy --exclude_on_edges --verbose --savedir D:\Cellpose\Masks\ --save_png
```

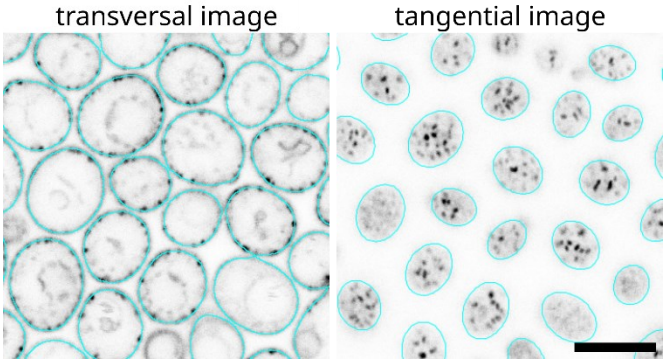


Figure 4. Guide to Segmentation. *Segmentation should be done in a way that the ROI edges are in the middle of the plasma membrane in transversal images (cell cross-sections) and just touch the outside of the objects in tangential images (cell surfaces). Yeast cultures were grown in synthetic complete media for 24 h and Nce102-GFP (a yeast plasma membrane microdomain protein) was imaged. Scale bar: 5 µm. Data from (Zahumenský et al., 2022).*

In the command, substitute the "D:\Cellpose" folder for where your exported images are. Also substitute values for *diameter*, *mask_threshold* and *flow_threshold* based on the parameters you obtained using the GUI. Note that the export of Masks does not work if there are any spaces in the path to your files. To circumvent possible issues, we find it best to have a dedicated folder for Cellpose segmentation (such as in the example), move the exported images there, and move them back to their original location after the segmentation, together with the *Masks* folder. For the "--use_gpu" option to work, additional steps are required before running the segmentation (see Cellpose documentation).

Preparation of regions of interest (ROIs) and verification of segmentation

Before analysis, the segmentation mask images obtained in Cellpose need to be converted to regions of interest (ROIs) that Fiji can use and these need to be verified for accuracy. To perform this, run the "*2. ROI_prep.ijm*" macro in Fiji. This macro is used for both tasks and needs to be run twice.

In the first run, select the "Convert Masks to ROIs" in the process option. This will convert all Masks of the selected image type into ROIs and save these in a newly created "ROIs" or "ROIs-tangential" folder (see Fig. 3). The process runs in batch mode; therefore, no images will be shown to the user. If you are working with budding yeast or another cell type with a simple round shape, it is advantageous to use the "Convert ROIs to ellipses" option. This is also useful for images of nuclei but is not suitable for cells with complex morphology (e.g., neurons). The "Channel and "Blind experimenter" option has no effect at this point. Once *OK* is pressed, a warning is displayed informing the user that if ROIs have already been created, these will be overwritten. Checking and adjusting ROIs (below) can be quite time-consuming and this would annihilate the results of previous work.

Disregard this message if you have not previously converted the segmentation masks to the ROIs. The macro automatically removes objects that are too close to the image edges and are therefore likely to be incomplete.

Once the macro is finished, run it again, selecting "Check ROIs" in the process option. Use the same options as in the first run. Using the “Blind experimenter” option randomizes the order in which the images are displayed and hides the filename and the location of the file, which reduces bias. The channel option specifies which channel is shown to the user. Once *OK* is pressed, an example of properly made ROIs in yeast cells is displayed to the user as an aid. Images of the selected type are displayed one by one to the user together with existing ROIs. These can then be removed or adjusted to correspond to the example as closely as possible (can be displayed again using the “Help” button in the dialog window). There are options to change the size and position of all ROIs at the same time. To add a new ROI, draw it in the image (the “Make ellipse” tool is preselected) and press *t* to add it to the ROI manager. All changes are automatically saved when *OK* is pressed in the dialog window. There is an option to return to the previous image if so desired. To increase the visibility of all features, the inverted grey LUT (lookup table) is used to display the images, combined with yellow outlines of the ROIs. At this stage, files can be excluded from the analysis by clicking the “exclude from analysis checkbox” – once *OK* is pressed, the current image will be closed and moved to the “data-exclude” folder within the current structure. Works even when the experimenter is blinded.

