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

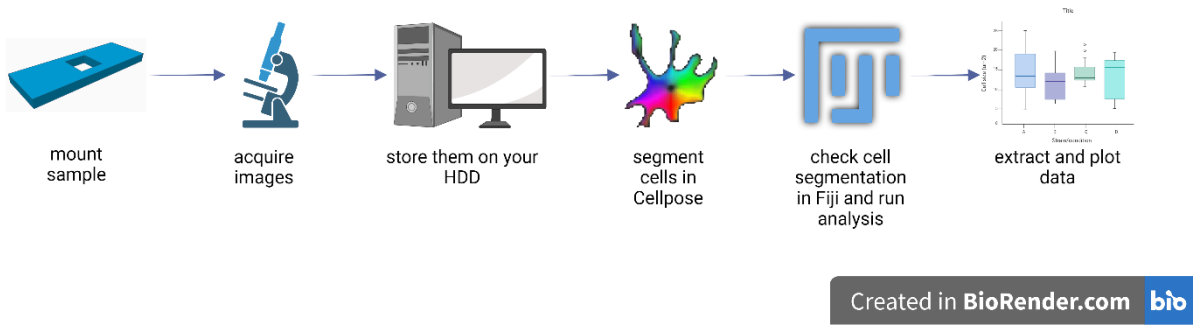
Abstract

Fluorescence microscopy images of biological samples contain valuable information but require rigorous analysis for accurate and reliable determination of changes in protein localization, fluorescence intensity and morphology of the studied objects. Traditionally, cells for microscopy are immobilized using chemicals, which can introduce stress. Analysis often focuses only on colocalization and involves manual segmentation and measurement, which are time-consuming and can introduce bias. Our new workflow addresses these issues by gently immobilizing cells using a small agarose block on a microscope cover glass. This allows for live cell imaging under conditions close to their natural environment and enables the addition of chemicals during time-lapse experiments. We use Cellpose software for cell segmentation and custom-written Fiji (ImageJ) macros for automated analysis of various cell parameters. The results can be easily processed using the provided R markdown scripts or available graphing software. Our method facilitates unbiased batch analysis of large datasets, improving the efficiency and accuracy of fluorescence microscopy research.

The reported sample preparation protocol and Fiji macros were used in our recent publications: *Microbiol Spectr* (2022), DOI: 10.1128/spectrum.01961-22; *Microbiol Spectr* (2022), DOI: 10.1128/spectrum.02489-22; *J Cell Sci* (2023), DOI: 10.1242/jcs.260554.

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

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, often of a single cell, is usually shown. This results in a lot of important information not being communicated to the reader. This can be redeemed by performing a formal rigorous statistical analysis of all acquired data and reporting information on variation across biological replicates and cells in the form of plots and statistical analysis. In 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 the formal analysis of microscopy images is performed, it is often limited to the quantification of colocalization where applicable. When additional analysis is performed, the cells are segmented and analyzed manually (e.g.,[1]). 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 cells adhere to glass surfaces via physical interactions. In our approach, (cell-walled) cells in thick suspensions are placed on a microscopy cover glass and overlaid with 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, 2). In this approach, the cells are gently immobilized on the cover glass just by mild mechanical forces, which does not induce a cellular stress response, as documented by the normal actin structure in *C. elegans*[2] and by the absence of stress granules *S. cerevisiae* cells[3,4]. 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 agarose is permeable to small molecules, cells can be treated by directly adding chemicals to the agarose block (when using an inverted microscope) during time-lapse experiments[3–5]. Adding a cover glass on the other side of the sample holder (Fig. 2) prevents water evaporation and shrinkage 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[6] with automatic cell-by-cell analysis performed by custom Fiji (ImageJ) macros. This approach allows for the processing of large microscopy datasets from unrelated experiments in batch mode, in a consistent and unbiased fashion. In addition to 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 analyzed to generate graphs and perform statistical analyses using provided custom R scripts. Alternatively, the Results table can be processed manually using the Multiple variables data table in the GraphPad Prism graphing software, or in Microsoft Excel/LibreOffice Calc – or by writing one's own macros. We have successfully used the reported protocol for sample preparation and Fiji macros in our recent publications: *Microbiol Spectr* (2022), DOI: 10.1128/spectrum.01961-22[7]; *Microbiol Spectr* (2022), DOI: 10.1128/spectrum.02489-22[8]; *J Cell Sci* (2023), DOI: 10.1242/jcs.260554[9].

