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Intracellular and plasma membrane cholesterol labeling and quantification using filipin and GFP-D4 probes.

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Abstract

Cholesterol, a major component of biological membranes, is rapidly trafficked and unevenly distributed between organelles. Anomalies of intracellular cholesterol distribution are the hallmark of a number of lysosomal lipid storage disorders. A major methodological obstacle for studying cholesterol trafficking is tracing this molecule in situ. The use of fluorescent probes that specifically bind cholesterol, allows the visualization and imaging of cellular cholesterol. Here, we describe a series of assays optimized for quantifying free cholesterol in cell populations and at the single cell level, both at the plasma membrane and inside cells. These methods use two fluorescent probes: the D4 fragment of perfringolysin O fused to GFP (GFP-D4); and the polyene macrolide filipin. First, we report a robust method for quantifying plasma membrane cholesterol by flow cytometry using the GFP-D4 probe. Second, to optically distinguish and quantify intracellular cholesterol accumulation, we have adapted the classical filipin cholesterol staining protocol. Indeed, we observed that treatment of living cells with methyl- β -cyclodextrin, a chemical known to extract cholesterol from the plasma membrane, improves the visualization of the intracellular cholesterol pool with filipin. To complement these staining procedures, we developed an image analysis protocol based on image segmentation to quantify, in a robust manner, intracellular cholesterol stained with filipin. Thus, this chapter is a guideline for cellular cholesterol staining and signal quantification.

1 Introduction

Cholesterol is a key molecule in biology, as a major structural component of cell membranes, and as a precursor of other vital compounds, such as hormones and bile [1, 2]. Interestingly, sterol levels are regulated at the subcellular scale. Indeed high, intermediate, and low levels of sterol are found in the plasma membrane, endosome/Golgi apparatus, and mitochondria/endoplasmic reticulum, respectively [3, 4]. This uneven sterol distribution between organelles results both from vesicular and non-vesicular transport mechanisms [5]. In particular, a class of proteins named Lipid Transfer Proteins (LTPs) plays a crucial role in the latter process. LTPs are cytosolic proteins which have a pocket that accommodates a lipid molecule; LTPs can capture, move, and deliver this lipid molecule within the cell [6-8]. The study of sterol transport inside cells faces a major obstacle, which is the ability to trace this type of molecule *in situ*. However, the use of natural fluorescent sterols that closely mimic cholesterol, fluorophore-labeled sterols, or fluorescent probes that can trace cholesterol distribution in cells, have advanced the understanding of sterol transport [9]. In this chapter, we describe the use of two fluorescent probes, GFP-D4 and filipin, to label and quantify plasma membrane and intracellular cholesterol levels.

The GFP-D4 probe is a fusion between the fluorescent protein GFP and the D4 fragment of perfringolysin O (referred to as θ toxin), a pore-forming toxin from the bacterium *Clostridium perfringens*. Perfringolysin O is a cholesterol-dependent cytolysin secreted as a water-soluble monomer that recognizes and binds cholesterol-rich membranes where it oligomerizes and creates pores. The D4 fragment of perfringolysin O is able to bind cholesterol-rich membranes (>30 mol% cholesterol) but is devoid of pore-forming activity [10-13]. Interestingly, mutants of GFP-D4, able to bind membranes with less cholesterol, have been generated [14, 15]. In this chapter, we describe the use of recombinant GFP-D4 to stain intracellular cholesterol, and to label plasma membrane cholesterol followed by its quantification by flow cytometry.

Filipin is a polyene macrolide extracted from the bacterium *Streptomyces filipinensis* [9, 16]. Filipin is naturally fluorescent and specifically binds to sterols but not to esterified sterols. Owing to its toxicity, filipin is used on fixed cells. The use of filipin to label and quantify intracellular cholesterol is technically challenging: first, the probe is very sensitive to photobleaching which impedes its use for quantification; and second, it labels total cellular cholesterol, and especially the plasma membrane, making intracellular cholesterol difficult to image. Thus, to bypass these technical limits, we set up a new protocol, described in this chapter, which allows the imaging and quantification of intracellular cholesterol using filipin [17]. To avoid filipin photobleaching, we used a new generation confocal microscope equipped with a 355 nm UV-laser and ultrasensitive detectors. In addition, to visualize intracellular cholesterol pools, we treated live cells with methyl- β -cyclodextrin (M β CD), a molecule able to deplete cholesterol from the plasma membrane [18].

