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Methods for Visualizing and Quantifying Cholesterol Distribution in Mammalian Cells using Filipin and D4 Probes.

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Abstract:

Cholesterol is a key component of biological membranes and, like many cellular lipids, is unevenly distributed among organelles. Disruptions in cholesterol trafficking are associated with various pathologies, including lysosomal lipid storage disorders, often characterized by intracellular cholesterol accumulation. A significant challenge in studying cholesterol trafficking is the ability to trace this molecule *in situ*. Fluorescent probes that specifically bind cholesterol have enabled the visualization and imaging of cholesterol distribution within cells. This chapter details optimized methods for visualizing and quantifying free cholesterol at the plasma membrane and intracellular levels in cell populations and individual cells. These methods use two fluorescent probes: the D4 fragment of perfringolysin O fused to monomeric EGFP (mEGFP-D4 and the more sensitive mutant mEGFP-D4H) and the polyene macrolide Filipin. We describe robust methods for quantifying plasma membrane cholesterol by flow cytometry, and to visualize intracellular cholesterol pools. Furthermore, we introduce a refined Filipin staining protocol that enhances intracellular cholesterol detection. For precise quantification, we developed an automated image analysis pipeline. This chapter provides a comprehensive guide for the staining and quantification of cellular cholesterol, offering valuable tools for studying cholesterol dynamics in mammalian cells.

1 Introduction

Cholesterol is a crucial lipid in mammalian cells, serving as a major structural component of cell membranes and a precursor of essential compounds, such as steroid hormones, vitamin D, and bile acids [1, 2]. Sterol levels are tightly regulated at the subcellular level, resulting in an uneven distribution across different organelles. The plasma membrane contains high concentrations of sterols (40 mol%), intermediate levels are found in endosomes and the Golgi apparatus, and low levels are present in mitochondria and the endoplasmic reticulum (5 mol%) [3, 4]. To ensure the proper distribution of cholesterol within cells, two transport pathways exist: vesicular and non-vesicular transport, with the latter mostly occurring at inter-organelle contacts [5, 6]. A specific class of proteins, named Lipid Transfer Proteins (LTPs), plays a crucial role in non-vesicular sterol transport. LTPs are cytosolic or organelle-bound proteins with a hydrophobic pocket or channel, that accommodates one or several lipid molecules. These proteins can bind, transport, and deliver this lipid molecule within the cell [7–9]. The study of intracellular sterol transport faces the challenge of tracking this type of molecule *in situ*. Interestingly, the use of natural fluorescent sterols that closely mimic cholesterol, fluorophore-labeled sterols, or fluorescent probes have advanced our understanding of sterol transport [10, 11]. In recent years, new probes have been developed, such as GRAM-W, a probe derived from GRAM domain of GRAMD1b, which allows for the visualization of accessible cholesterol in cells [12], and the mushroom-derived protein, maistero-2 [13]. Additionally, other probes have been identified for detecting cholesterol in different membrane contexts; for instance, pleurotolysin 2 (PlyA2), nakanori and ostreolysin A (OlyA) are mushroom-derived proteins that bind to complexes of sphingomyelin, a major sphingolipid in mammalian cells, and cholesterol [14–16]. This chapter details the use of a series of cholesterol fluorescent probes, GFP-D4-derived probes and Filipin, to label and measure cholesterol level at the plasma membrane and inside cells. While these probes are useful tools, they have limitations [17, 18] making it essential to use them in appropriately calibrated experiments with suitable controls.

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 that, when secreted as a water-soluble monomer, recognizes and binds to cholesterol-rich membranes. It then oligomerizes to form pores. The D4 fragment of perfringolysin O retains the ability to bind cholesterol-rich membranes (>30 mol% cholesterol) but is devoid of pore-forming activity [19–22]. Derivatives of perfringolysin O have been employed to localize and quantify membrane cholesterol in cells in culture as well as in organs such as brain and liver [23]. In recent years, mutants of GFP-D4 that bind to membranes with lower cholesterol content have been developed [24–26]. One of these mutants, termed GFP-D4H (D4 with higher affinity), has a substitution of the aspartic residue at position 434 with a serine (D434S), which increases the probe's affinity for cholesterol (> 20 mol% cholesterol) [27]. This chapter describes two methodological indications for the use of recombinant mEGFP-D4 probes i) to stain and visualize intracellular and plasma membrane cholesterol in fixed cells, ii) to quantify plasma membrane cholesterol using flow cytometry.

Filipin is a polyene macrolide antibiotic extracted from the bacterium *Streptomyces filipinensis* [10, 28]. Filipin is naturally fluorescent (excitation at 355 nm, emission between 385–470 nm) and specifically binds to free sterols, but not to esterified sterols. As Filipin permeabilizes the cell membrane, it is not suitable for live cell experiments. Using Filipin to label and quantify intracellular cholesterol presents several technical challenges: first, the probe is very sensitive to photobleaching, complicating its use for quantification; second, it strongly labels the plasma membrane, which can obscure intracellular cholesterol signals and make them difficult to image. Thus, to bypass these technical limits, we set up a protocol, described in this chapter, which allows the imaging and quantification of intracellular cholesterol using Filipin [29]. To minimize Filipin photobleaching, we utilized a confocal microscope equipped with a 355 nm UV-laser and ultrasensitive detectors. In addition, to visualize intracellular cholesterol, before fixation and cholesterol labeling with Filipin, we treated live cells with methyl- β -cyclodextrin (M β CD), a molecule able to deplete cholesterol from the plasma membrane and thus eliminate the plasma membrane signal and allow the detection of intracellular cholesterol pools [30].