Materials and Methods

Biological materials

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

Agarose blocks for microscopy sample preparation

1% nonfluorescent agarose was prepared in 50 mM potassium phosphate (KP_i) buffer (pH 6.3) prepared by mixing 7.75 ml of 1 M KH_2PO_4 with 2.25 ml of 1 M K_2HPO_4 and mixing it with distilled water to 200 ml. After boiling, 10 ml of this solution was poured into a 90 mm diameter Petri dish (and stored at 4 °C for up to a month after the petri dish was sealed with Parafilm). The agarose was cut into ~1×1 cm squares with a scalpel blade, and a small spatula (Fig. 1B) was used to manipulate the blocks.

For specific purposes, 1% conditioned agarose was made as follows: When harvesting (yeast) culture by centrifugation, 3 mL of the supernatant was removed into a fresh 15 mL Falcon tube containing 0.03 g of agarose. The mixture was closed firmly, placed in a tall glass beaker and heated carefully at small intervals in a microwave oven at a low intensity until the agarose had dissolved completely. After cooling down to touch, chemicals were added as required for the respective experiment, and the mixture was poured into a 35 mm diameter Petri dish placed on a level surface. Using conditioned agarose ensured that the environment in which the cells were grown was retained, minimizing stress exposure. This is especially important when changes in either 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 when the budding of yeast cells is to be monitored[1,4,9].

Microscopy sample preparation

Following cultivation at desired conditions, the yeast culture was transferred to a 50 ml Falcon tube and centrifuged for 2 min at 1430 rcf at room temperature. Most of the supernatant was removed by pipetting or decanting and the pellet vortexed in 3 one-second pulses (850 rpm; alternatively, the supernatant was kept and the pellet gently disturbed by pipetting – especially when exponential cultures were used). 1 µL of the cell suspension was pipetted onto a clean 0.17 mm thick microscopy cover glass, which was covered with a block of 1% agarose gel prepared with either KP_i buffer or cultivation media (conditioned agarose, see above), using the small flat spatula (Fig. 1B) to manipulate the agarose block. The cover glass was placed into the sample holder (Fig. 1A; a Tinkercad file for 3D printing can be downloaded here), with the agarose block inside the chamber and taped to the holder with Scotch tape. For time-lapse experiments and when a microscope stage incubator was used to control temperature, the sample holder was flipped upside down onto a lens cleaning paper and another cover glass was taped onto the other side (this prevents the agarose block from drying out during longer experiments). A visual guide is depicted in Fig. 2 and step-by-step instructions can be found at protocols.io[13].

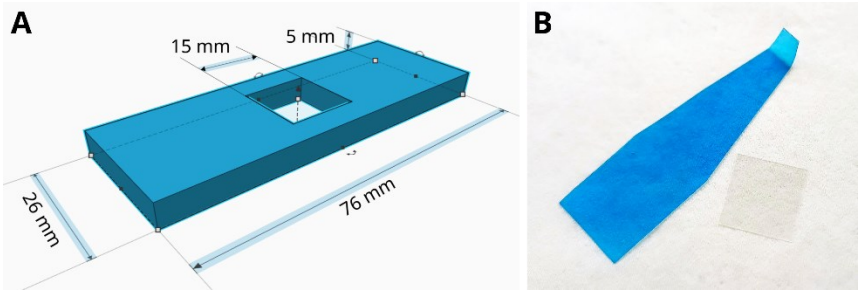


Figure 1. Equipment for microscopy sample preparation. A) Image of the custom-made microscopy sample holder; the Tinkercad file for 3D printing can be downloaded here. B) A spatula made of old Western blot film (a 22×22 mm cover glass for scale).