2 Materials

2.1 Chemicals and buffers

1. U18666A [(3 β)-3-[2-(Diethylamino)ethoxy]androst-5-en-17-one hydrochloride]: 1 mg/mL in water (stock solution). Store at -20 °C.
2. Methyl- β -cyclodextrin (M β CD) solution: Dissolve 660 mg of M β CD powder in 1 mL of water. Heat at 65°C for 15 min to dissolve, and vortex several times. Store this stock solution (500 mM) at -20 °C. Heat at 65°C for 15 min before use.
3. M β CD-cholesterol complex (M β CD/cholesterol molar ratio: 6.5/1) solution: Dissolve 2.65 g of M β CD in 25 mL of buffer (150 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, pH 7.5 adjusted with NaOH). Add 0.119 g of cholesterol powder (Sigma), mix, sonicate 3 times (10 min each) and heat (15 min at 65°C) to solubilize cholesterol. Store this stock solution (80 mM) at -20 °C. Heat at 65°C for 15 min before use.
4. Phosphate-buffered saline (PBS) 1X: 134 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4.
5. Paraformaldehyde (PFA) stock solution: 16% (w/v) in water (EMS). Store at room temperature (see Note 1).
6. PFA solution (4% (w/v) in PBS 1X). Mix 10 mL of PFA stock solution (16%) with 4 mL of PBS 10X and 26 mL of water. Store at 4°C (see Note 1).
7. Filipin solution. To prepare 50 mL of filipin solution (0.1 mg/ml), first dissolve 5 mg of filipin powder in 200 μ L of dimethyl sulfoxide (DMSO). Add this solution dropwise to 50 mL of PBS 1X under constant vortexing. Keep filipin protected from light. Aliquot this solution and store at -20°C.
8. 10% (w/v) bovine serum albumin (BSA) solution: Dissolve 1 g of BSA (Fraction V) in 10 mL of PBS 1X. Store the stock solution at -20 °C.
9. Blocking solution: 1% (w/v) BSA in PBS 1X.
10. Cell culture medium with and without serum. Trypsin solution (~30 μ g/mL) and Trypsin-EDTA (~30 μ g/mL – 0.53 mM).

2.2 GFP-D4 production

1. GFP-D4 expression plasmid (pET28/His6-EGFP-D4; RIKEN BioResource Research Center; RDB13961). This plasmid can be amplified in *E. coli* DH5 α bacteria.
2. *E. coli* BL21 (DE3) competent bacteria.
3. LB broth and LB agar plates containing 40 μ g/mL kanamycin.
4. IPTG (isopropyl β -D-1-thiogalactopyranoside): A 1 M stock solution is prepared by dissolving IPTG in water, and stored at -20°C.
5. Conical flasks.
6. Lysis buffer: 50 mM NaH₂PO₄/Na₂HPO₄, 300 mM NaCl, 10 mM imidazole, pH 8. Add 58.25 mL of Na₂HPO₄ (0.2 M), 4.25 mL of NaH₂PO₄ (0.2 M), 15 mL of NaCl (5 M) and 2.5 mL of imidazole (1 M) to a graduated cylinder. Add water to a volume of 250 mL, mix and store at 4°C. Before use, add 1 tablet of EDTA-free complete protease inhibitor cocktail (Roche) in 50 mL of lysis Buffer.
7. His-Select Nickel Affinity Gel (Sigma).
8. Elution buffer: 20 mM NaH₂PO₄/Na₂HPO₄, 250 mM imidazole, pH 7.4. Add 1.935 mL of Na₂HPO₄ (0.2 M), 0.565 mL of NaH₂PO₄ (0.2 M) and 6.25 mL of imidazole (1 M) to a graduated cylinder. Add water to a volume of 25 mL, mix and store at 4°C.
9. Phosphate buffer (PB): 20 mM NaH₂PO₄/Na₂HPO₄ pH 7.4.
10. Ultrafiltration units (Ultra-15 Amicon, MWCO 10 kDa, Millipore).
11. Glycerol (molecular biology grade).
12. Sonicator (Sonics Vibra-Cell 750 W equipped with a 13 mm probe).
13. Chromatography column (Bio-Rad Poly-Prep chromatography column).
14. Peristaltic pump (Gilson).
15. Spectrophotometer (Nanodrop, Thermo Scientific).

2.3 Flow cytometry and imaging

1. Round cover glasses (diameter 12 mm, N^o. 1.5H).
2. Superfrost microscope slides.
3. 24-well plates
4. P150 plates
5. Propidium iodide (PI) (50 μ g/mL stock solution, stored at 4°C) is diluted in PBS to a final concentration of 0.35 μ g/mL.
6. TO-PRO-3 iodide solution: Dilute a 1 mM stock solution in DMSO (ThermoFisher scientific, stored at -20°C) in PBS to a final concentration of 0.35 μ g/mL.
7. Hoechst-33258 solution. Prepare a stock solution of the dye at 10 mg/mL in water. Dilute the dye in PBS to a final concentration of 10 μ g/mL.
8. Anti-CD63 mouse monoclonal antibody (Developmental Studies Hybridoma Bank (DSHB) H5C6; deposited to the DSHB by August, J.T./Hildreth, J.E.K.). The hybridoma supernatant is diluted 100 times in blocking solution.
9. Mounting medium (ProLong Gold, Invitrogen).
10. Confocal laser scanning microscope (Leica SP8 UV) equipped with a 355 nm optically pumped semiconductor laser (Coherent), super-sensitive detectors (HyD, Leica) and a 63x objective (oil, NA 1.4).
11. Image analysis software: Fiji software (<http://fiji.sc>) or CellProfiler (<http://cellprofiler.org/>)
12. Flow cytometer (BD FACS Celesta) or similar.
13. Flowjo software (FlowJo LLC) or similar.

