Chapter 7

Measuring and Manipulating Membrane Cholesterol for the Study of Hedgehog Signaling

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Abstract

Cholesterol is an abundant lipid in mammalian plasma membranes that regulates the reception of the Hedgehog (Hh) signal in target cells. In vertebrates, cell-surface organelles called primary cilia function as compartments for the propagation of Hh signals. Recent structural, biochemical, and cell-biological studies have led to the model that Patched-1 (PTCH1), the receptor for Hh ligands, uses its transporter-like activity to lower cholesterol accessibility in the membrane surrounding primary cilia. Cholesterol restriction at cilia may represent the long-sought-after mechanism by which PTCH1 inhibits Smoothened (SMO), a cholesterol-responsive transmembrane protein of the G protein-coupled receptor superfamily that transmits the Hh signal across the membrane.

Protein probes based on microbial cholesterol-binding proteins revealed that PTCH1 controls only a subset of the total cholesterol molecules, a biochemically defined fraction called *accessible cholesterol*. The accessible cholesterol pool coexists (and exchanges) with a pool of *sequestered cholesterol*, which is bound to phospholipids like sphingomyelin. In this chapter, we describe how to measure the accessible and sequestered cholesterol pools in live cells with protein-based probes. We discuss how to purify and fluorescently label these probes for use in flow cytometry and microscopy-based measurements of the cholesterol pools. Additionally, we describe how to modulate accessible cholesterol levels to determine if this pool regulates Hh signaling (or any other cellular process of interest).

Key words Cholesterol, Sphingomyelin, Hedgehog signaling, Ostreolysin A, Anthrolysin O, Perfringolysin O, Primary cilia, Smoothened, Patched-1, Signal transduction, Lipids

1 Introduction

Cholesterol is a second messenger that controls the activity of the G protein-coupled receptor Smoothened (SMO), the protein that transmits the Hedgehog (Hh) signal across the membrane [1–4]. SMO activity is controlled by Patched-1 (PTCH1), a putative sterol

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transporter. Current data supports a model whereby PTCH1 prevents SMO activation by limiting its access to cholesterol, keeping the Hh pathway off. Signaling is initiated when secreted Hh ligands bind to and antagonize PTCH1, allowing cholesterol levels to rise and then bind to SMO, resulting in receptor activation [5, 6].

We have recently proposed the concept that PTCH1 regulates SMO by controlling accessible cholesterol—a biochemically and thermodynamically distinct pool of cholesterol [6]. Accessible cholesterol is the fraction of cholesterol molecules that have high chemical activity and can interact with proteins to control cell signaling events [7]. Accessible cholesterol can interconvert with a pool of cholesterol called sequestered cholesterol, which has lower chemical activity due to its interaction with phospholipids like sphingomyelin (SM) [8–10]. Recent development of protein-based probes has enabled quantification and visualization of the accessible and SM-sequestered cholesterol pools in live cells [11–16]. Accessible cholesterol can be detected by bacterial toxins such as Perfringolysin O (PFO) or domain 4 of Anthrolysin O (ALOD4) [17–21]. SM-sequestered cholesterol can be detected by Ostreolysin A (OlyA), a fungal toxin that binds SM/cholesterol complexes [22, 23]. OlyA(E69A) is a useful point mutant of OlyA that can bind SM free from cholesterol in addition to SM complexed to cholesterol, thus enabling visualization of total SM [24].

Using these tools, we showed that the primary cilia, the organelle where PTCH1 controls SMO activity, is depleted of accessible cholesterol compared to the plasma membrane [6]. Inhibition of PTCH1 with Hh ligands causes an increase in accessible cholesterol specifically in the ciliary membrane. Previously, the reason why the vertebrate Hh pathway has evolved to require primary cilia was unknown. We propose that the membrane of this organelle provides a cholesterol-scarce environment where PTCH1 can use cholesterol as a second messenger to regulate SMO without disrupting other cholesterol-dependent processes in cells. In summary, two properties of cholesterol enable it to act as a second messenger for the Hh pathway: first, only a fraction of cholesterol molecules are biochemically active, and second, its levels in primary cilia are low enough to be controlled by a cholesterol transporter.