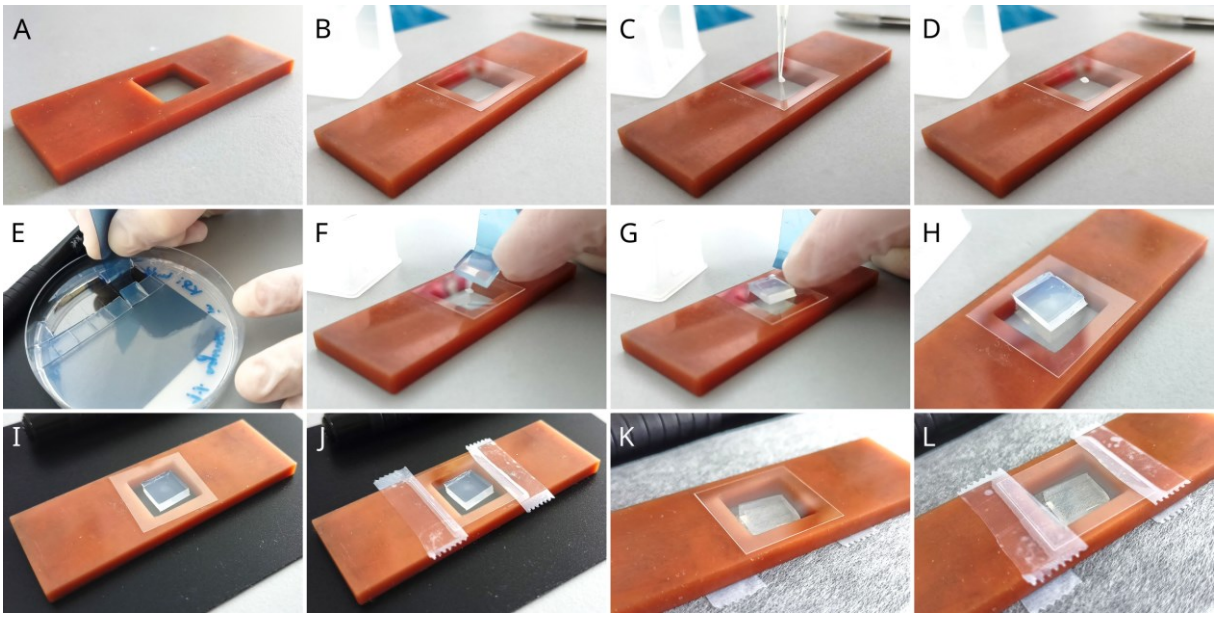


Figure 2. Microscopy specimen preparation. A) The sample holder. B) Place cover glass on a level surface, e.g., the sample holder. C-D) Pipette 1 µL of the cell suspension on the cover glass. E) Use the spatula to lift a small block (~1×1 cm) of pre-cut agarose from the Petri dish. F-H) Cover the cell suspensions with the agarose block. I) Turn the glass with 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 (putting it on a non-abrasive surface) and tape another cover glass to seal the chamber.

Software

- Our custom Fiji macros and R scripts written for this protocol have been deposited to GitHub: https://github.com/jakubzahumensky/microscopy_analysis.
- 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

- Fiji (ImageJ 1.53t or higher; https://imagej.net/software/fiji/downloads) with additional 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[10] (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)
- Up-to-date version of R (including R Studio) – recommended for automatic data processing
- Prism 8 or higher (GraphPad) – *optional* – for semi-automatic data processing
- Microsoft Excel/LibreOffice Calc – *optional* – for manual data processing (not recommended)

Microscopy image acquisition

In microscopy experiments, it is important to observe a certain set of rules to ensure that the downstream data analysis works properly and that the acquired data are valuable. Here we give the following 7 "microscopy commandments" to ensure that the acquired images are of high enough quality (for a more detailed description see e.g.,[11]):

1. Thou shall not overexpose your images.
2. Thou shall set offset properly (when using a photomultiplier 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 the focus in one image area and imaging another area directly adjacent, e.g., using the 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 take images with high noise.
   Note: While noise is always present in microscopy images, high noise levels can create issues for the segmentation of cells and obscure subcellular 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 the 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/cell type, 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 the mean intensity in the plasma membrane to that in the 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 Discussion.