3 Methods

3.1 GFP-D4 production and purification

1. Transform competent *E. coli* BL21 (DE3) cells with the pET28/His6-EGFP-D4 vector according to the manufacturer's instructions.
2. Spread bacteria on LB agar plates with kanamycin and incubate overnight at 37°C.
3. Transfer one colony to 10 mL of LB broth with kanamycin. Incubate 16 h at 37°C under agitation.
4. Transfer this pre-culture to a 5 L conical flask containing 1 L of LB broth with kanamycin.
5. Incubate at 37°C under agitation. Measure the optical density (OD_{600nm}) at a wavelength of 600 nm with a spectrophotometer. When the OD_{600nm} has reached 0.4-0.6, cool down the culture to 18°C.
6. Add IPTG to a final concentration of 0.4 mM.
7. Incubate under agitation for 16 h at 18°C.
8. Transfer the culture medium to centrifugation tubes. Pellet the cells by centrifugation at 3,500 \times g for 15 min.
9. Discard the supernatant. Resuspend the cell pellet in 100 mL of lysis buffer.

10. Sonicate the suspension for 10 min (40% amplitude, on/off cycles 10s/20s) on ice under constant stirring.
11. Centrifuge twice at $3,500 \times g$ for 15 min at 4°C . Keep the supernatant.
12. Centrifuge twice at $11,000 \times g$ for 30 min at 4°C . Keep the supernatant.
13. Add 1.5 mL of nickel-charged resin into a chromatography column. Connect the column to a peristaltic pump (see Note 2).
14. Wash the resin with 16 mL of lysis buffer (flow rate $\sim 1\text{mL}/\text{min}$).
15. Load the supernatant on the resin (see Note 3).
16. Wash the column with 30 mL of lysis buffer.
17. Elute the GFP-D4 probe with 6 mL of elution buffer.
18. The buffer is then exchanged and the probe concentrated with an ultrafiltration unit: first, equilibrate the membrane of a centrifugal filter by adding 5 mL of phosphate buffer. Centrifuge for 10 min at $3,500 \times g$. Discard the liquid.
19. Add the GFP-D4 probe to the ultrafiltration unit. Spin at $4,500 \times g$ for 30 min in a fixed-angle rotor (see Note 4). Discard the flow-through.
20. Add 7 mL of PB buffer and spin at $4,500 \times g$ for 30 min (see Note 4). Discard the flow-through.
21. Repeat step 20 in section 3.1 two times.
22. Final spin at $4,500 \times g$ and stop when the probe is concentrated in ~ 1 mL. Recover the probe and measure its concentration with a spectrometer (molar extinction coefficient $\epsilon_{280\text{nm}}=65,320 \text{ M}^{-1}\cdot\text{cm}^{-1}$; Molecular weight: 42.1 kDa).
23. Add 1 mL of glycerol and store the probe at -20°C .

3.2 Plasma membrane cholesterol staining with the GFP-D4 probe, followed by confocal imaging

1. Day 0: Plate cells on a glass coverslip (see Note 5 and 6).
2. Day 1: Incubate with the GFP-D4 solution (1: 200 in PBS containing 1% BSA) for 30 min at 37°C (see Note 7 and Note 8).
3. Rapid wash with 1 mL of PBS at room temperature (see Note 9).
4. Fix the cells with 0.5 mL of PFA solution for 10 min at room temperature.
5. Wash with 1 mL of PBS for 5 min at room temperature.
6. Stain nuclei with the DNA marker Hoechst-33258 for 5 min.
7. Wash with 1 mL of PBS for 5 min at room temperature.
8. Mount the sample (see Note 10).
9. Acquire images with a confocal laser scanning microscope equipped with an Argon Laser (488 nm line for GFP excitation) and a 405nm diode laser (Hoechst-33258 excitation) (Fig. 1a).