In this chapter, we discuss methods to manipulate and visualize the lipids that control the Hh signaling pathway. We describe how to purify and label the accessible cholesterol-binding toxin ALOD4, the SM/cholesterol complex-binding toxin OlyA, as well as the total SM-binding toxin OlyA(E69A). We discuss how to deliver cholesterol and cholesterol-binding proteins to live cells in order to visualize and control accessible cholesterol levels and modulate Hh signaling output. Finally, we describe how to measure outer leaflet accessible cholesterol and SM at primary cilia, the organelle required for Hh signal transduction. These methods can

be applied beyond Hh signaling to interrogate the roles of these lipid pools in any cellular process of interest.

2 Materials

2.1 Materials for Expression and Purification

1. Expression plasmids.
   (a) pOlyA-His$_6$ in the pET21C+ expression vector, encodes Ostreolysin A (OlyA) (aa 1–138) from *Pleurotus ostreatus* along with a COOH-terminal His$_6$ epitope tag. The two native cysteines in OlyA (C62 and C94) were mutated to serine and a new cysteine was introduced near the COOH-terminus (S151C) to allow for maleimide labeling at a position far from OlyA's binding site for sphingomyelin/cholesterol complexes [24].
   (b) pOlyA(E69A)-His$_6$ is the same as pOlyA-His$_6$ described above except that it contains a mutation (E69A) which abolishes the cholesterol requirement for OlyA binding and allows binding to sphingomyelin in complexes with cholesterol as well as free sphingomyelin [24].
   (c) pHis$_6$-ALOD4, in the pRSET B expression vector, encodes the cholesterol-binding domain (domain 4) of Anthrolysin O (ALO) from *Bacillus anthracis* (aa 404–512) with a NH_2-terminal His$_6$ epitope tag. The sole native cysteine in ALOD4 (C472) was mutated to alanine and S404 was mutated to cysteine to allow for maleimide labeling at a position far from the cholesterol-binding site in ALOD4 [19].
   (d) pHis$_6$-ALOD4 (Mut) is the same as pHis$_6$-ALOD4 described above except that it contains six mutations (G501A, T502A, T503A, L504A, Y505A, and P506A) to abolish cholesterol binding [19].
   (e) pHis$_6$-PFO*, in the pRSET B expression vector, encodes Perfringolysin O (PFO) from *Clostridium perfringens* with a NH_2-terminal His$_6$ epitope tag. The sole native cysteine in PFO (C459) was mutated to alanine. PFO* harbors a key mutation (Y181A) that prevents pore formation by this protein upon binding cholesterol in membranes at 4 °C [17]. A detailed protocol for expression and labeling of this protein was previously published [25].
2. BL21 (DE3) pLysS competent cells.
3. SOC medium.
4. Ampicillin: prepared as a 100 mg/mL (1000×) stock solution.
5. Luria broth agar plates supplemented with 100 μg/mL ampicillin.
6. Luria Broth (LB).

7. Isopropyl β-D-thiogalactopyranoside (IPTG): prepared as a 1 M (1000×) stock solution.
8. Buffer A: 50 mM Tris–HCl, 150 mM NaCl, and 1 mM TCEP, pH 7.5.
9. Buffer B: 50 mM Tris–HCl, 150 mM NaCl, and 1 mM TCEP, 500 mM imidazole pH 7.5.
10. Lysozyme: prepared as a 10 mg/mL solution (10×).
11. Phenylmethanesulfonyl fluoride (PMSF): prepared as a 20 mg/mL solution (1000×).
12. Protease inhibitor tablets.
13. Dounce: 100 mL glass Dounce tissue grinder.
14. Sonifier.
15. Fast protein liquid chromatography (FPLC) such as an AKTA purifier or equivalent.
16. HisTrap HP column: prepacked 1 or 5 mL HisTrap column.
17. Centrifugal filters: such as Amicon Ultra-4 10-kDa cutoff.
18. Superdex 200 column or equivalent.
19. 15% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels.
20. Quick Coomassie stain or similar product.
21. 5x SDS loading buffer: 10% (w/v) SDS, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris–HCl, pH 6.8, 0.05% (w/v) bromophenol blue.
22. Spectrometer: such as a NanoDrop.
23. Thiol-reactive dye: such as the Alexa Fluor maleimide.
24. Dithiothreitol (DTT): prepared as a 1 M solution in water.
25. Disposable column: such as Poly-Prep Columns.
26. 37 °C incubator.
27. Centrifuge.
28. Liquid nitrogen.
29. 50 mL Falcon tubes.
30. Orbital shaker.
31. 50 mL Glass beaker.
32. Low-protein-binding filter (0.22 μm pore size).
33. 50 mL or 150 mL superloop.
34. NiNTA column.
35. NiNTA beads.

