Published in final edited form as:

Curr Protoc Microbiol. 2012 November ; CHAPTER: Unit11A.2. doi:
10.1002/9780471729259.mc11a02s27.

Spingolipid trafficking and purification in *Chlamydia trachomatis*-infected cells

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Abstract

Chlamydia trachomatis is an obligate intracellular human pathogen, which lacks a system that allows genetic manipulation. Therefore, chlamydial researchers must manipulate the host cell to better understand chlamydial biology. Host-derived lipid acquisition is critical for chlamydial survival within the host. Hence, the ability to track and purify sphingolipids in/from chlamydial infected cells has become an integral part of pivotal studies in chlamydial biology. This Unit outlines protocols that provide details about labeling eukaryotic cells with exogenous lipids to examine Golgi-derived lipid trafficking to the chlamydial inclusion and then performing imaging studies or lipid extractions for quantification. Details are provided to allow these protocols to be applied to subconfluent, polarized or siRNA knockdown cells. In addition, one will find important experimental design considerations and techniques. These methods are powerful tools to aid in the understanding of mechanisms which allow *C. trachomatis* to manipulate and usurp host cell trafficking pathways.

Keywords

Chlamydia trachomatis; sphingomyelin; lipid extraction

INTRODUCTION

It is well established that chlamydial acquisition of host-derived lipids is essential for chlamydial survival and infectivity (vanOoij et al., 2000). Therefore the ability to examine lipid trafficking events in the context of a chlamydial infection is vital to furthering our understanding of chlamydial biology. Working with labeled lipids (for example, C₆-NBD-ceramide or ¹⁴C-ceramide) has helped to define discrete pathways and sources for lipids being trafficked to the chlamydial inclusion. This Unit addresses the basics of experimental design, labeling of cells with traceable lipid, principles in live cell imaging and lipid extraction. Support Protocols 1, 2 and 3 cover the basics of tissue culture relative to experimental set-up. Also provided is a table (Table 1) which matches the described methods with the options for experimental design and set-up. Specifically, Support Protocols 1 and 2 outline how to set-up experiments using non-polarized and polarized cells, respectively. Support Protocol 3 describes a method of delivery of siRNA, which will knockdown the expression of a specific gene of interest. Basic Protocol 1 and Alternative Protocol 1 delineate the methods used for labeling chlamydial infected monolayers with

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Janet Sager contributed to the development of *Support Protocol 4*, and Emily McDowell contributed to the development of the modifications found in *Basic Protocol 3*.

either a fluorescent ceramide (C₆-NBD-ceramide) or with radioactive ceramide (N-1-[¹⁴C]oleoy-D-sphingosine), respectively. Basic Protocol 2 describes the procedure involved in live cell imaging of monolayers labeled with fluorescent lipid. To quantitate changes in lipid trafficking due a chlamydial infection, Basic Protocol 3 outlines the procedure for lipid extraction and resolution of the extracts by thin layer chromatography. In some instances, it might be necessary to examine the lipid content of chlamydial organisms in the absence of host cell contaminants. For this purpose, Support Protocol 4 describes steps involved in a small scale chlamydial purification.

CAUTION: When working with *Chlamydia trachomatis*, you must minimize the risk of producing aerosols. Additionally, when performing procedures involving sonication, and centrifugation (steps in which aerosols can be generated) it is highly important to wear appropriate PPE, including lab coat, gloves, safety glasses and masks. With the exception of centrifugation steps, all steps involving live organisms must be performed in a BSL-2 biosafety cabinet unless otherwise stated.

CAUTION: *Chlamydia trachomatis* is a Biosafety Level 2 (BSL-2) organism. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A:1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: When working with radioactivity, one must follow institutional and federal guidelines for record keeping, hygiene and safety. Use of radioactive materials in this protocol will also label microorganisms, therefore, you must exercise extreme vigilance and caution when working with *Chlamydia trachomatis* and radioactivity.

CAUTION: All work utilizing chloroform or molybdenum must be performed within the confines of a chemical fume hood. The ventilation of a chemical fume hood is fundamentally different than a BSL-2 biosafety cabinet and will not protect an individual from aerosols of live organisms. While chloroform will effectively kill *C. trachomatis*, extra care not generate aerosols when dealing with live microorganisms must be taken.