3.3 Plasma membrane cholesterol staining with the GFP-D4 probe, followed by flow cytometry analysis

1. Day 0: Plate 600,000 cells in a P150 plate.
2. Day 1: Rinse the cells with PBS. Trypsinize the cells and resuspend them in 5 mL of PBS (see Notes 11 and 6).
3. Centrifuge for 5 min at $230 \times g$.
4. Resuspend the cells in 800 μL of PBS. Aliquot in 3 tubes (250 μL in each tube) to have a technical triplicate.
5. Add 250 μL of GFP-D4 (10 $\mu\text{g}/\text{mL}$) diluted in PBS containing 2% of BSA in each tube (see Notes 7 and 12).
6. Incubate for 30 min at 37°C under gentle agitation.
7. Fix the cells by adding 500 μL of PFA working solution. Mix gently. Incubate 10 min at room temperature (see Note 1).
8. Pellet the cells by centrifugation for 5 min at $230 \times g$.
9. Resuspend the cells in 1 mL of PBS.
10. Analyze GFP-D4 fluorescence on individual cells by flow cytometry.
11. Single cells are discriminated from doublets using forward scatter height (FSC-H) and forward scatter area (FSC-A) measures. FCS-A and FCS-H are plotted and cells along the diagonal are gated. GFP fluorescence is measured on individual cells using blue laser (488 nm) excitation and a FITC filter (band pass filter 530/30 nm). A minimum of 10,000 events must be counted.
12. Analyze the results with Flowjo software: Viable cells are manually gated using SSC (Side-scattered) and FSC (Forward-scattered) parameters. Data are represented as single-parameter histograms showing GFP fluorescence intensity (x-axis) and the number of events (y-axis). The mean of GFP fluorescence is also measured (Fig. 1c, d).

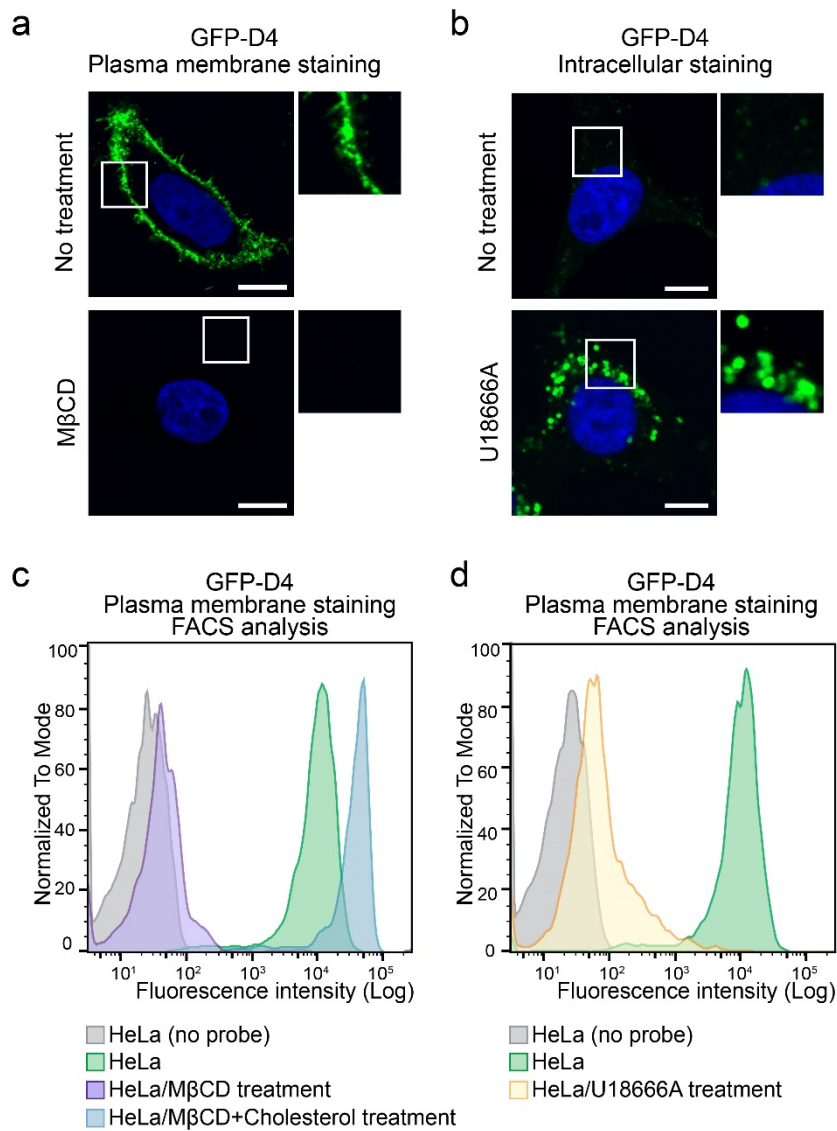


Figure 1: Plasma membrane and intracellular cholesterol staining with the GFP-D4 probe

