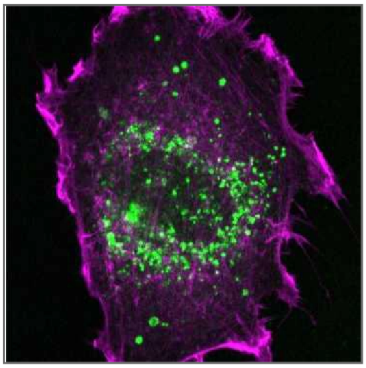

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Protocol status: Working
We use this protocol and it's working

Lysosome analysis with confocal microscopy_V2 V.1

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ABSTRACT

This protocol provides an overview of lysosomal analysis using confocal microscopy and Fiji

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Keywords: Lysosomes, Confocal microscope, Analysis

MATERIALS

Staining
- LAMP1 (D2D11) mAb rabbit
- Secondary Ab anti-rabbit
- GOLD SEAL circular cover glass (Electron Microscopy Sciences)
- Phalloidin-iFluor™ 488 Conjugate (AAT Bioquest)
- Microscope slides

Cell-culture
- 24-well plate for cell culture purposes
- cell-culture media (DMEM/RPMI) with FBS

Abs-staining buffer (0.2 µm-filtered, frozen aliquots)
- Glycine 1.5 g (Bachem, Cat no. 4030676)
- BSA 30 g (Fisher Scientific, Cat no. 11413164)
- 1000 mL sterile 1X PBS (pH 7.5)
- Filtered with a 0.22 µm Stericup (Millipore, Cat no. SCGPU02RE)

Saponin (Santa Cruz Biotechnology, sc-280079)
- 5% (w/v) dissolved in PBS and filtered over a 0.2 µm filter.

Confocal microscope:
63X zoom and NA of 1.4 is recommended

Standard cell-culture equipment

Computer and FiJi

Sterilize glass cover slips

1 Put the glass coverslips in a 24-well plate, submerge the coverslips in 70% ethanol, and then expose them UV light in a tissue culture hood for between 20 and 30 minutes. 30m

2 Remove the ethanol, wash the wells containing coverslips three times with sterile PBS, and remove the PBS Add 450 µL of cell-culture media to the well. 2m

Seed, treat and fixate cells

16h 35m

3 Make a suspension of your cells (1.200.000 cells/mL) and seed these at 60.000 cells per well - add 50 16h 10m

Note: This cell number is an empirically observed amount with minimal contact between relatively large cells (Fibroblast/Macrophages). However, optimal seeding densities might vary per cell type.

Alternatively, you could use confluence and the area of a cell-culture dish and aim for 50% confluency. This might require adding extra volume to make the total volume 500 µL.
Example: you have a 1mL suspension of a 100% confluency T75-flask.
The area of a 24-well plate well = ~1.9cm^2
(1.9/75)*0.5 = 0.0126 mL = 12.6 µL cell suspension

Incubate the cells overnight at 5%CO2 and 37 ºC, to let them adhere.

4 The next day, refresh the media to remove any dead cells and treat the attached cells. Wash the cells with 15m
500 µL 1X sterile PBS 3 times and fixate the cells 15 min at 4C with 2-4% PFA

5 After fixation, wash the wells 4 times with 500 µL 1X sterile PBS. Add 500 µL 1X sterile PBS to the well an 10m
store them at 4ºC until use.

Stain glass coverslips

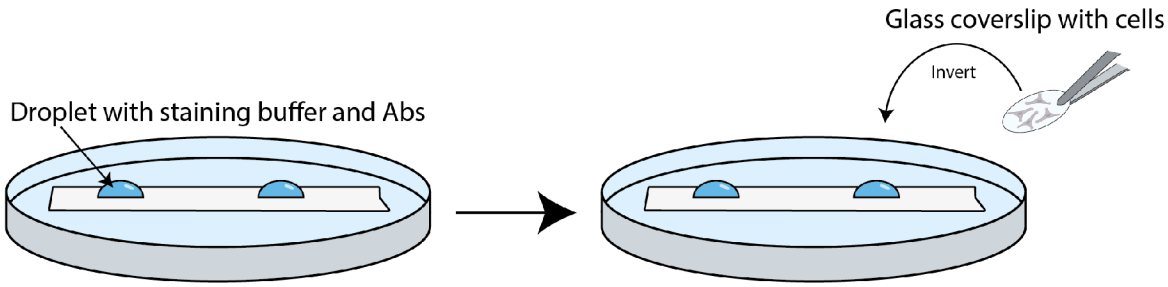
30m

6 Remove PBS and add Abs-staining buffer supplemented with 0.1 % Saponin and incubate at room temperature for 30 min. 30m