Data analysis

Here, we report our custom Fiji (ImageJ) macros that facilitate automatic analysis of large numbers 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, their circumference and for 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 folder and all its subfolders (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. The 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. The user is informed when the macro run is finished with a message (“Finito!”). If there are any irregularities during ther macro run, the message also provides 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 to process data from multiple experiments at the same time, it is advantageous to use experimental (accession) codes for your experiments. These codes also facilitate cross-referencing between digital data and hard-copy lab notebooks (as well as between related experiments). We use experiment codes following the “JZ-FM-αβγ” format, where JZ are initials of the experimenter (here, “Jakub Zahumensky”, FM denotes “fluorescence microscopy” and αβγ is a three-digit number). All the 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 YYMMDD format to keep the folders ordered chronologically. In each replicate folder, separate folders for transversal (images of cell cross-sections)

and tangential (depicting cell surfaces) images need to be created, as required. In these, a folder called "data" is used for storing the actual data to be analyzed. All additional folders that are required are created by the provided macros. If images require preprocessing, such as drift correction or z-projection, the use of an additional "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 and temperature (if multiple options exist) and time, condition/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. Images from time-lapse experiments and z-stacks need to be corrected for drift and bleaching (e.g., using the "0. correct and project.ijm" macro) before the analysis. Time-series also need to be separated into individual images. As the Results table is comma-separated, the information in filenames also 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 R/GraphPad Prism), as well as manual processing (Microsoft Excel/LibreOffice Calc). Experiments destined for analysis can be temporarily moved to a single folder and analyzed in a single run.

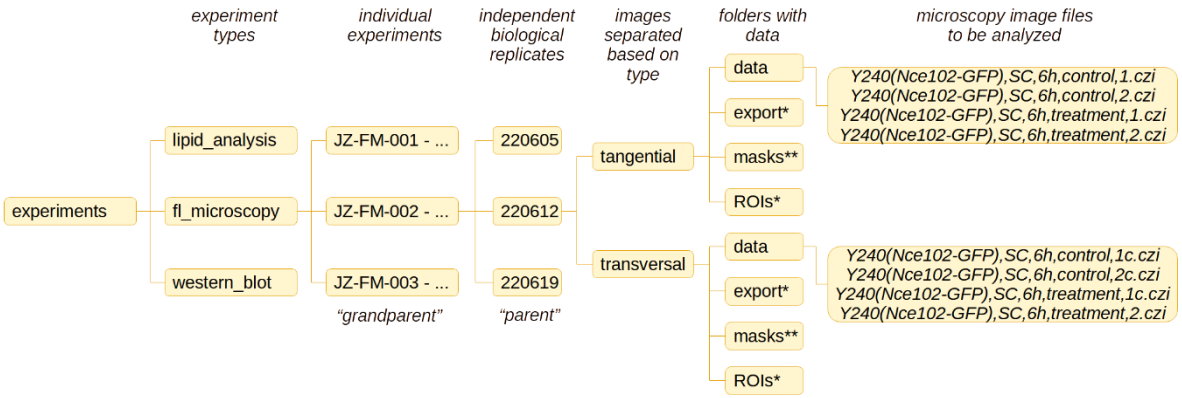


Figure 3. Example data organization structure. The folders marked with a single and double asterisk are created automatically by the provided Fiji macros and Cellpose, respectively.

Preparation for analysis – image export and object segmentation

To segment objects (typically cells and/or nuclei) in microscopy images, we use Cellpose 2.0[6] (see Software). Other segmentation options are available in Fiji (ImageJ) or Cell Profiler[12], but these options are not covered in this protocol. Cellpose 2.0 can be used either through its graphical user interface (GUI) or via the command line (Anaconda; see Software). The GUI is useful for determining the segmentation parameters for a 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. To export all images across biological replicates (and/or experiments), run the "1. export.ijm" macro. For 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 be used only 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 channels with brighter fluorescence signals and clearly visible plasma membranes. The analysis (described below) processes a single frame at a time. Therefore, for time-lapse experiments, images need to be split into individual frames.

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 estimate of the 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 (a detailed description of the 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 such a way that their edges are placed in the middle of the plasma membrane. For tangential images, masks should be just as large 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 for cell segmentation using Cellpose:

```
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
```

In the command, substitute the "D:\Cellpose" folder for where your exported images are. Additionally, 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 segmentation, together with the masks folder (needs to be called "masks" for downstream automatic processing). For the "--use_gpu" option to work (i.e., use the graphical processing unit for calculations; optional), additional steps are required before running the segmentation (see Cellpose documentation).