NOTE: When working with living cells, all solutions and equipment must be sterile and aseptic technique must be used.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator.

BASIC PROTOCOL 1. LABELING CELLS WITH C₆-NBD-CERAMIDE TO EXAMINE TRAFFICKING OF GOLGI-DERIVED SPHINGOLIPIDS TO THE CHLAMYDIAL INCLUSION

The addition of a fluorescent NBD (N-[7-(4-nitrobenzo-2-oxa-1,3-diazole) moiety to any lipid allows the tracking of the lipid within and out of a eukaryotic cell. C₆-NBD-ceramide indiscriminately labels cellular membranes at 2–4°C, but upon a shift of temperature to 37°C, it becomes a vital stain of the Golgi (Lipsky and Pagano, 1983, 1985b). Within the Golgi, C₆-NBD-ceramide is metabolized into NBD-glucosylceramide and NBD-sphingomyelin, which are subsequently trafficked to the plasma membrane. Addition of bovine serum albumin (BSA) to basal medium (for example, unamended Dulbecco's Modified Eagle's Medium) allows for the 'back-exchange' or extraction of NBD-lipids from the plasma membrane (Lipsky and Pagano, 1985a, b). The back-exchange process allows for the examination of lipids delivered to the plasma membrane and sequesters these lipids from sphingomyelinases, which revert the lipids back to ceramide and allow them reenter trafficking pathways. Therefore, when C₆-NBD-ceramide is added to cells, one can monitor

its metabolism in the Golgi and track the delivery of its derivatives to the cell surface. Previous studies have demonstrated that in chlamydial infected cells labeled with C₆-NBD-ceramide, the chlamydial inclusion intercepts Golgi-derived NBD-sphingomyelin (Hackstadt et al., 1996; Hackstadt et al., 1995; Moore et al., 2008; Wolf and Hackstadt, 2001). Most recently, methods developed from these initial studies helped to identify a role in SRC family kinases in the trafficking of sphingomyelin to the chlamydial inclusion (Mital and Hackstadt, 2011). Basic Protocol 1 outlines the steps for labeling cells with C₆-NBD-ceramide to study lipid transfer and metabolism. Prior to attempting Basic Protocol 1, Support Protocols 1, 2 or 3 and Table 1 should be consulted for proper experimental design and set-up.

Materials

Seeded, infected tissue culture cells (see Support Protocols 1, 2 or 3)

Eagle's Minimum Essential Medium (EMEM) (ATCC, 30-2003)

EMEM supplemented with 0.7% defatted-BSA (dfBSA) (see recipe)

EMEM supplemented with 0.035% dfBSA (see recipe)

5 mM C₆-NBD-ceramide (Invitrogen, N1154) (see recipe)

15-ml sterile conical tubes

Low-speed refrigerated bench-top centrifuge with tissue culture plate adaptors

1. Pre-cool the infected monolayer by placing the tissue culture plate(s) in pre-cooled refrigerated bench-top centrifuge set at 4°C to 12°C for 15 to 30 minutes.

Some tissue culture cells are sensitive to temperatures around 4°C, but typically can maintain homeostasis at 12°C. If the cell line that you are using is not sensitive to low temperatures, you may alternatively incubate cells on ice in a 4°C refrigerator.

2. Immediately before use, dilute C₆-NBD-ceramide to 5 μM in EMEM supplemented with 0.035% dfBSA. Vortex solution vigorously and keep on ice.
3. Remove cells from centrifuge and place on ice. Wash cells twice with unamended EMEM, before placing the appropriate amount of labeling medium onto cells.

For cells seeded in 24-well plates, use 1 ml of labeling medium/well. For cells in 100 mm dishes, use 5 ml of labeling medium/well. For cells in a T-150 flask, use 10 ml of labeling medium/flask.

4. Return cells to pre-cooled refrigerated bench-top centrifuge for 40 minutes to an hour.

The purpose of this step is to fully label intracellular membranes with C₆-NBD-ceramide, prior to the temperature shift to 37°C. Thus maximizing the amount of C₆-NBD-ceramide that labels the Golgi apparatus and synchronizing its metabolism within the Golgi. Vesicular trafficking and labeling of the Golgi with C₆-NBD-ceramide is inhibited by temperatures below 14°C.