7 - Dilute your LAMP1 (D2D11) mAb rabbit (1:200) in Abs-staining buffer supplemented with 0.1 % Saponin 5m
for 30 µL per sample. put on ice

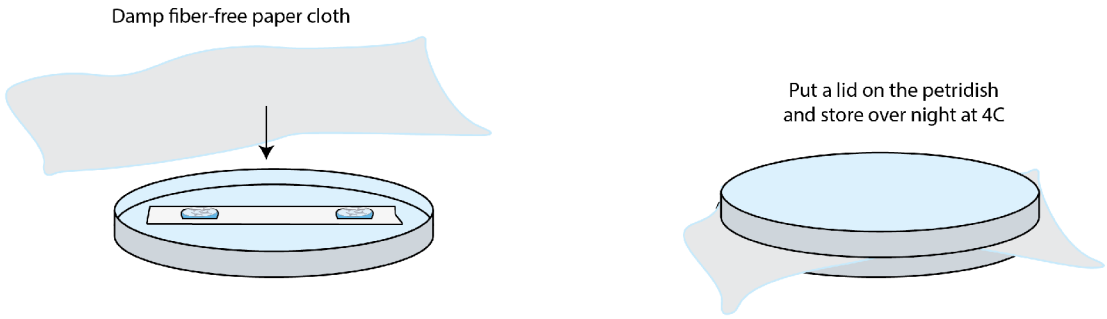
8 Put parafilm in a petridish. Add 30 µL droplets of Abs-staining buffer containing the primary Ab 5m

9 Invert the glass coverslips onto the droplets, facing cells down into the droplet 5m



Schematical overview of inverting glass coverslips samples on to droplets on parafilm containing LAMP1 Ab in staining buffer

10 Add a damp lint-free tissue to the Petri dish and store the sample overnight at 4°C. 16h



Schematical overview of contraption to store microscopy samples overnight, make sure that the damp cloth is not touching the sample

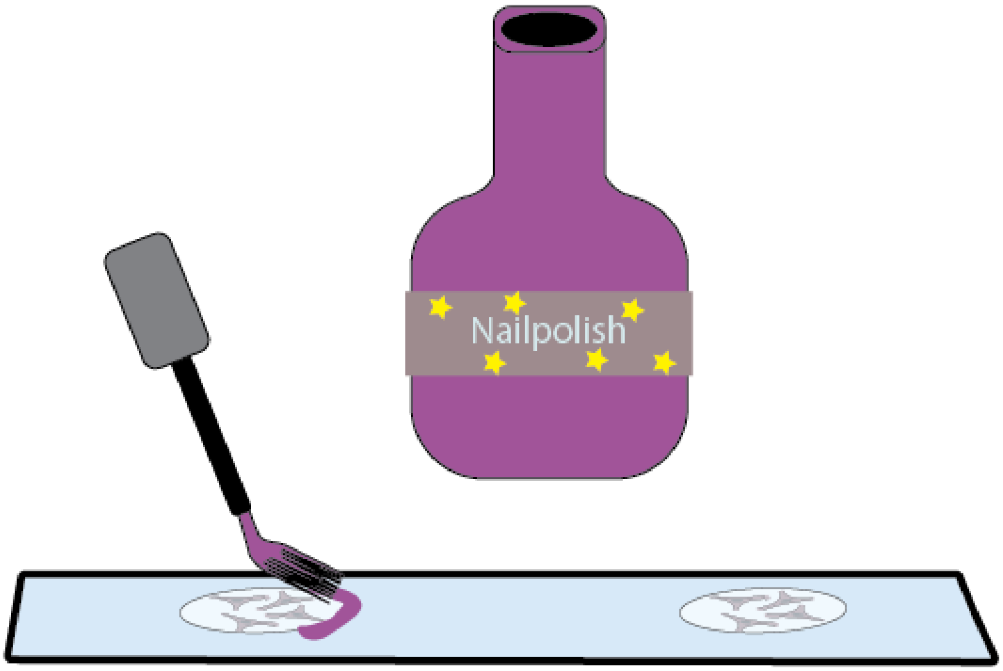
11 The next day, invert the glass coverslips back into a 24-well plate and wash two times with cold 1X PBS 10m

12 Repeat steps 7, 8, and 9, but now instead of the primary Ab, use the secondary against the primary Ab and other markers like DAPI (5 µg/mL) or phalloidin. Make sure to incubate the sample in the dark at room temperature for 45-60 min 30m