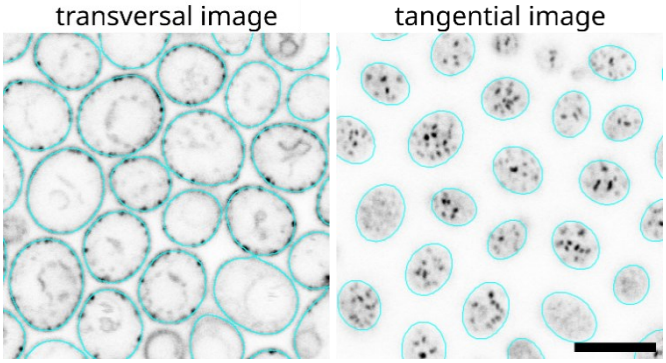


Figure 4. Guide to Segmentation. Segmentation should be performed in a way that the ROI edges are in the middle of the plasma membrane in transversal images (cell cross-sections) and just touch the outside of the objects in tangential images (cell surfaces). Yeast cultures were grown in synthetic complete media for 24 h and Nce102-GFP (a yeast plasma membrane microdomain protein) was imaged. Scale bar: 5 µm. Raw data from Zahumensky et al., 2022[7].

Preparation for analysis – regions of interest (ROIs) and segmentation verification

Before analysis, the segmentation mask images obtained in Cellpose need to be converted to regions of interest (ROIs) that Fiji can use, and the accuracy of these ROIs needs to be verified. 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.

Conversion of masks to ROIs – In the first macro run, select the “Convert Masks to ROIs” in the process option. This macro converts all Masks of the selected image type into ROIs and saves these in a newly created “ROIs” folder in the form of zip files called RoiSets (see Fig. 3). The process runs in batch mode; therefore, no images are 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 approach is also useful for images of nuclei but is not suitable for cells with complex morphologies (e.g., neurons). The “Blind experimenter” option has no effect at this point. If ROIs have already been created, a warning is displayed upon pressing OK, as these will be overwritten if the macro is run, and all changes made previously to the ROIs will be lost. The macro automatically removes objects that are too close to the image edges and are therefore likely to be incomplete.

ROI verification – Once the macro is finished, run it again, selecting "Check ROIs" in the process option and using the same options as in the first run. Selecting the “Blind experimenter” option can be used to reduce experimenter bias. Specifically, it randomizes the order in which the images are displayed and hides the filenames and the location of the files from the status message. 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. Then, images of the selected type are displayed one by one to the user together with existing ROIs. These can be removed or adjusted to correspond to the example as closely as possible (the example 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’ on the keyboard 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 one of the previous images, or jump ahead, if desired. To

increase the visibility of all features, the inverted gray lookup table (LUT) is used to display the images, combined with red outlines of the ROIs. In this step, files can be excluded from the analysis (e.g., if bleached or focused incorrectly) 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. This feature works even when the experimenter is blinded.

Note that the ROIs defined in this step will be used for the analysis of all available channels in the subsequent steps. If, for example, whole cells are to be analyzed in one channel and nuclei in another, it is recommended to split the images into separate channels and analyze them independently.

Running the analysis

During the analysis, all images within the specified folder and subfolders are successively opened along with the respective RoiSet (in batch mode, i.e., no images are displayed). Each ROI is then analyzed separately, and the corresponding data is written in a log file that is saved in the form of a csv (comma-separated values) table whenever an image is closed. This enables resumption of an interrupted analysis run. Additional log windows appear, informing the user about the analysis progress (including rough estimate of the remaining time), listing processed images and images that were not analyzed to absence of defined ROIs.

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 folder from which the analysis is to start (the Results will be saved here). 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 (use a comma to specify multiple channels or 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 (information written here will be used as column titles in the Results 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, ensure that the naming is consistent across all the 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). 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). The "Help" button can be used 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 a dialog window appears (with a message saying: “Finito!”) with additional information in case of irregularities. The following files are created by the macro:

- “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” – the "Results table" containing the results of the analysis for the specified channel CH. The numbers in the brackets specify, in the format shown, the date and time when the analysis was concluded.

- "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 if the analysis has been interrupted for any reason. This file is converted to the "Results table" file once the analysis of all images is completed.
- "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 prepared, the analysis can be resumed (by running it again with the same parameters).
- "processed_files.tsv" – lists the files that have been analyzed. If an incomplete analysis is resumed, these are skipped.