Analysis

Running the quantification

Once you have checked and adjusted the ROIs, run the “*3. Quantify.ijm*” macro. In the dialog window that appears, choose the directory from which you wish to start the analysis (the Results will be saved here). This needs to be at least one level above your “data” folder (i.e., ”*parent*” in Fig. 3). If you only want to process images of a particular yeast strain (cell line), or condition, use the “subset” option – only filenames containing the specified string of characters will be processed. Select which channel(s) you want to analyze (you can use a comma to specify multiple channels and a dash to define a range; a separate Results table will be created for each channel). Specify the naming scheme you are using for your filenames (whatever you write here will be used as column titles in the Result table). As explained above, the details in the filenames need to be comma-separated to facilitate automatic downstream filtering and processing. Before running the macro, make sure that the naming is consistent across all data that are included in the analysis. Specify the format of the experiment code (accession code) you are using and select the proper image type (transversal/tangential). Specify the lower (min) and upper (max) limit for cell area (in μm^2; as appears in the microscopy images). Only cells within this range will be included in the analysis. The coefficient of variance option can be used to automatically filter out cells without structure (i.e., probably dead cells). If your images have been deconvolved, select *yes* in the respective option. This changes a few parameters in the analysis so that smooth, noise-free intensity distributions are successfully processed. If you use this option, note that intensity readings do not provide useful information and verify that other parameters make sense in the context of your experiment. Use the "Help" button to get information on individual parameters on the fly without needing to consult the protocol. The actual analysis can take a long time, depending on how many images and cells are being analyzed. With big datasets, it is advised to run the analysis overnight (once you have established that there are no issues).

Once the analysis is finished, you will be informed with a dialog window. This will show additional information in case of irregularities. Three files are created by the macro in the Directory specified by the user when the analysis is started.

- “*Results of tangential image analysis, channel <CH> (YYYY-MM-DD,HH-MM-SS).csv*” or “*Results of transversal image analysis, channel <CH> (YYYY-MM-DD,HH-MM-SS).csv*” (for an example, see Supplementary tables 1 and 2).– the "*Results table"* containing the results of the analysis for the specified channel CH. Numbers in the brackets specify, in the format shown, the date and time when the analysis was concluded. The Results table contains a header with basic information on the macro run: macro version, date and time when the analysis was started, which channel was analyzed and a handful of interesting parameters used for the analysis. The header lines start with the hash symbol (#) and are ignored by automatic processing when using unix bash scripts and R.

- "*results-temporary_<image_type>_channel_<CH>.csv*" – a temporary file updated every time the analysis of all cells in a single image is concluded. Together with the two files listed below, this file is used to resume an incomplete analysis, e.g., if some images do not have prepared ROIs for analysis, or the analysis has been interrupted (by the user, due to an error in the macro run, a power outage, etc). This file is converted to the "*Results table*" file once the analysis of all images is finished.
- "*files_without_ROIs.tsv*" – lists files from the "*data*" folder that have no ROIs in the respective "ROIs" folders (and are, therefore, skipped in the analysis). Once these are made, the analysis can be resumed."*processed_files.tsv*" – lists the files that have been analyzed. If an incomplete analysis is resumed, these are skipped.

Note: To count the number of bright fluorescence foci (microdomains), and nowhere else, the analysis uses non-linear adjustments. All intensities are analyzed and reported from the raw data.

Extracting numbers and plotting

The Results table can be further processed in multiple ways, from automatic filtering and copy-pasting in Excel/LibreOffice Calc to fully automatic mean and SD calculation and plotting in R (including statistical analysis).

<u>bash</u> – use the provided Unix shell script – copy the Results file into the processing folder. make sure that only a single Results file is present in this folder. Navigate to this folder in bash and run the "sh extract.bash" command. If you want to process data from a single biological replicate, e.g., a representative time-lapse, run "sh extract-single.bash". A new folder is created for each experiment. The Results table is further automatically filtered for biological replicates, strains, conditions, etc. and Means and SDs are calculated for each unique combination for each parameter. The results are reported in separate files for each quantified parameter. If your filenames do not include information on strain, medium, time, condition or frame, these will be substituted with a full stop (.) in the files with Means and SDs.

<u>GraphPad Prism</u> – click *File → New → New Project File →* Create *Multiple variables* and copy-paste the whole Results table here. Then click *Analyze → Data Processing → Extract and Rearrange*, select which parameters you want to extract (we typically use BR_date, strain, condition + the graphed variable, such as cell intensity), select *Format of results table* (e.g., Grouped for two-way ANOVA) and *Data arrangement* for plotting and statistical analysis. The exact choices depend on your actual data.

<u>Microsoft Excel/LibreOffice Calc</u> – when opening the Results file in Excel/Calc, make sure to select that commas are used as column separators. Once open, select the row with the column headers (exp_code, BR_date, etc.), then click *Data → AutoFilter* in Calc or *Data → Filter* in Excel (a funnel icon in both). Now, clicking on each column title you can select which lines to display. These can then be moved/copied elsewhere to calculate mean and SD within each biological replicate, strain and condition.