(a) Plasma membrane cholesterol staining of HeLa cells with the GFP-D4 probe. Live cells were left untreated (top image) or treated with M β CD (10 mM in medium without serum for 30 min at 37°C; bottom image), then stained with the GFP-D4 probe and fixed. The nucleus staining is shown in blue. Scale bar: 10 μ m. (b) Intracellular cholesterol staining of HeLa cells with the GFP-D4 probe. Live cells were left untreated (top image) or treated with U18666A (2.5 μ g/mL in normal medium for 16 h at 37°C; bottom image), fixed, permeabilized, and then stained with the GFP-D4 probe. Nuclei staining is shown in blue. Scale bar: 10 μ m. In panel (a) and (b), a confocal section image and a higher magnification (2.5 \times) image of the area outlined in white are displayed. (c) Flow cytometry analysis of plasma membrane cholesterol staining with the GFP-D4 probe. Live HeLa cells were left untreated (green curve), or were treated with M β CD (10 mM for 30 min at 37°C; purple curve) or with M β CD complexed to cholesterol (500 μ M for 2 h at 37°C; blue curve). (d) Flow cytometry analysis of live HeLa cells which are untreated (green curve), or treated with U18666A (2.5 μ g/mL for 16 h at 37°C; yellow curve). After the different treatments, the cells were stained with the GFP-D4 probe, fixed and GFP-D4 staining intensity was analyzed by flow cytometry. These representative histograms display the number of cells analyzed (normalized to mode) as a function of GFP-D4 fluorescence (log intensity). Unstained cells (grey curve) are shown. Note that HeLa cells are strongly labeled with the GFP-D4 probe; M β CD and M β CD/cholesterol treatments prior to labeling strongly decrease and increase GFP-D4 signal intensity, respectively; U18666A treatment decreases GFP-D4 signal intensity. Indeed, U18666A was shown to induce cholesterol accumulation in the endocytic pathway at the expense of the plasma membrane.

3.4 Intracellular cholesterol labeling with the GFP-D4 probe

1. Day 0: Plate ~10,000–15,000 cells on a glass coverslip (see Note 5).
2. Day 1: Wash with 1 mL of PBS at room temperature for 2 s (see Note 13).
3. Fix the cells with 500 μ L of PFA solution at room temperature for 10 min (see Note 1).
4. Wash twice with 1 mL of PBS at room temperature (5 min). Keep the cells in PBS.
5. Permeabilize the cells using a liquid nitrogen bath: Take the coverslip out of the 24-well plate, remove the excess of PBS and put the coverslip in liquid nitrogen. Wait for 5 s. Place the coverslip back into the 24-well plate in PBS.
6. Incubate with 500 μ L of blocking solution at room temperature for 30 min, under gentle agitation.

7. Incubate with the GFP-D4 solution (1:200 in PBS containing 1% BSA) for 30 min at 37°C (see Notes 7 and 12).
8. Rapid wash with 1 mL of PBS at room temperature (see Note 9).
9. Fix the cells with 500 μ L of PFA solution for 10 min at room temperature (see Note 1).
10. Wash with 1 mL of PBS at room temperature for 5 min (see Note 14).
11. Stain nuclei with 1 mL of Hoechst-33258 solution.
12. Wash with 1 mL of PBS at room temperature for 5 min.
13. Mount the sample (see Note 10).
14. Acquire images with a confocal laser scanning microscope equipped with an Argon Laser (488 nm line for GFP excitation) and a 405nm diode laser (Hoechst-33258 excitation) (Fig. 1b).

3.5 Cholesterol labeling with filipin, followed by confocal imaging and image analysis

3.5.1 Labeling and image acquisition

1. Day 0: Plate \sim 10,000–15,000 cells on a glass coverslip (see Note 5).
2. Day 1: Wash twice with 1 mL of PBS at room temperature (2 s each).
3. In order to facilitate intracellular cholesterol visualization, remove plasma membrane cholesterol with M β CD: Dilute the M β CD stock 50 times with pre-heated culture medium without serum, to obtain a 10 mM M β CD concentration. Add 500 μ L of M β CD solution onto the cells, and incubate at 37°C for 30 min.
4. Wash twice with 1 mL of PBS at room temperature (2 min each).
5. Fix the cells with 500 μ L of PFA solution at room temperature for 10 min (see Note 1).
6. Wash with 1 mL of PBS at room temperature (5 min).
7. Incubate with 500 μ L of filipin solution at room temperature for 30 min, under gentle agitation (see Note 7).
8. Remove the filipin solution and wash cells with 1 mL of PBS at room temperature for 2 min (see Notes 15 and 16).