In the updated version of this methods chapter [31], we have, in particular, included the mEGFP-D4H mutant probe, which has increased sensitivity for cholesterol in intracellular membranes, and improved the image analysis pipeline.

2 Materials

2.1 Chemicals and buffers

1. U18666A [(3 β)-[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 working 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 100 mL of Filipin solution (0.5 mg/ml), first dissolve 50 mg of Filipin powder in 2 mL of dimethyl sulfoxide (DMSO). Then, add this solution dropwise to 100 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. Prepare fresh.
10. Cell culture medium with and without serum. Trypsin solution (~30 μ g/mL) and Trypsin-EDTA (~30 μ g/mL – 0.53 mM). Store at 4°C.
11. Digitonin stock solution (50 mg/mL): Dissolve 10 mg of Digitonin (D141; Sigma) in 20 mL of water. Store at -20°C.

2.2 Monomeric EGFP-D4 production

1. Monomeric EGFP-D4 (mEGFP-D4) and monomeric EGFP-D4H (mEGFP-D4H) expression plasmids (pET28 His6-mEGFP-D4 Addgene 226372 <https://www.addgene.org/226372/>; pET28 His6-mEGFP-D4H; Addgene 226373 <https://www.addgene.org/226373/>). These constructs encode the D4 [22] and the D4H [27] probes fused to monomeric EGFP (A206K mutant), respectively. Alternatively, plasmids encoding the D4 probe fused to EGFP (pET28/His6-EGFP-D4; RIKEN BioResource Research Center; RDB13961; <https://dna.brc.riken.jp/en/>) or to mCherry (pET28/His6-mCherry-D4; RIKEN BioResource Research Center; RDB14300) can be used [16, 19]. These plasmids can be amplified in *E. coli* DH5 α bacteria.
2. *E. coli* BL21 (DE3) competent bacteria.
3. LB broth, Auto inducible medium (Terrific Broth Base including Trace elements, Formedium, AIMTB0260), and LB agar plates containing 40 μ g/mL kanamycin.
4. Conical flasks.
5. Lysis buffer: 50 mM NaH₂PO₄/Na₂HPO₄, 150 mM NaCl, 35 mM Imidazole, pH 8. Add 58.25 mL of Na₂HPO₄ (0.2 M), 4.25 mL of NaH₂PO₄ (0.2 M), 7.5 mL of NaCl (5 M) and 8.75 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.
6. 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.
7. Phosphate buffer (PB): 20 mM NaH₂PO₄/Na₂HPO₄, 150 mM NaCl, pH 7.4. Add 7.74 mL of Na₂HPO₄ (0.2 M), 2.26 mL of NaH₂PO₄ (0.2 M) and 3 mL of NaCl (5 M) to a graduated cylinder. Add water to reach a total volume of 100 mL, mix well, and store at 4°C.

8. Vacuum filtration holder (500 mL) for 47 mm membranes, compatible with a 45 mm neck ISO flask.
9. Filter of 47 mm diameter with 0.45 μm pore size (Millipore Express PLUS Membrane Filter hydrophilic polyethersulfone).
10. Ultrafiltration units (Ultra-15 Amicon, MWCO 30 kDa, Millipore).
11. Glycerol (molecular biology grade).
12. Cell Disruptor TS SERIES (Constant Systems Ltd.).
13. Affinity chromatography column (HisPur™ Ni-NTA Chromatography Cartridge, ThermoFisher Scientific) or similar.
14. ÄKTA Start protein purification system (GE Healthcare Life Sciences) or similar.
15. Spectrophotometer (Nanodrop, Thermo Scientific).

2.3 Flow cytometry and Imaging

1. High Precision round cover glasses (diameter 12 mm, N°. 1.5H; 0117520; Marienfield).
2. Superfrost Plus Adhesion microscope slides (J1800AMNZ; EpreDia).
3. 24-well culture plates (Corning).
4. P100 culture dishes (Corning).
5. Alexa Fluor 555- and Alexa Fluor 647-Phalloidin (ThermoFisher Scientific).
6. Anti-LAMP1 mouse monoclonal antibody H4A3 (Developmental Studies Hybridoma Bank (DSHB), deposited to the DSHB by August, J.T. / Hildreth, J.E.K.; RRID:AB_2296838).
7. Donkey anti-mouse secondary antibody Alexa Fluor 555 (RRID: AB_2762848) or Alexa Fluor 647 (RRID: AB_162542).
8. 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 $\mu\text{g/mL}$.
9. SiR-DNA solution (Spirochrome). Prepare a stock solution of the dye at 1 mM in anhydrous DMSO.
10. Mounting medium (ProLong Gold, Invitrogen).
11. 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).
12. Spinning-disk confocal microscope (CSU-X1; Nikon, 100 \times , NA 1.4).
13. Image analysis software: Fiji software (<http://fiji.sc/>) and CellProfiler (<http://cellprofiler.org/>).
14. Flow cytometer (BD FACS Celesta) or similar.
15. Flowjo software (FlowJo LLC) or similar.