2.2 Materials for Cell Culture and Hedgehog Signaling Assays

1. Cholesterol powder.
2. Methyl-beta-cyclodextrin powder.
3. Micro-tip sonicator.
4. Glass vials: wider cap is preferred to avoid breaking with micro-tip sonicator.
5. Water bath sonicator with heating control.
6. Chloroform.
7. Methanol.
8. Optimem, stored at 4 °C.
9. Myriocin, prepared as a 30 mM stock in dimethyl sulfoxide (DMSO) and then aliquoted and stored at −80 °C.
10. Egg sphingomyelin powder, stored at −20 °C.
11. Fatty Acid Free Bovine Serum Albumin powder, stored at −20 °C.
12. Bovine Serum Albumin powder, stored at −20 °C.
13. Staurosporine, prepared as 1 mM stock in DMSO and stored at −20 °C.
14. Sonic Hedgehog, recombinant protein.
15. NIH/3T3 Flp-In cell line or other Hedgehog signaling-responsive cell line.
16. HyClone High Glucose Dulbecco's Modified Eagle Medium (DMEM).
17. Fetal bovine serum.
18. 5% Lipoprotein-depleted fetal bovine serum (LDS) DMEM: High Glucose DMEM with added L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin, and LDS added to 5%. Filter sterilize through 0.2 μm filter and store at 4 °C. *See* **Note 1**.
19. L-Glutamine.
20. Penicillin/Streptomycin.
21. Non-essential amino acids.
22. Lipoprotein-depleted fetal bovine serum (LDS).
23. U18666A, prepared as 10 mM stock in DMSO and stored in the dark at −20 °C.
24. Matrigel.
25. Paraformaldehyde, prepared as 20% solution and stored in 1 mL aliquots at −80 °C.
26. Prolong Diamond Antifade Mountant.
27. Nitrogen gas.
28. Tissue culture hood.

29. 0.2 μm Filters.
30. Trypsin.
31. Tissue culture dishes including 24-well tissue culture dishes.
32. Tissue culture incubator.
33. Materials for Western Blotting or quantitative Real-Time PCR.
34. Phosphate-buffered saline (PBS).
35. Flow cytometer.
36. Probe Blocking Buffer (PBB): 1x PBS with 10 mg/mL bovine serum albumin, filter sterilized through 0.2 μm filter.
37. Aluminum foil.
38. GFP-tagged ARL13B (or other fluorescently labeled cilia-resident protein).
39. Glass coverslips.
40. Glass slides.
41. Metal rack.
42. Parafilm.

3 Methods

3.1 Generation of Lipid-Binding Toxins

3.1.1 Expression Protocol

1. Transform ALOD4 and OlyA plasmids into BL21 (DE3) pLysS *Escherichia coli* competent cells according to the manufacturer instructions. Plate transformed cells on LB agar plates containing 100 μg/mL ampicillin. Incubate for 16 h at 37 °C.
2. Inoculate 160 mL Luria broth (LB) containing 100 μg/mL ampicillin with one transformed colony from **step 1** for each construct. Incubate for 12 h at 37 °C, 250 rpm. ALOD4 expression will be problematic if starter culture grows beyond an OD_{600} of 1.0 [19].
3. Inoculate 1 L LB containing 100 μg/mL ampicillin with 20 mL of the starter culture. Incubate at 37 °C, 250 rpm and monitor growth by checking the OD_{600}.
4. When OD_{600} reaches 0.4–0.6, reduce temperature to 18 °C and induce protein expression with addition of IPTG (1 mM final concentration) for 18–24 h.
5. Harvest bacteria by centrifugation at 4000 rpm, or about 5800 ×*g*, for 10 min at 4 °C, then flash freeze bacterial pellets in liquid nitrogen and store at −80 °C for up to 3 months.

3.1.2 Purification Protocol for ALOD4 and OlyA

1. Thaw bacterial pellet on ice for 10 min.
2. Suspend in 20 mL buffer A containing 1 mg/mL lysozyme, 20 μg/mL PMSF, and one protease inhibitor tablet per 1 L bacterial culture pellet.