The tables generated by the bash script can be used in GraphPad Prism, Sigmaplot (or another scientific graphing software) to make graphs and perform statistical analysis. Before meaningful plots can be generated and statistical analysis performed, certain data (chiefly fluorescence intensity) need to be normalized – see general note 1. As a good guide on how to plot your data, we recommend a recent paper that described what the authors termed SuperPlots. Using these plots, information on individual cells, including their respective biological replicates, is visible (Lord et al., 2020).

Validation of protocol

1. This protocol or its parts have been used and validated in the following research articles:

- Zahumensky et al. (2022). Microdomain Protein Nce102 Is a Local Sensor of Plasma Membrane Sphingolipid Balance. *Microbiology Spectrum*. 10(4):e0196122 (Figure 1, panels C-E; Figure 2, panels B-D; Figure 3, panels C-H; Figure 4, panels B, D, E and G; Figure 5, panels B-D; Figure 6, panels B-F; Figure 8, panels B, D, E; Supplementary Figures S1-S5).

- Balazova et al. (2022). Two Different Phospholipases C, Isc1 and Pgc1, Cooperate To Regulate Mitochondrial Function. *Microbiology Spectrum*. 10(6):e0248922 (Figure 4, panel F)
- Vesela et al. (2023). Lsp1 partially substitutes for Pil1 function in eisosome assembly under stress conditions. *Journal of Cell Science*. 136(3):jcs260554 (Figure 1, panels B-D; Figure 3-5, panels B-D in each; Figure 6, panels B, C; Supplementary Figures S1-S3)
- note that the macros have been updated after these papers were published to incorporate new features (e.g., resume incomplete analysis, analyze multiple channels in a single run, blind experimenter) and get rid of known issues (macro running commands before the previous one has been completed). Additional approaches for the quantification of plasma membrane microdomains have also been implemented.
- For additional validation, we include the analysis of a microscopy image of a neuron as an example of a morphologically complex cell, to demonstrate the robustness of the macros (Fig. 5; Supplementary table 3).

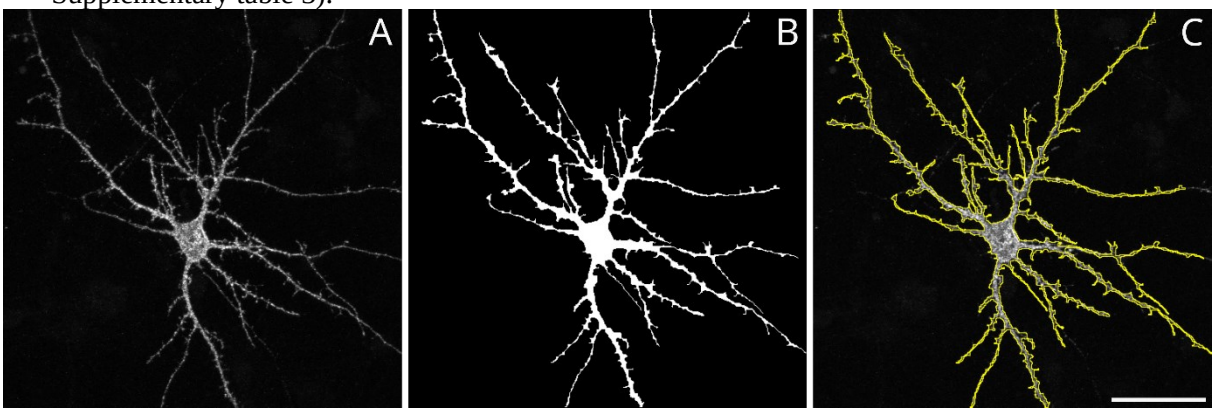


Figure 5. Segmentation of a neuron for protocol validation. *A) raw image B) manually created binary mask for segmentation (Fiji). C) region of interest (ROI) created from the mask in B. Primary hippocampal neuronal cultures were prepared from rats at embryonic day 18. Neurons were transfected with DNA vector containing gene for GFP-GluN3A subunit of N-methyl-D-aspartate receptors on in vitro day 13. Immunofluorescence labeling was performed 24 hours after transfection using primary rabbit anti-GFP and secondary anti-rabbit IgG conjugated Alexa 647 antibodies for surface receptors. Intracellular signal was enhanced using primary mouse anti-GFP and secondary anti-mouse IgG conjugated Alexa 488 antibodies after permeabilization. Scale bar: 50 µm. Image courtesy of Petra Zahumenska. Analysis results are available in Supplementary table 3.*

General notes and troubleshooting

General notes

Normalization to control - certain values (e.g., mean fluorescence intensity of a cell) need to be normalized before the statistical significance of the observed changes between strains/cell lines and/or treatments can be tested. A usual practice is to simply divide values belonging to a single biological replicate set by the value of the control sample. While useful, it hides the variability of the control sample and lowers the power of the statistical tests employed. To circumvent this, we suggest the following normalization procedure and provide an example (Table 3; assuming the same set of conditions is used in each biological replicate):