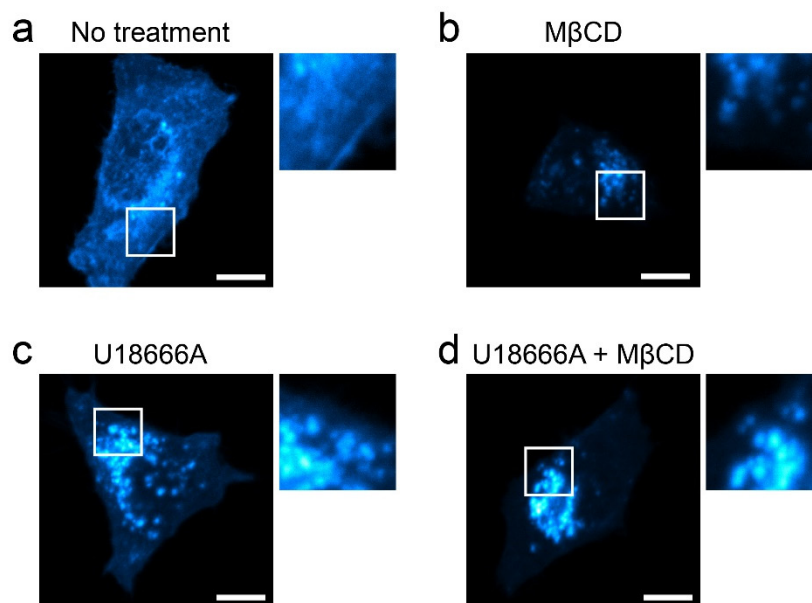


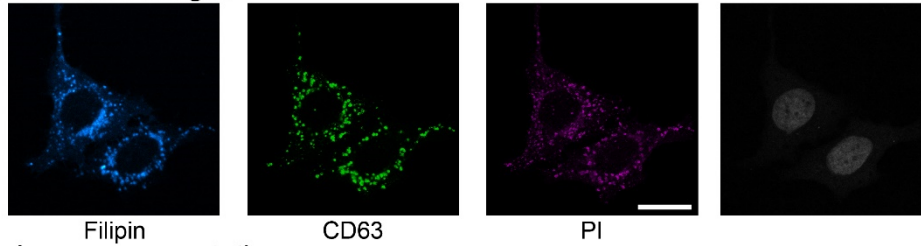
Figure 2: Whole-cell and intracellular cholesterol staining with filipin

(a). Live HeLa cells were fixed and stained with filipin. (b) Cells were treated with M β CD (10 mM in serum-free medium; 30 min at 37°C), before fixation and filipin staining. (c) Live HeLa cells were treated with U18666A to induce cholesterol accumulation in endosomes/lysosomes before fixation and filipin staining. (d) Cells were treated with U18666A and then with M β CD, before fixation and filipin staining. Each panel displays a confocal section image and a higher magnification (2.5 \times) image of the outlined in white. Scale bars: 10 μ m. Note that M β CD treatment, by removing cholesterol from the plasma membrane, improves the visualization of intracellular cholesterol pools.

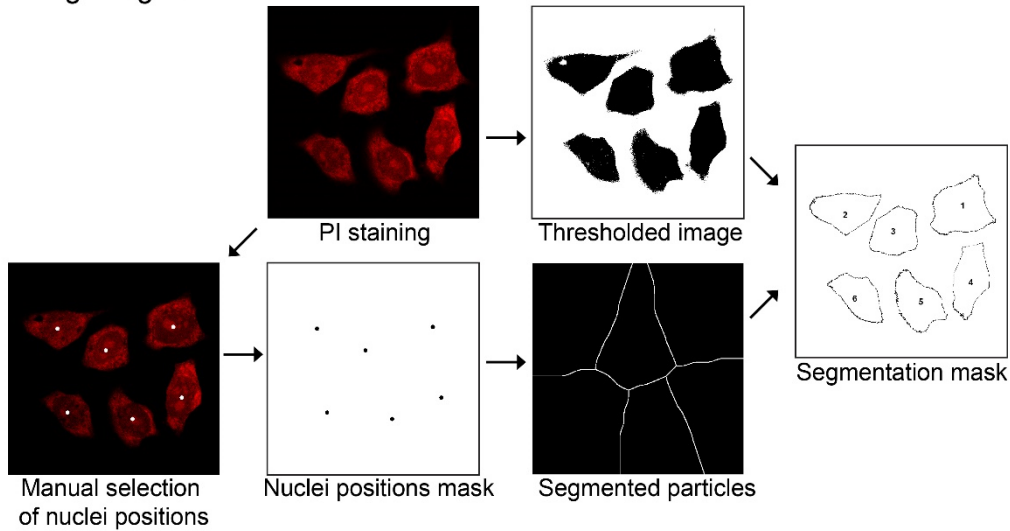
9. Cells are labeled with propidium iodide (PI) (0.35 μ g/mL in PBS) for 5 min, which labels nucleic acids (both DNA and RNA), and thus delineates the cell morphology, an important step for image analysis. Alternatively, cell nuclei are labeled with the DNA marker TO-PRO-3 iodide (0.35 μ g/mL in PBS) for 5 min.
10. Wash twice with 1 mL of PBS at room temperature (2 min each time).
11. Mount the sample (see Note 10).
12. Acquire images with a confocal laser scanning microscope equipped with a 355 nm optically pumped semiconductor laser to excite filipin. Acquisitions are made using the photon counting mode with a super-

sensitive detector to decrease light dosage and thus filipin photobleaching (Fig 2). Typically, a single optical section comprising multiple randomly chosen cells is acquired (Fig. 3a).