3. Pour cell lysate into a glass Dounce. Dounce 12 times, pour into a 50 mL falcon tube, and incubate on an orbital shaker at 4 °C. After 2 h, repeat Dounce step.
4. Pour cell lysate into a 50 mL glass beaker on ice. Sonicate with a digital sonifier: 40% amplitude, 3 min active (3 s on, 3 s off) followed by 6 min inactive. Repeat two additional times.
5. Subject cell lysate to centrifugation at 4 °C for 30 min and 25,000 ×*g*. Filter supernatant with a low-protein-binding filter (0.22 μm pore size) and discard pellet.
6. Add filtered supernatant to a superloop (either 50 mL or 150 mL superloop, depending on the volume of supernatant collected). Dilute with buffer A to fill the superloop.
7. Using a FPLC system, load contents of superloop into a 1 or 5 mL prepacked HisTrap column. Place buffer line A in buffer A and buffer line B in buffer B.
8. Wash NiNTA column with 20 column volumes buffer A, then 20 column volumes (CV) with 90% buffer A and 10% buffer B.
9. Elute NiNTA-bound protein with a linear gradient of buffer B from 10 to 60% (50 mM imidazole to 300 mM imidazole) over 20 CV.
10. Recover fractions with eluted ALOD4 or OlyA, run an aliquot of each fraction on a 15% SDS polyacrylamide gel to confirm correct fractions were collected and to determine the purity of protein.
11. Concentrate eluate with Amicon Ultra-4 10-kDa cutoff centrifugal filters, 3220 ×*g*, 4 °C. Overconcentration of ALOD4 protein can result in protein precipitation. To avoid this, protein concentration can be accomplished by ion exchange chromatography [19].
12. Subject concentrated eluate to centrifugation at 20,000 ×*g*, 30 min, 4 °C to prepare for gel filtration. After centrifugation, move the supernatant to a new tube. There should not be a visible pellet.
13. Inject 2 mL of eluate from **step 12** over a Superdex 200 (or equivalent) column equilibrated in buffer A, connected to an AKTA purifier FPLC (or equivalent). Collect fractions containing eluted protein.
14. Determine protein concentration using either a BCA assay or a spectrophotometer such as a NanoDrop according to manufacturer instructions.

3.1.3 Fluorescent Labeling of ALOD4 and OlyA

1. Combine 50 nanomoles of purified ALOD4 or OlyA with 500 nanomoles of Alexa Fluor maleimide in a final volume of 750μL of buffer A. Protect tube from light by wrapping in

aluminum foil. Incubate labeling reaction mixture for 16 h at 4 °C on an orbital shaker.

2. Quench labeling reaction mixture with addition of DTT (10 mM final concentration).
3. Add quenched labeling reaction to 2 mL NiNTA beads that have been equilibrated in buffer A in a chromatography column.
4. Wash with 10 CV buffer A followed by 10 CV 90% buffer A mixed with 10% buffer B (50 mM imidazole).
5. Elute labeled protein by adding 2 mL 40% buffer A mixed with 60% buffer B (300 mM imidazole).
6. Dilute eluate with 10 mL buffer A to lower imidazole concentration, concentrate with Amicon Ultra-4 10-kDa cutoff centrifugal filters, 3220 ×*g*, 4 °C until protein is 0.5–1 mg/mL. Store at 4 °C and use within 2 weeks. For long-term storage, mix concentrated eluate with glycerol (20% v/v final concentration), divide into 50–100μL aliquots, and flash freeze in liquid nitrogen. Store at −80 °C for up to 2 months. Thaw aliquots on ice and centrifuge (20,000 ×*g*, 30 min, and 4 °C) to remove aggregates before use.