1. Calculate the mean of all values within a biological replicate set.
2. Divide each value within the biological replicate set by the mean value.
3. Calculate the mean value of the control samples across replicates.
4. Divide all scaled values across replicates by the mean of the controls.

condition	raw data replicate 1	raw data replicate 2	raw data replicate 3	scaled replicate 1	scaled replicate 2	scaled replicate 3	normalized to control replicate 1	normalized to control replicate 2	normalized to control replicate 3
control	20	25	50	0.83	0.80	0.86	1.00	0.96	1.04
treatment 1	25	38	64	1.04	1.22	1.10	1.25	1.46	1.33
treatment 2	21	22	48	0.88	0.70	0.83	1.05	0.85	0.99
treatment 3	30	40	70	1.25	1.28	1.21	1.50	1.54	1.45

Table 3. Normalization example. The raw data are first scaled within their respective biological replicate groups and then normalized using the mean value of the control samples.

Troubleshooting

Problem 1: Cellpose does not export Masks properly.

Possible causes: The path to the images to be processed contains spaces.

Solution: Segment all images in a dedicated folder without any spaces anywhere in the path, e.g., "D:\segmentation\" and then move the "Mask" folder to the folder of the respective biological replicate.

Problem 2: Cellpose does not use GPU to calculate the segmentation masks

Possible causes:

a) The used PC is running an AMD-family GPU (under Windows).
b) Required dependencies are not satisfied.

Solution:

a) There is no known solution for the authors at the moment of writing, the Masks will be computed using the CPU, which will take longer; alternatively, check here for new developments on the topic.
b) Follow the instructions in the official documentation here.

Problem 3: no data reported in the Results table

Possible causes:

a) The required data structure is not in place.
b) Cells were not segmented and/or the segmentation masks were not converted into ROIs. In this case, you should get a message informing you that ROIs are missing, and a list of files affected by this.
c) You are running the macro directly from the "data" folder.

Solution:

a) Check the file structure, consulting Fig. 3. Note that the folder names of "*transversal*" and "*tangential*" are absolutely required. All other filenames are derived from these.
b) Check that there is a folder called "*ROIs*" and that the filenames correspond to those in the respective "*data*" folder (and have a "*-RoiSet.zip*" suffix)
c) Run the macro from the parent folder (i.e., the biological replicate folder), or higher

Problem 4: "*Numeric value expected in run() function*" error during the verification of segmentation.

Possible cause: Unknown; some manipulation causes this; hard to reproduce.

Solution: Unknown – save RoiSet manually and run the macro again. You can jump forward by an appropriate number of images to resume where you left off.

Problem 5: Currently hypothetical – "*file not found*" during conversion of Masks to ROIs

Possible cause: After publishing of this protocol, Cellpose developers have changed the naming of the exported segmentation masks.

Solution: Check that the filenames end with "_cp_masks.png". If they do not, replace the value assigned to the "*MasksSuffix*" variable in line 1 of the "*2. ROI_prep.ijm*" macro from "*_cp_masks.png*" to the new suffix (recommended) or rename the Masks files.

Author contributions

Conceptualization: J.Z., J.M.; Methodology: J.Z., J.M.; Software: J.Z.; Validation: J.Z., J.M.; Formal analysis: J.Z.; Investigation: J.Z.; Resources: J.M.; Data curation: J.Z.; Writing - original draft: J.Z.; Writing - review & editing: J.Z., J.M.; Visualization: J.Z.; Supervision: J.M.; Project administration: J.Z.; Funding acquisition: J.M.

Acknowledgments

Microscopy was performed at the Microscopy Service Centre of the Institute of Experimental Medicine CAS supported by the MEYS CR (LM2023050 Czech-Bioimaging). The macros provided here were originally written for (Balazova et al., 2022; Vesela et al., 2023; Zahumenský et al., 2022). We further acknowledge our colleagues Petra Zahumenska, MSc for providing us with a sample neuron image, and Dr Petra Vesela for helping with the preparation of images depicting detailed sample preparation and critical input on the manuscript.

Competing interests

The authors declare that they have no competing interests.

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