5. Wash cells twice with unamended EMEM. Add EMEM supplemented with 0.7% dfBSA (back-exchange medium) and transfer cells to a 37°C, 5%CO₂ incubator.

Refer to step 4 for appropriate volumes.

6. Proceed to Basic Protocol 2 or 3.

ALTERNATE PROTOCOL 1. LABELLING CELLS WITH ¹⁴C-CERAMIDE

While there are no physical hindrances to tracking a lipid by using a fluorescent moiety, such as NBD, there may be an experimental need to have greater sensitivity in quantification than densitometry of a thin layer chromatography plate will allow. However, unlike NBD-tagged lipids, there is no way to 'back-exchange' radiolabeled lipids from the plasma membrane. So, sphingomyelin that reaches the plasma membrane would be subjected to sphingomyelinases and converted back to ceramide, thereby complicating the ability to trace Golgi-derived lipids. The use of a radioactive lipid, in this case *N*-1-[¹⁴C]oleoy-D-sphingosine, ¹⁴C-ceramide, allows for the purification of chlamydial organisms or isolate specific subcellular fractions to quantify radiolabelled lipid content by scintillation counting. To confirm the presence of radioactive sphingomyelin as the sole lipid species found within the chlamydial organisms, one can run a thin layer chromatography plate (Basic Protocol 3) with the modifications associated with examining endogenous lipids outlined in the notes. Please consult Table 1 for proper experimental design preparation.

Materials

Seeded, infected tissue culture cells in 2–4 T-150 flasks/ condition (see Support Protocols 1, 2 or 3)

Seeded, uninfected tissue culture cells in 2–4 T-150 flasks for control

N-1-[¹⁴C]oleoy-D-sphingosine (50–60mCi/molar)

Normal growth medium supplemented with lipid-free (defatted) fetal bovine serum albumin (dfFBS)

50-ml sterile conical tubes

1. Dilute *N*-1-[¹⁴C]oleoy-D-sphingosine to 0.5 µCi/ml in normal growth medium supplemented with dfFBS.
2. Wash cells in plain medium.
3. Label cells with 12.5 ml medium. Transfer to CO₂ tissue culture incubator.
4. After 24 to 42 hours, proceed to Support Protocol 4.

SUPPORT PROTOCOL 1. SEEDING OF NON-POLARIZED EUKARYOTIC CELLS

Because *Chlamydia* are obligate intracellular bacteria, all assays must be performed within the context of a host cell. The proper methodology regarding chlamydial infections and additional protocols for culturing chlamydial organisms in tissue culture can be found in Current Protocols Unit 11A.1, 'Cultivation and Laboratory Maintenance of *Chlamydia trachomatis*' by Marci A. Scidmore (Scidmore, 2005). This protocol outlines the basic technique of seeding eukaryotic cells for a chlamydial infection.

Materials

Trypsinized eukaryotic cells (consult Table 1)

Tissue culture plate/ dish (consult Table 1)

Complete tissue culture medium (consult Table 1)

0.4% Trypan blue solution *or* Erythrocin B Stain

0.5 ml microcentrifuge tube (does not have to be sterile)

Hemocytometer (Bright-Line, Hausser Scientific, Horsham, PA)

Light Microscope with a 10X objective

Autoclaved glass coverslips, circles, 12mm diameter, 0.12 mm thickness (required for live cell imaging only)

1. Consult Table 1 to determine which end-assay you will be setting up.

If the end-assay is live cell imaging of non-polarized cells, cells need to be seeded onto coverslips. Take a pair of curved end forceps and soak in 70% ethanol. Use the sterilized forceps to carefully place glass coverslips into the wells of a 24-well plate. Then continue with the below protocol to seed cells.

2. Resuspend the trypsinized cells to 10 ml in complete medium.

The serum in complete medium inhibits the action of trypsin. If trypsin is left too long on cells, it will compromise their viability.

3. Remove a 100 μ l aliquot of cells and place in 0.5 ml microcentrifuge tube. Add 100 μ l of 0.4% Trypan blue solution or Erythrocin B Stain to the aliquot of cells. Mix well by pipetting up and down.

4. Add an aliquot of the cells to the hemacytometer and enumerate cells in the grid with a 20X objective on a light microscope.