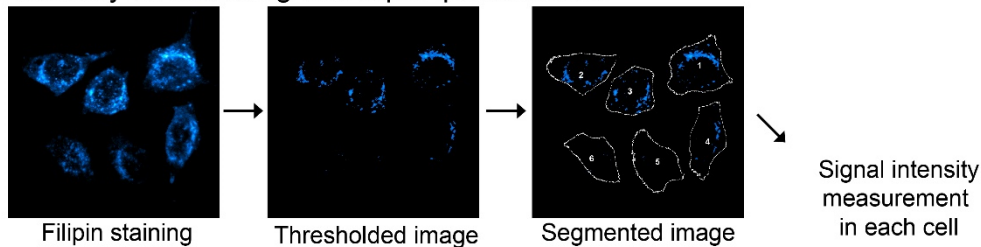
a Confocal images



b Image segmentation



c Intensity thresholding and filipin quantification



d CD63 mask and filipin quantification

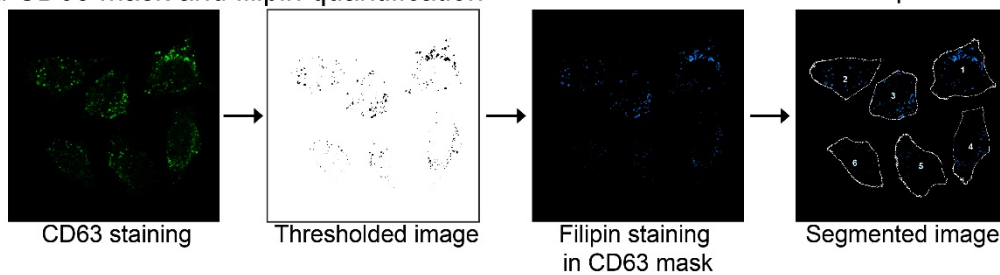


Figure 3: Filipin staining quantification by image analysis

(a) HeLa cells were stained with filipin (blue), anti-CD63 antibodies (green), and propidium iodide (PI, red). Images were acquired with a confocal microscope and analyzed with Fiji. Scale bar: 30 μm . (b) Image segmentation: First an intensity threshold is applied to determine the cell contours (Thresholded image). Nuclei positions (Nuclei positions mask), manually selected on the PI staining image (Manual selection of nuclei positions), are used to divide the image into discrete areas with a watershed algorithm (Segmented particles). Then, the thresholded and the segmented images are combined to generate a mask showing the contour of individual cells (Segmentation mask). (c) A threshold is applied on the filipin staining image to decrease the background signal and to focus the analysis on filipin bright spots corresponding to cholesterol accumulations. The segmentation mask is applied on this thresholded image, and filipin signal intensity is measured in each cell. (d) A threshold is applied on CD63 staining image to delineate late endosomes and lysosomes positive for this marker. The segmentation mask is applied on this thresholded image, and filipin signal intensity is measured in each cell.

3.5.2 Quantification of cholesterol in discrete subcellular structures

We use the Fiji software or CellProfiler to automate the analysis. The quantification of cholesterol accumulation in discrete subcellular structures is based on signal intensity thresholding

1. Individual cells are segmented on images using the PI staining (Fig. 3b). First an intensity threshold is applied to determine the cell contours. Nuclei positions, manually selected on the PI staining image, are used to divide the image into discrete areas with a watershed algorithm (Find maxima). Then, the thresholded and the segmented images are combined to build a segmentation mask showing the contour of individual cells.
2. Apply a threshold value to the filipin image (see Note 17).
3. Then, apply the segmentation mask and measure filipin staining intensity in individual cells (Fig. 3c).

3.5.3 Quantification of cholesterol in a specific subcellular compartment

The second method is based on the use of a mask to restrict the analysis to a specific cellular compartment (Fig. 3d; illustrated with a labeling of late endosomes/lysosomes with anti-CD63 antibodies).

1. Individual cells are segmented as described in step 1 in section 3.5.2
2. Apply an intensity threshold to the image of the subcellular compartment marker staining.
3. Generate a mask from this threshold.
4. Combine the threshold and the segmentation masks, and measure filipin staining intensity in individual cells.