13 Invert the glass coverslips back into a 24-well plate and wash three times with cold 1X PBS 5m

14 Add 5µl of Trolox media to a microscope glass slide. Take out the glass coverslip from the 24-well plate and briefly hold the side of the coverslip against a dry lint-free tissue to absorb as much liquid as possible before inverting the coverslip onto the Trolox media. 5m

15 Let the sample cure for an hour or two at room temperature in the dark and seal the sample with nail polish once the sample has dried to some extent and is not moving when touched gently. After sealing the sample, store it away at room temperature in the dark until image acquisition. 3h



Make sure to be careful not to spill nail polish everywhere

16 Before image acquisition, you should see a lot of dried salts when looking at your sample. Make sure to add some milliQ to the top of your sample and gently add a fibre-free cloth onto the water droplets to remove the dissolved salts. repeat this step a few times and wipe the samples dry afterwards, now we can go to the microscope for imaging

Imaging

17 Since not every lab has the same microscope, I feel it does not make sense to specify a microscope here. However, I recommend performing confocal microscopy with high magnification (63X, NA 1.4) to look at lysosomes, as these organelles are small and require some resolution to make sure lysosomes are distinguished enough for identification. 4h

Overall I try to have a sufficient amount of images for analysis (n=10-20 per condition times 3 technical repeats)

When an intensity quantification is desired to indicate protein levels or other stainings, I recommend resorting to flow cytometry or western blot techniques. If you still want to use microscopy as a quantification tool, at least make sure not to overexpose your images and saturate pixels.

Image analysis

18 Depending on the question that you might require different image analysis strategies are suitable for looking at lysosomes. In this section, I will explain the basal procedure that I use to analyse lysosomes. This protocol assumes that you stored your samples as a '.lif' file.

This tutorial is for users that run:
- Windows 11 version 23H2 (OS Build 22631.3007)
- ImageJ 1.54f

19 Open Fiji

20 Drag your '.lif' file into the program and select all samples of interest

21 Run this code in the ImageJ2 Script editor (ImageJ Macro), to get all images into a TIFF:

```
//Get image IDs of all open images
dir = getDirectory("Choose a Directory");

for (i=0;i        selectImage(i+1);
        title = getTitle;
        print(title);
        //ids[i]=getImageID;

        saveAs("tiff", dir+title);
}
run("Close All");
```

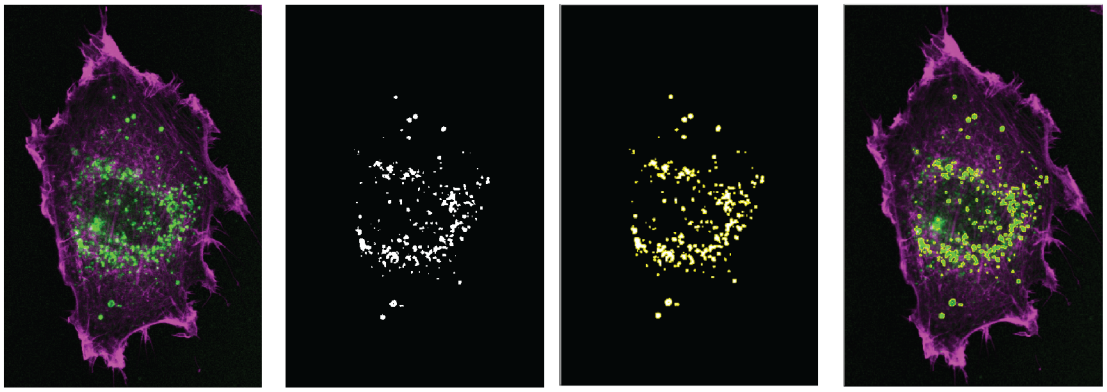
After you run this code, consider putting all TIFFs into a folder per condition

22 Now we want to get all images from a folder/condition, create a LAMP1 lysosome mask and analyse the structures:

```
dir = getDirectory("Choose a Directory");
for (i=0;i        selectImage(i+1);
        title = getTitle;
        run("Split Channels");
//create Lamp1 lysosome mask and analyse particles - change C3 to the
channel that is used to visualize LAMP1
        selectWindow("C3-" + title);
        run("Subtract Background...", "rolling=50 slice");
        run("Median...", "radius=0.5");
        setAutoThreshold("Otsu dark no-reset");
        setOption("BlackBackground", true);
//Make sure to check with a raw image if you agree with the mask
        run("Convert to Mask", "method=Otsu background=Dark only black");
        run("Analyze Particles...", "display exclude summarize overlay
composite");
        selectWindow("Results");
        saveAs("CSV", dir+"Results" + title);
        selectWindow("Summary");
        saveAs("CSV", dir+"Summary");
```

Now you should have all your data stored in your folders for further analysis

23



Example of analysis