3.2 Generation of Bioavailable Sterol Complexes

3.2.1 Cholesterol Complexation with Methyl-Beta Cyclodextrin (MβCD)

1. Dissolve cholesterol in a glass vial with a mixture of chloroform-methanol (2:1 vol/vol) to generate a 10 mg/mL stock solution (25.86 mM).
2. Add 336μL (8.7 μmol) of the 10 mg/mL stock solution to a new glass vial.
3. Stream nitrogen gas over the sterol solution until the solvent is evaporated completely, generating a thin film in the vial. You can also remove solvent under vacuum.
4. Dissolve methyl-beta-cyclodextrin (MβCD) in Optimem (in tissue culture hood) at a final concentration of 50 mg/mL (38 mM). Add 2 mL of this solution to the dried sterol film in the glass vial.
5. Sonicate in a room temperature water bath for 2–5 min to loosen sterol film from the walls of the glass vial.
6. Micro-tip sonicate the mixture until it becomes clear. Perform on ice to avoid heating. This takes ~30 rounds of 20 s sonication, allowing foam to clear, and sample to cool, in between each sonication step.
7. Filter solution through a 0.2 μm filter and store at 4 °C or freeze in liquid nitrogen and store in aliquots at −80 °C. The MβCD:cholesterol ratio is 8.8:1 in inclusion complexes. *See* **Note 2**.

3.3 Altering Accessible Cholesterol and Sphingomyelin Levels for Hedgehog Signaling Assays

The following experiments utilize NIH/3T3 Flp-In cells because they robustly respond to Hedgehog ligands. Other Hedgehog-responsive cell lines can also be used.

3.3.1 Culturing NIH/3T3 Flp-In Cells in Lipoprotein-Depleted Serum DMEM

1. Culture NIH 3T3 Flp-In cells for approximately 1 week in 5% LDS DMEM. If passaging cells is necessary, quench trypsin with 5% LDS DMEM, and seed in 5% LDS DMEM.
2. Seed cells for experimental analysis in 5% LDS DMEM and grow to confluency.
3. Serum starve cells overnight in supplemented DMEM containing 0.5% LDS, sterilized by passing through a 0.2 μm filter. Simultaneously add 1μM U18666A to prevent transport of cholesterol from the lysosome to the plasma membrane. At this time ligands such as Sonic Hedgehog can also be added.
4. Analyze Hedgehog signaling output by either Western Blotting or quantitative Real-Time PCR to measure Hedgehog target genes (see detailed protocol, [26]).

3.3.2 Depletion of Cellular Accessible Cholesterol with ALOD4

1. Grow NIH/3T3 Flp-In cells to confluency in 10% fetal bovine serum (FBS) supplemented DMEM. Other Hedgehog signaling-responsive cell lines can also be used.
2. Serum starve cells overnight in 0.5% FBS supplemented DMEM.
3. Add ALOD4 (purified in buffer B) diluted to 25 μM in 0.5% serum DMEM. For wells not receiving ALOD4, an equivalent amount of the buffer B was added as a control. Alternatively, the ALOD4 mutant that does not bind to cholesterol can be used as a control.
4. Add Sonic Hedgehog ligands 1 h later if measuring Hedgehog signaling output.
5. Incubate cells in tissue culture incubator for 5 h.
6. Wash cells three times with 1x PBS to remove unbound ALOD4 prior to analysis.
7. Immediately lyse cells for downstream analysis in appropriate buffer. Because of the short incubation with Sonic Hedgehog, target gene responses will be significantly less than in overnight treatments.
8. Analyze Hedgehog signaling output by either Western Blotting or quantitative Real-Time PCR to measure Hedgehog target genes (see detailed protocol, [26]).

3.3.3 Depletion of Cellular Sphingomyelin

1. Seed NIH/3T3 Flp-In cells in 10% FBS supplemented DMEM at a low enough density where the cells can grow for at least 3 days before reaching confluency (timing may vary for cell types). Other Hedgehog signaling-responsive cell lines can also be used.

2. Aspirate media once cells have attached to the culture dish (1–2 h), and add fresh media containing 40 μM myriocin. *See* **Note 3**.
3. Grow cells in myriocin-containing media for at least 48 h until confluent, and then aspirate media and replace with 0.5% FBS supplemented DMEM containing fresh 40 μM myriocin. If measuring Hedgehog signaling output, simultaneously add Sonic Hedgehog ligands. Incubate for 16–24 h prior to downstream analysis.
4. Analyze Hedgehog signaling output by either Western Blotting or quantitative Real-Time PCR to measure Hedgehog target genes (see detailed protocol, [26]).