3 Methods

3.1 mEGFP-D4 probes production and purification

1. Transform competent *E. coli* BL21 (DE3) cells with the pET28 His6-mEGFP-D4 or the pET28 His6-mEGFP-D4H vector according to the manufacturer's instructions.
2. Plate the transformed bacteria on LB agar plates with kanamycin and incubate overnight at 37°C.
3. Inoculate one colony into 10 mL of LB broth containing kanamycin and incubate for 16 h at 37°C under agitation.
4. Transfer the overnight culture to a 5 L conical flask containing 1 L of auto-inducible terrific broth with kanamycin.
5. Incubate at 37°C with agitation. Measure the optical density ($\text{OD}_{600\text{nm}}$) at 600 nm with a spectrophotometer. Once the $\text{OD}_{600\text{nm}}$ reaches 0.4-0.6, lower the temperature to 18°C.
6. Incubate under agitation for 16 h at 18°C.
7. Transfer the culture medium to centrifugation tubes. Pellet the cells by centrifugation at 3,500 \times g for 15 min.
8. Discard the supernatant. Resuspend the cell pellet in 100 mL of lysis buffer.

9. Sonicate the suspension for 10 min (40% amplitude, on/off cycles 10s/20s) on ice under constant stirring.
11. Centrifuge at $3,500 \times g$ for 15 min at 4°C . Keep the supernatant.
12. Centrifuge at $50,000 \times g$ for 30 min at 4°C . Keep the supernatant.
13. Pass the lysate through a $0.45 \mu\text{m}$ pore filter.
13. Assemble the affinity chromatography column onto the protein purification system (*see Note 2*).
14. Run an affinity chromatography program as follows: i) equilibrate the column with 20 mL of lysis buffer; ii) load the lysate onto the column; iii) wash the column with 30 mL of lysis buffer; and iv) elute the mEGFP-D4 probe with 6 mL of elution buffer, collecting 1 mL fractions (*see Note 3*).
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 PB. Centrifuge for 10 min at $3,500 \times g$. Discard the liquid.
19. Add the mEGFP-D4 probe to the ultrafiltration unit. Spin at $3,500 \times g$ for 10 min in a fixed-angle rotor (*see Note 4*). Discard the flow-through.
20. Add 7 mL of PB buffer and spin at $3,500 \times g$ for 10 min (*see Note 4*). Discard the flow-through, , leave 2 mL in the upper chamber. Repeat this step twice to ensure proper buffer exchange.
21. Finally, spin at $3,500 \times g$ until the volume in the upper chamber reaches ~ 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).
22. Add 1 mL of glycerol and store the probe at -20°C .

3.2 Plasma membrane cholesterol staining with the mEGFP-D4 probes, followed by confocal imaging

1. Day 0: Plate 20,000 cells on a glass coverslip (*see Note 5 and 6*).
2. Day 1: Incubate with 280 μL mEGFP-D4 solution (100 $\mu\text{g}/\text{mL}$ in blocking solution) for 30 min at 37°C (*see Note 7 and Note 8*).
3. Rapid wash with 1 mL of PBS 1X at room temperature (*see Note 9*).
4. Fix the cells with 300 μL of PFA working solution for 20 min at room temperature.
5. Wash with 1 mL of PBS 1X 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 1X for 5 min at room temperature.
8. Mount cover slip on a microscopy slide (*see Note 10*).
9. Image the cells using a Spinning-disk confocal microscope (CSU-X1; Nikon, 100 \times , NA 1.4; Gataca systems) equipped with Lasers exciting the following wavelengths: 405 nm line for nuclei labelling, 488 nm line for GFP excitation. (Fig. 1a, b).