  Disclaimer: To count the number of bright fluorescence foci (microdomains), and nowhere else, the analysis uses nonlinear adjustments.

Structure of the results table

- Header - 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. Each line of the header starts with the pound (#) sign so that it is automatically ignored by the provided R scripts.
- Columns – The first several columns (up to the frame column) contain descriptive parameters, i.e., information about the analyzed sample, derived from the file names. The columns from "mean background" onward contain quantification parameters, i.e., numbers extracted from the microscopy images by the "Quantify" macro. The quantified parameters are described in detail in the "results_table_legend.md" file in the GitHub repository (see *Data and code availability* section).
- Rows – Each row contains data pertaining to a single cell (ROI)

Extracting numbers and plotting

The Results table can be further processed in multiple ways. We recommend either taking advantage of the provided R script, which enables fully automated processing, plotting and statistical analysis, or using GraphPad Prism.

R scripts – Our custom R scripts can be found here (in the form of a markdown file) and used as follows: place the Results table to be processed into a folder together with the provided R markdown file ("microscopy_analysis_data_processing.Rmd"). Open the markdown in R studio and run the scripts (organized into code chunks with descriptions). A new folder called "analysis" is created with a subfolder for each experiment. The Results table is automatically filtered (grouped) based on all possible unique combinations of descriptive parameters (biological replicates, strains, conditions, etc.). Means and standard deviations (SDs) are calculated and reported in separate tables for each quantified parameter across these groups.

Boxplots showing the median, range, and individual data points for biological replicates are generated for each parameter. The user is prompted to select the independent (x), grouping (factor) and faceting variables from sample description parameters if multiple choices exist. The user can also

select quantification parameters for which to plot intricate violin plots, including statistical analyses of differences (Holm- or Bonferroni-corrected multiple *t-tests*).

Independent statistical analysis is performed on data with at least 3 biological replicates (after automatic filtering). Normality (Shapiro–Wilk) and equal variance (Levene's test) are tested, followed by multiple unpaired t-tests (using Bonferroni and Holm corrections) and ANOVA with Tukey's HSD (honestly significant difference) post-hoc test. The results are reported for each quantified parameter in a separate text file.

<u>GraphPad Prism</u> – Load the Results table by clicking File → New → New Project File → Create Multiple variables and copy-paste the whole Results table here. 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 the actual data.

<u>Microsoft Excel/LibreOffice Calc</u> – When opening the Results file in Excel/Calc, ensure 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 the mean and standard deviation (SD) within each biological replicate, strain and condition.

Note: The tables containing means and SDs generated by the R script can be used directly in GraphPad Prism, SigmaPlot (or another scientific graphing software) to create 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 Discussion). 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 readily visible[13].

Discussion

Key capabilities

We report a simple sample preparation method that enables live cell-walled cells to be gently immobilized in a monolayer, imaged under almost natural conditions and treated directly during a time-lapse experiment. We developed a Fiji-based analysis technique for budding yeast with GFP-tagged proteins that enables batch analysis of large microscopy datasets. Its use is not limited by any imaging platform, microscopy type, labeling or organism, as we demonstrate below. Multiple parameters are reported for each object, including size, fluorescence intensity in the plasma membrane and cytosol and the number of microdomains (i.e., areas of high local intensity). The analysis is largely automated and requires user input only to verify the accuracy of segmentation, with clear rules and an option for blinding this step. Hence, our approach allows for unbiased batch analysis of large datasets, greatly improving the efficiency and accuracy of fluorescence microscopy research. Combined with

the provided R scripts, which enable streamlined processing of the data extracted from the microscopy images to report summary data (means and standard deviations), plot graphs and perform statistical analysis, it represents a very powerful tool that can be used wherever microscopy images are acquired and analyzed.

Validation of the analysis workflow

We have previously successfully employed the sample preparation method and Fiji macros described here in multiple studies, validating this approach[7–9]. We have updated the macros after these papers were published to incorporate new features (e.g., resume incomplete analysis, analyze multiple channels in a single run, blind experimenter, additional approaches for the quantification of plasma membrane microdomains) and fix known issues (e.g., macro running commands before the previous one has been completed). In addition, we created R scripts that greatly facilitate downstream processing, graphing and statistical analyses of the data. To demonstrate the universality and robustness of the reported Fiji macros, we include the analysis of a microscopy image of a neuron as an example of a morphologically complex cell (Fig. 5).