3.3.4 Increasing Cellular Sphingomyelin

1. Grow NIH/3T3 Flp-In cells in 10% FBS supplemented DMEM until confluent. Other Hedgehog signaling-responsive cell lines can also be used.
2. Serum starve cells in 0.5% FBS supplemented DMEM. Simultaneously treat with Sonic Hedgehog and either 50 nM staurosporine or 30 μM egg sphingomyelin:fatty acid free BSA complexes [27]. Incubate for 16–24 h prior to downstream analysis.
3. Analyze Hedgehog signaling output by either Western Blotting or quantitative Real-Time PCR to measure Hedgehog target genes (see detailed protocol, [26]).

3.4 Flow Cytometry Measurement of Outer Leaflet Cholesterol, Sphingomyelin, or Cholesterol/Sphingomyelin Complexes

3.4.1 Staining Cells with Lipid-Binding Probes for Flow Cytometry

1. Grow NIH/3T3 Flp-In cells in a 24-well tissue culture dish in 10% FBS supplemented DMEM until confluent. *See* **Note 4**. Other Hedgehog signaling-responsive cell lines can also be used.
2. Serum starve cells in 0.5% FBS supplemented DMEM overnight to match conditions used for Hedgehog signaling assays. Drugs to increase or decrease cellular cholesterol or sphingomyelin are added at this point (*see* Subheading 3.3).
3. Wash cells briefly with 1x PBS and then trypsinize for 5 min at 37 °C.
4. Quench trypsin with 1 mL ice-cold 0.5% serum DMEM, pipetting up and down to generate a single cell suspension.
5. Pellet cells by spinning at 4 °C and 1000 ×*g* for 5 min.
6. Decant media, and then resuspend cells in 0.5 mL ice-cold Probe Blocking Buffer (PBB) pipetting up and down to generate a single cell suspension.
7. Incubate in PBB on ice for 5 min, and then pellet cells by centrifugation at 4 °C, 1000 ×*g* for 5 min.

8. Carefully remove PBB, and then resuspend cells in PBB containing desired fluorescently labeled probes at the following final concentrations: 5 μg/mL PFO*, 2 μM ALOD4, and 2 μM OlyA. Cell pellet sizes may vary, but typically 20μL is a sufficient volume of diluted probe for cell staining.
9. Incubate cells with probes on ice for 30 min, covered with aluminum foil to protect from light.
10. Briefly wash cells twice with 100μL PBB, spinning each time at 4 °C and 1000 ×*g* to remove PBB and unbound probes. Place cell pellets on ice and only resuspend in the final volume of PBB immediately prior to flow cytometry.

3.4.2 Flow Cytometry Measurement

1. During the flow cytometry experiment, keep cells on ice and shielded from light with aluminum foil.
2. Select live, singlet cell populations based on forward and side scatter and analyze without further gating.
3. Adjust laser power as needed to avoid saturation of the detector. For example, if measuring outer leaflet cholesterol with PFO* or ALOD4, begin with cholesterol-treated samples to ensure the detector is not saturated.
4. For probes labeled with 647 nm dyes, we used a 638 nm laser for excitation. Emission was measured by first excluding wavelengths lower than 639 nm and higher than 685 nm and then collecting using a 665/30 nm bandpass filter.

3.5 Microscopy Imaging of Primary Cilia Stained with Lipid-Binding Probes

3.5.1 Staining Primary Cilia with PFO*

1. Seed NIH/3T3 Flp-In cells stably expressing GFP-tagged ARL13B (or other fluorescently labeled cilia-resident protein) on acid-washed coverslips treated with Matrigel to aid in adherence. *See* **Note 5**. Myriocin can be added at this time. *See* **Note 6**. Other Hedgehog signaling-responsive cell lines can also be used.
2. Grow cells to confluency in 10% FBS supplemented DMEM.
3. Serum starve cells in 0.5% FBS supplemented DMEM for 16–24 h to induce ciliation. MβCD:cholesterol complexes can be added at this point (concentrations between 0.3 mM and 1 mM MβCD:cholesterol are ideal, *see* **Note 2**).
4. To measure the increase in ciliary cholesterol resulting from Patched-1 inactivation, add Sonic Hedgehog ligands directly to cells 15–30 min before beginning the staining procedure.
5. Aspirate media out of wells, and add coverslip containing cells to a metal rack placed on ice. It is useful to place parafilm on top of the rack prior to adding the coverslips to aid in the removal of the coverslips at the end of the protocol.