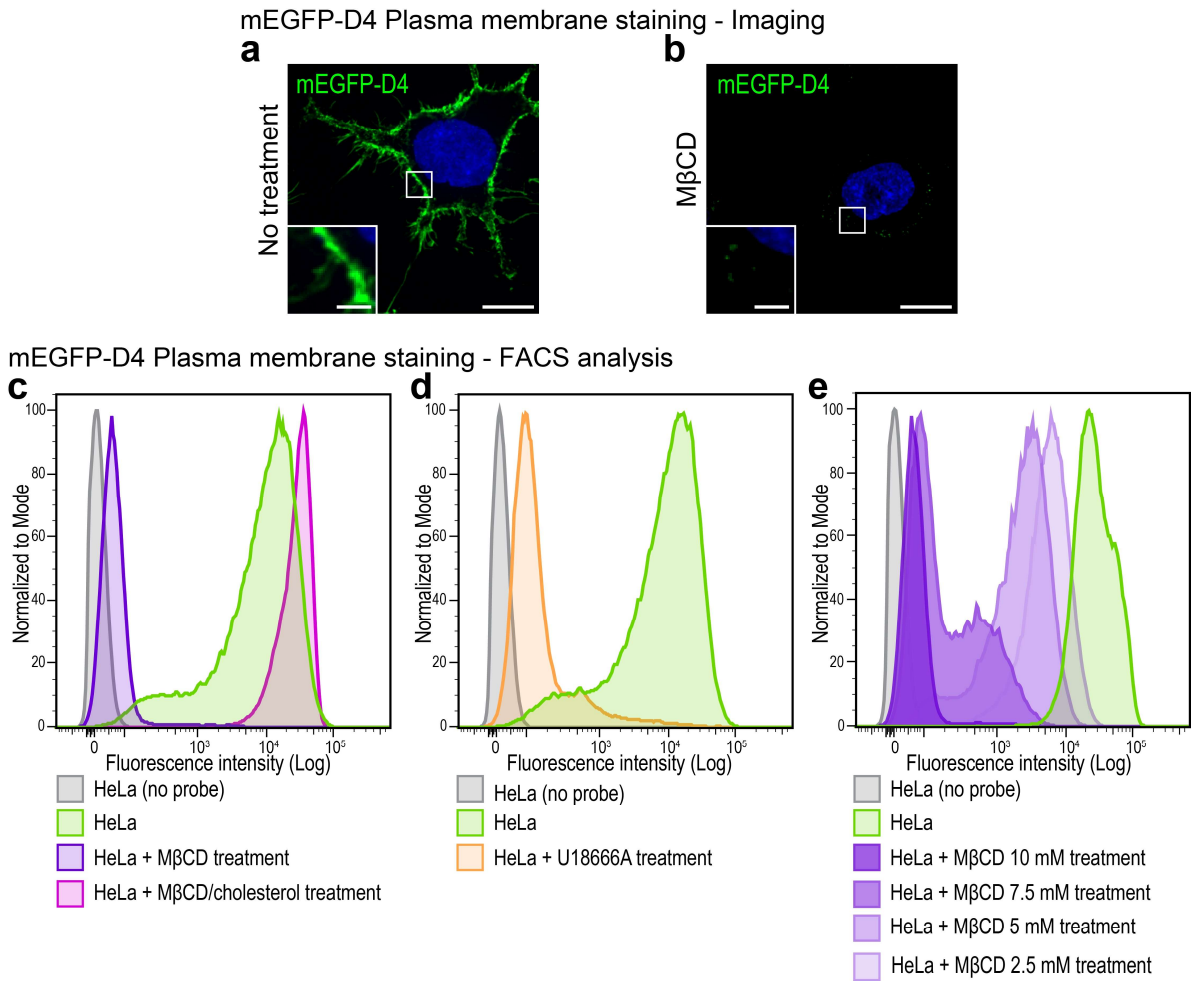


Fig. 1: Plasma membrane cholesterol staining with the mEGFP-D4 probe

(a, b) Plasma membrane cholesterol staining of HeLa cells using the mEGFP-D4 probe. Live cells were either left untreated (a) or treated with MβCD (10 mM in serum-free medium for 15 min at 37°C) (b), followed by staining with the mEGFP-D4 probe (green) and fixation. The nucleus staining is shown in blue. Scale bars: 10 μm. The inset shows a magnified view of the indicated area; scale bars: 2 μm.

(c-e) Flow cytometry analysis of plasma membrane cholesterol staining with the mEGFP-D4 probe. Live HeLa cells were left untreated (green curve), or treated with MβCD (10 mM for 15 min at 37°C; purple curve), MβCD complexed with cholesterol (500 μM for 2 h at 37°C; pink curve) (c), U18666A (2.5 μg/mL for 16 h at 37°C; orange curve) (d), or various concentrations of MβCD (10, 7.5, 5, and 2.5 mM for 15 min at 37°C; curves in shades of purple) (e). After treatment, the cells were stained with the mEGFP-D4 probe, fixed and mEGFP-D4 staining intensity was analyzed by flow cytometry. These representative histograms display the number of cells analyzed (normalized to mode) as a function of mEGFP-D4 fluorescence (log intensity). Unstained cells (gray curve) are also shown. Note that HeLa cells exhibit strong labeling with the mEGFP-D4 probe; MβCD and MβCD/cholesterol treatments prior to labeling strongly decrease and increase mEGFP-D4 signal intensity, respectively; U18666A treatment reduces mEGFP-D4 staining intensity. U18666A is known to induce cholesterol accumulation in the endocytic pathway at the expense of the plasma membrane; the mEGFP-D4 probe is sensitive to the cholesterol concentration gradient induced by different concentrations of MβCD.

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

1. Day 0: Plate 1,200,000 cells in a P100 dish.
2. Day 1: Rinse the cells with 1 mL Trypsin. Trypsinize the cells with 2 mL and resuspend them in 5 mL of culture medium (see Notes 6 and 11).
3. Centrifuge for 5 min at $230 \times g$.
4. Resuspend the cells in 800 μL of PBS 1X. Aliquot in 3 tubes (250 μL in each tube) to have a technical triplicate.
5. Add 250 μL of mEGFP-D4 (1:100 or ~200 μg/mL) diluted in PBS 1X containing 2% of BSA in each tube (see Notes 7 and 11).
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 low speed centrifugation for 5 min at $230 \times g$.
9. Resuspend the cells in 1 mL of PBS 1X.
10. Analyze mEGFP-D4 fluorescence on individual cells by flow cytometry.
11. Single cells are distinguished from doublets using forward scatter height (FSC-H) and forward scatter area (FSC-A) measurements. FCS-A and FCS-H are plotted, and cells along the diagonal are gated. GFP fluorescence is measured on individual cells using a 488 nm excitation wavelength laser and a FITC filter (bandpass filter 530/30 nm). A minimum of 100,000 events should be recorded.
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 with modal normalization (y-axis). The mean of GFP fluorescence is also measured (Fig. 1c-e).