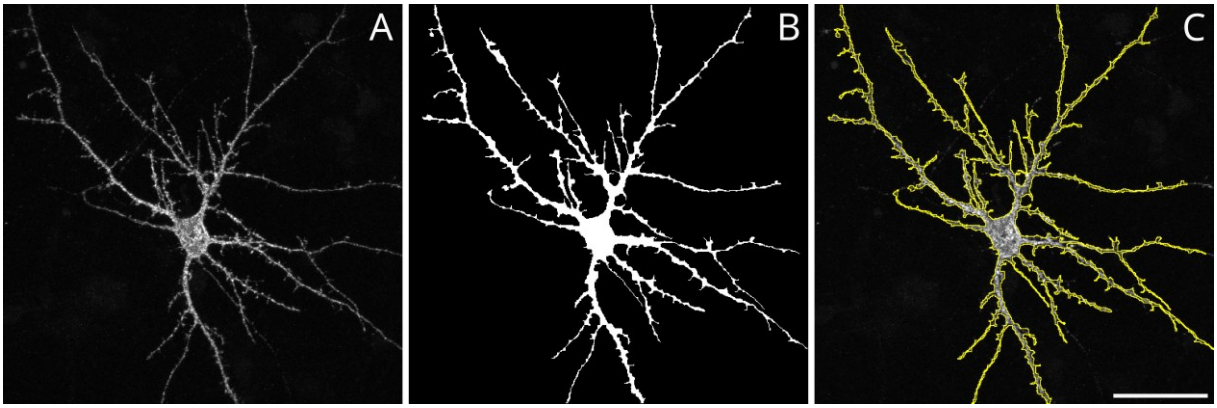


Figure 5. Segmentation of a neuron for protocol validation. A) Raw image B) Segmentation mask creating by thresholding image A followed by manual adjustments (Fiji). C) Region of interest (ROI) created from the mask in B. Primary hippocampal neuronal cultures were prepared from rats at embryonic day 18. The neurons were transfected with a DNA vector containing gene encoding the 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. The 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. The analysis results are available in the Zenodo repository (see *Data and Code availability* section).

Troubleshooting

There are several issues one can run into when using our analysis macros. The known common pitfalls are listed below and mostly involve incorrect paths to folders. Should any additional issues arise, the readers are welcome to contact the authors.

Issue	Possible cause(s)	Solution(s)
Cellpose does not export Masks properly	The path to the images to be processed contains spaces	Segment all images in a dedicated folder without any spaces anywhere in the path, e.g., “D:\segmentation\” and then move the

		"masks" folder to the folder of the respective biological replicate
Cellpose does not use GPU to calculate the segmentation masks	The used PC is running an AMD-family GPU (under Windows)	There is no solution known to 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.
	Required dependencies are not satisfied.	Follow the instructions in the official documentation here
No data reported in the Results table	The required data structure is not in place.	Check the file structure, consulting Fig. 3. Note that the folders called "transversal", "tangential" and "data" are absolutely required.
	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.	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).
Currently hypothetical – "file not found" during conversion of Masks to ROIs	After publishing of this protocol, Cellpose developers have changed the naming of the exported segmentation masks.	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.

Table 1: Troubleshooting of the most common analysis issues

Note on normalization

Certain values (e.g., the mean fluorescence intensity of a cell) need to be normalized before the statistical significance of the observed changes between strains/cell lines and/or conditions/treatments can be tested. The 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 itself 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 this 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.

	raw data			scaled			normalized to control		
condition	replicate 1	replicate 2	replicate 3	replicate 1	replicate 2	replicate 3	replicate 1	replicate 2	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 2. 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.

Author contributions

Jakub Zahumensky – Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. **Jan Malinsky** – Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing - review & editing

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Data and code availability

Code described in this article and sample data have been deposited to GitHub: https://github.com/jakubzahumensky/microscopy_analysis and Zenodo (https://zenodo.org/records/11517778), respectively.

Competing interests

The authors declare that they have no competing interests.

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