6. Dilute fluorescently labeled PFO* in ice-cold 0.5% FBS supplemented DMEM to 5–10 μg/mL. Add 50μL of diluted PFO* to coverslips for 30 min. Cover with foil to protect fluorophore from light.
7. Aspirate PFO* off of cells, taking care to avoid disturbing cells. Wash cells once with ice-cold 1x PBS by adding it slowly and then immediately aspirating it off.
8. Fix cells in ice-cold 4% paraformaldehyde diluted in 1x PBS for 10 min.
9. Wash coverslips carefully with ice-cold 1x PBS three times before mounting on glass slides containing ProLong Diamond Antifade Mountant.
10. Cure slides overnight at room temperature in the dark before imaging.

3.5.2 Staining Primary Cilia with OlyA

1. Seed NIH/3T3 Flp-In cells stably expressing GFP-tagged ARL13B (or other fluorescently labeled cilia-resident protein) on acid-washed coverslips treated with Matrigel to aid in adherence. Other Hedgehog signaling-responsive cell lines can also be used.
2. Grow cells to confluency in 10% FBS supplemented DMEM.
3. Serum starve cells in 0.5% FBS supplemented DMEM for 16–24 h to induce ciliation. MβCD:cholesterol complexes can be added at this point (concentrations between 0.3 mM and 1 mM MβCD:cholesterol are ideal). *See* **Notes 2 and 7**.
4. Dilute OlyA to 2 μM in room temperature 0.5% FBS supplemented DMEM.
5. Cover the bottom of a dish or chamber used to stain coverslips with fresh parafilm. Aspirate media out of wells, and add the coverslips.
6. Add 50μL of diluted OlyA to coverslips and incubate for 10 min at room temperature.
7. Aspirate OlyA off of coverslips and then wash once with 1x PBS by adding it carefully to the coverslips and then immediately aspirating it off.
8. Fix cells in room temperature 4% paraformaldehyde diluted in 1x PBS for 10 min.
9. Wash coverslips carefully with room temperature 1x PBS three times before mounting on glass slides containing ProLong Diamond Antifade Mountant.
10. Cure slides overnight at room temperature in the dark before imaging.

4 Notes

1. The fetal bovine serum used in typical cell culture experiments is replete with cholesterol-rich lipoproteins, eliminating the need for cells to synthesize cholesterol de novo. We have observed that culturing cells in lipoprotein-depleted media decreases the basal signaling levels observed in Hedgehog signaling assays, increasing the dynamic range of pathway activity (unpublished data).
2. We typically report the concentration of methyl-beta-cyclodextrin (MβCD) delivered to cellular assays, rather than the amount of cholesterol. This is because the concentration of cholesterol in the complex is less exact than the MβCD concentration. If perfect complexation is achieved, the cholesterol concentration is 8.8 times less than the MβCD concentration.
3. Myriocin is dissolved in DMSO to generate 40 mM stocks. This generates a clear solution, which may turn cloudy after multiple freeze-thaws, so it is best to aliquot small volumes. Regardless of the apparent clarity of myriocin stocks, upon addition to cells, we often notice that there are particulates of myriocin (observed with a 5× microscope). These typically go into solution after ~24 h.
4. Clonal cell lines can have significantly different background fluorescence. If you wish to make cross comparisons between different clonal cell lines, plate an additional well of cells from each background to use for normalization. Subject these control cells to the same trypsinization, wash and incubation steps as those receiving fluorescent probes, excluding the probe addition. The fluorescence measured for these cells can be regarded as the autofluorescence for all related cells, and used to normalize values.
5. Traditional methods of antibody-based staining for immunofluorescence microscopy require fixation and permeabilization. This method is not amenable to staining with lipid-binding probes because permeabilization extracts the lipids that the probes bind to. Therefore, cells are stained live, and then fixed after probe addition. In order to identify cilia, we generated cell lines stably expressing either GFP- or RFP-tagged ARL13B, a cilia resident protein.
6. PFO* binds to accessible cholesterol in membranes. Primary cilia are depleted of accessible cholesterol, and thus do not show PFO* binding unless accessible cholesterol is increased through the addition of exogenous MβCD:cholesterol or through sphingomyelin depletion with drugs like myriocin.

7. In NIH/3T3 Flp-In cells, we only observed robust wild-type OlyA staining upon cholesterol addition to cells. OlyA(E69A), which binds both free sphingomyelin and cholesterol-complexed sphingomyelin, can be observed without perturbations.

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