3.4 Intracellular cholesterol labeling with the mEGFP-D4 probes

1. Day 0: Plate 20,000 cells on a glass coverslip in a well of a 24-well plate (*see Note 5*).
2. Day 1: Fix the cells with 300 μL of PFA working solution at room temperature for 20 min (*see Note 1*).
4. Wash twice with 1 mL of PBS 1X at room temperature (5 min). Add 1 mL PBS 1X.
5. Permeabilize the cells using a liquid nitrogen bath: Take the coverslip out of the 24-well plate with a tweezer, remove the excess of liquid on a tissue and maintain the coverslip in liquid nitrogen for 3 seconds or until no more bubbles are produced. Place the coverslip back into the 24-well plate with the cells facing up in PBS 1X.
6. Incubate with 300 μL of blocking solution at room temperature for 30 min, under gentle agitation.
7. Incubate with the mEGFP-D4 probe (100 $\mu\text{g}/\text{mL}$ in 300 μL of blocking solution) for 1 h at room temperature (*see Notes 7*).
8. Rinse the cells with 1 mL of PBS 1X at room temperature for a few seconds (*see Note 9*).
9. Fix the cells a second time with 300 μL of PFA working solution for 10 min at room temperature (*see Note 1*). This step is crucial to ensure that the probe remains bound to the sample.
10. Wash with 1 mL of PBS 1X at room temperature for 5 min.
11. Permeabilize the cells a second time with Digitonin (50 $\mu\text{g}/\text{mL}$ in 300 μL of PBS 1X) for 10 min at room temperature. This step is necessary for effective immunolabeling with certain antibodies such as anti-LAMP1 antibodies.
12. Wash twice with 1 mL of PBS 1X at room temperature (5 min).
13. Incubate with 300 μL of blocking solution at room temperature for 10 min, under gentle agitation.
14. Incubate with 250 μL of anti LAMP1 antibody (1:100 in blocking solution) at room temperature for 3h, under gentle agitation (*see Note 14*).
15. Wash twice with 1 mL of PBS 1X at room temperature (5 min).
16. Incubate with 250 μL of secondary antibody coupled to AlexaFluor 555 (1:1000), AlexaFluor 647 phalloidin (1:1000) and Hoechst-33258 in PBS 1X at room temperature for 30 min, under gentle agitation.
17. Wash twice with 1 mL of PBS 1X at room temperature for 5 min.
18. Mount the sample (*see Note 10*).
19. Acquire images with a Spinning-disk confocal microscope (CSU-X1; Nikon, 100 \times , NA 1.4; Gataca systems) equipped with Lasers exciting the following wavelengths: 405nm line for nuclei labelling, 488 nm line for GFP excitation, 555 nm line for LAMP1 labelling, 647 nm for phalloidin labelling.