4 Notes

1. Manipulate PFA under a fume hood. Wear protective gloves, protective clothing, and eye protection.
2. Alternatively, use an automated purification system with a pre-packed immobilized metal ion affinity chromatography (IMAC) column.
3. The green color of GFP facilitates the purification since the protein can easily be followed when bound to the resin and during elution.
4. Adjust the centrifugation time to the volume remaining on the filter which should not be lower than ~1 mL.
5. Under sterile condition, put a glass coverslip in each well of a 24-well plate. Add 500 μ L of pre-heated medium and the cell suspension (~10,000--15,000 cells). The confluency should be low (around 20--30%) to have isolated cells.
6. For cells which have a tendency to form clusters, trypsin EDTA can be used to detach and dissociate cells before the plating.
7. Protect the samples from light during all steps, once filipin or GFP-D4 has been added.
8. Optional: The cells can be pretreated with M β CD to remove cholesterol from the plasma membrane as negative control. Dilute the M β CD stock 50 times with pre-heated medium without serum, to obtain a 10 mM M β CD solution in culture medium. Wash the cells twice with PBS. Add 0.5 mL of M β CD solution onto the cells, and incubate at 37°C for 30 min. Remove the M β CD solution and wash the cells once with PBS.
9. This step allows the removal of the excess probe. Remove the probe solution, add 1 mL of PBS at room temperature, and immediately remove it. A longer incubation in PBS would enable the GFP-D4 probe to detach from the membrane before the fixation step.
10. Apply a drop of mounting medium on a glass slide. Take out the cover glass carefully from the well, remove the excess PBS, and carefully place over the mounting media with the cells face down. Incubate at least 2 h at room temperature before use. Slides can be store at 4°C protected from light.
11. On Day 1, positive and negative control samples can be obtained by adding or removing cholesterol from the plasma membrane before trypsinisation. Plasma membrane cholesterol levels can be reduced by M β CD or U18666A treatment, or increased by M β CD-cholesterol treatment. M β CD treatment: Dilute the M β CD stock 50 times with pre-heated medium without serum, to obtain a 10 mM M β CD solution in culture medium; wash the cells twice with PBS; add 5 mL of M β CD solution onto the cells and incubate at 37°C for 30 min. U18666A treatment: Wash the cells twice with PBS; treat the cells with U18666A (2.5 μ g/mL) in normal culture medium for 16 h at 37°C. M β CD-cholesterol treatment: Dilute the M β CD-cholesterol complex stock 160 times with pre-heated medium without , to obtain a 500 μ M M β CD-cholesterol complex solution in culture medium; wash the cells twice with PBS; add 5 mL of M β CD-cholesterol complex solution onto the cells, and incubate at 37°C for 2 h.
12. The concentration of GFP-D4 has to be adjusted to the samples analyzed. Increasing the concentration of GFP-D4 will increase the fluorescent signal proportionally.

13. On Day 1, positive control samples can be obtained by treating cells with U18666A to induce cholesterol accumulation in endosomes. Treat the cells with U18666A (2.5 µg/mL) in normal culture medium for 16 h at 37°C, and then wash with PBS.
14. At this step, an immunofluorescence staining can be done to detect the protein of interest in addition to cholesterol.
 - a) Incubate the cells with 500 µL of blocking solution at room temperature for 30 min, under gentle agitation.
 - b) Add appropriate primary antibody diluted in 250 µL of blocking solution, and incubate for 3 h at room temperature, or overnight at 4°C, under gentle agitation.
 - c) Wash 3 times with 1 mL of PBS (5 min each time).
 - d) Add fluorescently-conjugated secondary antibodies (Alexa Fluor 488), and incubate 1 h at room temperature, under gentle agitation.
 - e) Wash 3 times with 1 mL of PBS at room temperature (5 min each time).
15. At this step, an immunofluorescence staining can be done to detect the protein of interest in addition to cholesterol.
 - a) Incubate the cells with 500 µL of blocking solution at room temperature for 30 min, under gentle agitation.
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 - c) Wash 3 times with 1 mL of PBS (5 min each time).
 - d) Add fluorescently-conjugated secondary antibodies (Alexa Fluor 488), and incubate 1 h at room temperature, under gentle agitation.
 - e) Wash 3 times with 1 mL of PBS at room temperature (5 min each time).
 - f) Incubate the cells with 500 µL of filipin solution at room temperature for 30 min, under gentle agitation.
 - g) Wash twice with 1 mL of PBS at room temperature (5 min each time).
16. Since filipin permeabilizes membranes, it allows antibody entry inside cells. Note that only some antibodies work in the presence of filipin; this has to be tested for your specific application.
17. The choice of the threshold value is somehow arbitrary. However, in order to allow a comparison between experiments, the threshold can be determined in control cells (no cholesterol accumulation) as the 99th percentile of pixel intensity values. Evaluate this value for all images of control cells and calculate a mean intensity which will be defined as the threshold value.

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