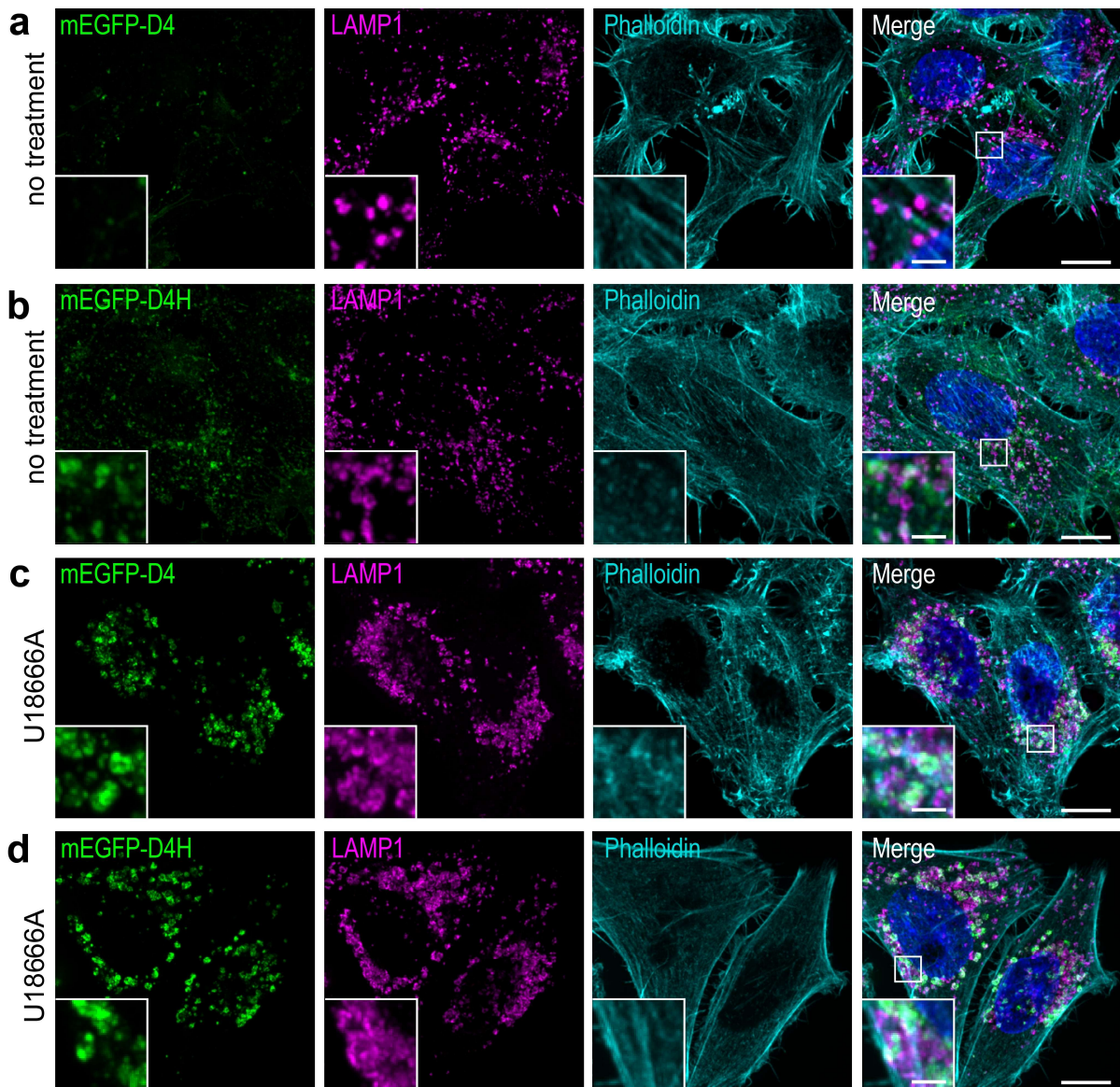


Fig. 2: Intracellular cholesterol staining with the mEGFP-D4 probe

(a-d). Live HeLa cells were left untreated (a, b), or treated with U18666A (2.5 $\mu\text{g}/\text{mL}$ in normal medium for 16 h at 37°C) to induce cholesterol accumulation in late endosomes/lysosomes (c, d), before fixation and staining with the mEGFP-D4 (a, c) and the mEGFP-D4H (b, d) probe. Late endosomes/lysosomes were labeled with anti-LAMP1 antibodies (magenta). Whole-cell staining was performed using phalloidin (cyan), and nuclei were stained with Hoechst-33258 (blue). Scale bars: 10 μm . The inset shows a magnified view of the indicated area, Scale bars: 2 μm .

Note that the mEGFP-D4H probe stained more intracellular structures than the mEGFP-D4 probe, showing that it detects intracellular cholesterol pools with greater sensitivity. Both mEGFP-D4 and mEGFP-D4H similarly stained the outer leaflet of the plasma membrane, as visualized by microscopy or flow cytometry (data not shown).

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

3.5.1 Labeling and image acquisition

1. Day 0: Plate 20,000 cells on a glass coverslip (*see Note 5*).
2. Day 1: Wash twice with 1 mL of pre-heated culture medium without serum 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 (1:50) with pre-heated culture medium without serum, to obtain a 10 mM M β CD concentration. Add 300 μ L of M β CD solution onto the cells, and incubate at 37°C for 10-15 min.
4. Fix the cells with 300 μ L of PFA working solution at room temperature for 20 min (*see Note 1*).
5. Wash with 1 mL of PBS 1X at room temperature (5 min).
6. Incubate with 300 μ L of Filipin solution at room temperature for 30 min, under gentle agitation (*see Note 7*).
7. Remove the Filipin solution (it is reusable) and wash cells with 1 mL of PBS 1X at room temperature for 2 min (*see Note 12*).
8. Incubate with 300 μ L of blocking solution at room temperature for 30 min, under gentle agitation.
9. Incubate with 250 μ L of anti LAMP1 antibody (1:100, in blocking solution) at room temperature for 3h, under gentle agitation.
10. Wash twice with 1 mL of PBS 1X at room temperature (5 min).
11. Incubate with 250 μ L of secondary antibody coupled to AlexaFluor 488 (1:1000), AlexaFluor 555 phalloidin (1:1000) and Sir-DNA (Dilute the dye 1000 times to a final concentration of 500 nM) in PBS 1X at room temperature for 30 min, under gentle agitation.
12. Wash twice with 1 mL of PBS 1X at room temperature (5 min).
13. Incubate the cells with 300 μ L of Filipin solution at room temperature for 30 min, under gentle agitation.
14. Remove the Filipin solution and wash three times with 1 mL of PBS 1X at room temperature.
15. Mount the sample (*see Note 10*).
16. Acquire images with a confocal laser scanning microscope equipped with a 355 nm optically pumped semiconductor laser to excite Filipin. Images are acquired with a super-sensitive detector to minimize light exposure and reduce Filipin photobleaching (Fig 3). Typically, a single optical section containing several randomly chosen cells is acquired (Fig. 4a).

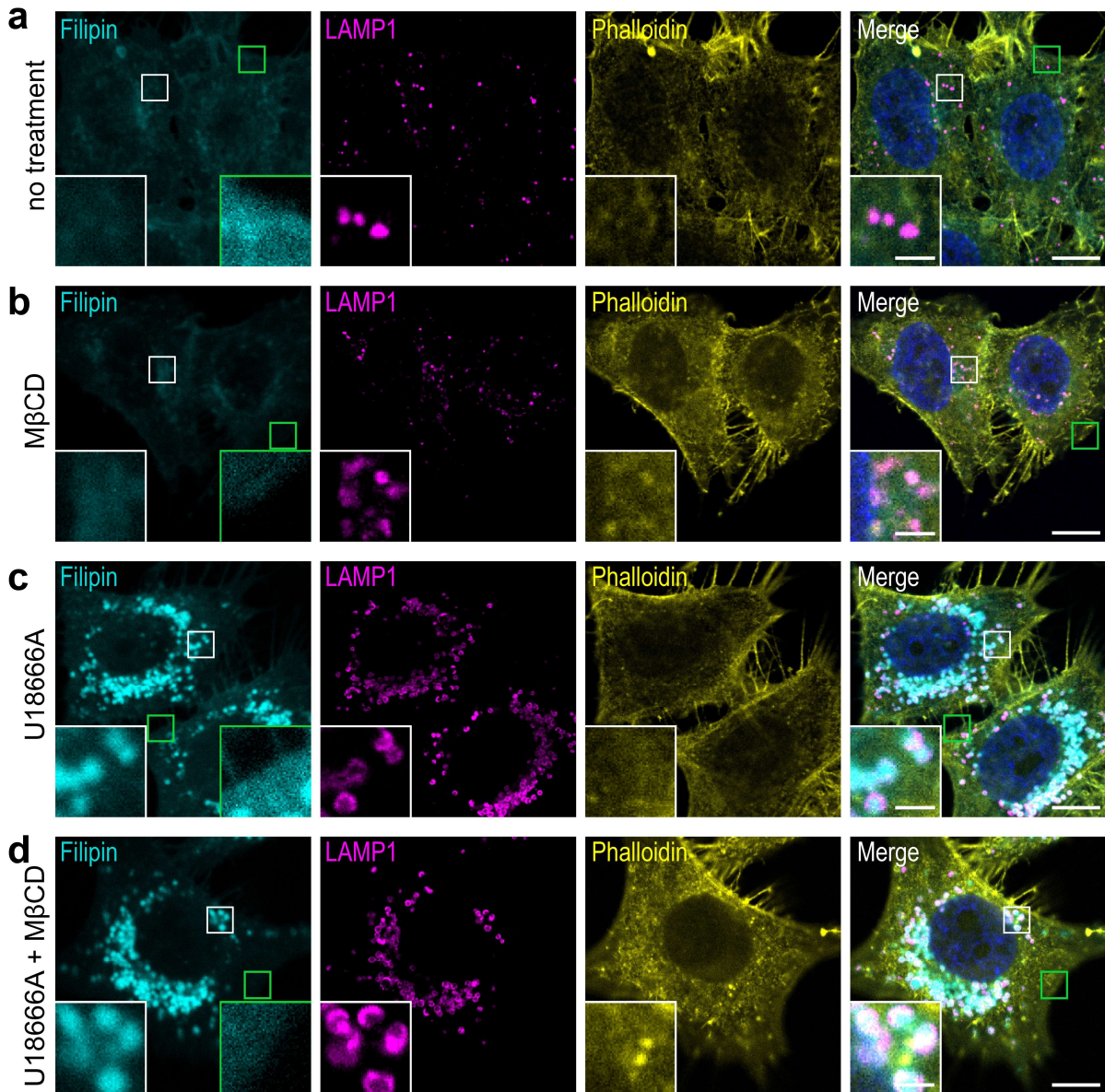


Fig. 3: Whole-cell and intracellular cholesterol staining with Filipin

(a-d). Live HeLa cells were left untreated (a), treated with M β CD (10 mM in serum-free medium; 15 min at 37°C) (b), treated with U18666A (2.5 μ g/mL in normal medium for 16 h at 37°C) to induce cholesterol accumulation in late endosomes/lysosomes (c), and treated with U18666A followed by M β CD (d), before fixation and Filipin staining (cyan). Late endosomes/lysosomes were labeled with anti-LAMP1 antibodies (magenta), the actin cytoskeleton was stained with fluorescent phalloidin (yellow), and nuclei with Sir-DNA (blue).

Scale bars: 10 μ m. The insets show magnified views of the indicated areas, Scale bars: 2 μ m. The signal intensity of Filipin in the green-highlighted inset has been increased to facilitate the visualization of the M β CD effect.

Note that M β CD treatment, by removing cholesterol from the plasma membrane, improves the visualization of intracellular cholesterol pools. See also [29]

3.5.2 Quantification of cholesterol in a specific subcellular compartment

We use CellProfiler software to automate the analysis. Filipin staining quantification is achieved using a mask to restrict the analysis on specific cellular compartments, as illustrated in Figure 4, where late endosomes/lysosomes (LE/Lys) are labeled with anti-LAMP1 antibodies. The CellProfiler pipeline is available on GitHub <https://github.com/JulieEich/Filipin-quantification>, along with a set of sample images for testing.

1. Individual cells are segmented by combining information from nuclei position and cell contours obtained from Phalloidin images (Fig. 4a, b).
2. Late endosomes/lysosomes are segmented from LAMP1 images using StarDist, which was trained on a custom dataset [32].

3. The segmentation masks are then applied to the Filipin staining image
4. Perform multi-parameter measurements, including the number of Late Endosomes/Lysosomes, Filipin intensity per cell and per LE/lys, and other relevant metrics.

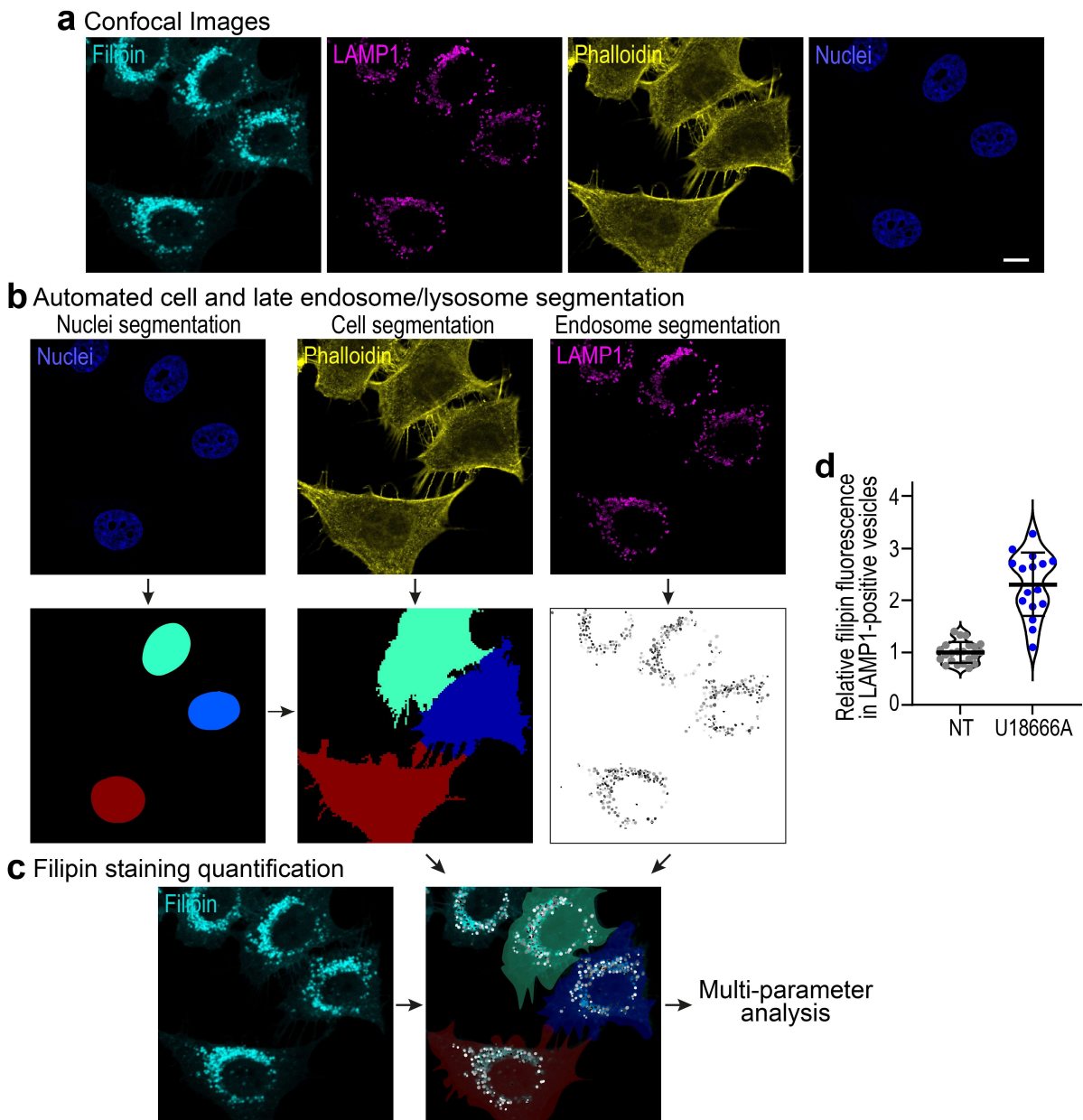


Fig. 4: Quantification of Filipin staining by image analysis

(a) HeLa cells treated with U18666A were labelled with Filipin (cyan), anti-LAMP1 antibodies (magenta), fluorescent phalloidin (yellow) and SiR-DNA (blue). Images were acquired with a confocal microscope and analyzed with CellProfiler. Scale bar: 10 μm .

(b) Image segmentation: cell positions and contours were determined by combining the segmentation of nuclei and phalloidin images. LE/Lys were segmented based from LAMP1 images using StarDist, which was trained on a custom dataset [32].

(c) Filipin staining quantification: using the segmentation data of cells and LE/Lys, a multi-parameter analysis was conducted to measure Filipin intensity in individual objects.

(d) Example of Filipin intensity quantification: Filipin intensity was measured in LAMP1-positive objects in cells that were either untreated or treated with U18666A. Each data point represents the mean Filipin intensity in LAMP1-positive vesicles within individual cells. Means and standard deviations (SD) are shown as black bars. Note that Filipin intensity in LE/Lys is highly increased in U18666A-treated cells compared to non-treated cells.

4 Notes

1. Manipulate PFA under a fume hood. Wear protective gloves, protective clothing, and eye protection.
2. Alternatively, use a manual purification system with a chromatography column (Bio-Rad Poly-Prep), His-Select Nickel Affinity Gel (Sigma), and a peristaltic pump.
3. The green color of GFP facilitates the purification, as the protein can easily be tracked when bound to the resin and during elution.
4. Adjust the centrifugation time based on the volume remaining on the filter, ensuring it is not less than ~1 mL.
5. Under sterile condition, place a glass coverslip in each well of a 24-well plate. Add 1 mL of pre-heated medium and the cell suspension (adjusting the number of cells according to the cell type).
6. For cells that tend to form clusters, trypsin-EDTA can be used to detach and dissociate the cells before plating. In this case, remove EDTA by centrifugation, and resuspend the cells in an EDTA-free medium before plating.
7. Protect the samples from light during all steps after adding Filipin or mEGFP-D4.
8. Optional: The cells can be pretreated with M β CD to remove cholesterol from the plasma membrane as a 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, and incubate at 37°C for 15 min. Remove the M β CD solution and wash the cells once with PBS.
9. This step removes the excess probe. Remove the probe solution, add 1 mL of PBS 1X at room temperature, and immediately remove it. A longer incubation in PBS could cause the mEGFP-D4 probe to detach from the membrane before the fixation step.
10. Apply a drop of mounting medium to a glass slide. Carefully remove the coverslip from the well, remove the excess PBS, and gently place it over the mounting medium with the cells face down. Incubate at least overnight at room temperature in the dark before use. Slides can be stored at 4°C, protected from light.
11. Adjust the concentration of mEGFP-D4 according to the samples analyzed. Increasing the concentration of mEGFP-D4 will proportionally increase the fluorescent signal.
12. As Filipin permeabilizes membranes, it allows antibody entry inside cells. Note that only some antibodies work in the presence of Filipin; this must be tested for your specific application.

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