Using the hemacytometer described, you will see 4 countable areas/grid. Count all four areas, take the average number, multiply by 20 $\times 10^3$ (conversion for cells/ml assigned to the hemacytometer), and then by 10 (or total volume in which the cells are resuspended). This will give you the number of cells per 10 mls. This will allow you to set up a ratio to determine how many ml's of cells you need for your assay. Consult Table 1 for appropriate densities and volumes.

5. Based on the cell count, dilute cells to the appropriate density and seed in appropriate tissue culture plate/dish (see Table 1).

Typically, you culture HeLa cells for 24 hours and C2BBel cells for 48 hours prior to chlamydial infection. Please consult Current Protocols Unit 11A.1, 'Cultivation and Laboratory Maintenance of Chlamydia trachomatis' by Marci A. Scidmore (Scidmore, 2005) for proper methodology regarding chlamydial infections.

SUPPORT PROTOCOL 2. POLARIZATION OF EPITHELIAL CELLS

The plasma membranes of polarized cells are organized into distinct apical and basolateral domains (Bacallao et al., 1989). Lipid and protein trafficking to these domains is highly regulated to maintain these distinct membranes. Therefore, studying lipid trafficking in polarized cells can distinguish which exocytic pathway (apical or basolateral) is involved in trafficking of specific lipids to specific compartments. In the case of *C. trachomatis* serovar L2, a polarized cell model was used to demonstrate that the chlamydial inclusion intercepts basolaterally-targeted, sphingomyelin containing exocytic vesicles (Moore et al., 2008). This protocol describes the methods used to obtain and measure a polarized cell monolayer. After the monolayer is polarized, it can be labeled with lipid per Basic Protocol 1 or Alternative Protocol 1, and processed accordingly. Consult Table 1 in order to seed cells in the appropriate sized insert for the desired experiment.

Materials

Trypsinized eukaryotic cells (consult Table 1)

Tissue culture inserts coated with fibrillar collagen type I and pore size 0.4 micron (BD Biosciences, Bedford, MA) (consult Table 1 for size)

Complete tissue culture medium (consult Table 1)

Intestinal Differentiation Media Pack (BD Biosciences)

0.4% Trypan blue solution *or* Erythrocin B Stain

0.5 ml microcentrifuge tube (does not have to be sterile)

Hemocytometer (Bright-Line, Hausser Scientific, Horsham, PA)

Light Microscope with a 10X objective

EVOM Ohm meter (World Precision Instruments, Sarasota, FL)

STX2, EVOM chopstick electrodes (World Precision Instruments)

1. Incubate the tissue culture inserts in appropriate amount of medium (insert and underlying well) for a minimum of 30 minutes at 37°C, 5%CO₂.

This step is absolutely required to guarantee maximum cell binding to the filter substrate. Always seed an 'empty' insert, an insert with medium, but no cells. This is important for accurate transepithelial resistance readings (see Step 7).

Inserts are a necessary platform to cultivate polarized cells. Once tight junctions form, the apical and basolateral membranes are effectively separated from each other, and without access to medium the basolateral membrane will 'dry up'. The pores in the inserts allow the basolateral membranes access to medium and nutrients, which is essential for maintaining a healthy, polarized monolayer.

2. Resuspend the trypsinized cells to 10 ml in complete medium.

The serum in complete medium inhibits the action of trypsin. If trypsin is left too long on cells, it will compromise their viability.

3. Remove a 100 µl aliquot of cells and place in 0.5 ml microcentrifuge tube. Add 100 µl of 0.4% Trypan blue solution or Erythrocin B Stain to the aliquot of cells. Mix well by pipetting up and down.

4. Add an aliquot of the cells to the hemacytometer and enumerate cells in the grid with a 20X objective on a light microscope.

Using the hemacytometer described, you will see 4 countable areas/grid. Count all four areas, take the average number, multiply by 20 × 10³ (conversion for cells/ml assigned to the hemacytometer), and then by 10 (total volume in which the cells are resuspended). This will give you the number of cells per 10 mls. This will allow you to set up a ratio to determine how many ml's of cells you need for your assay. Consult Table 1 for appropriate densities and volumes.

5. Based on the cell count, dilute cells to the appropriate density (see Table 1) in 'basal seeding medium' from Intestinal Differentiation Media Pack. Remove medium from insert and replace with appropriate volume of cells. Incubate for 24 hours at 37°C, 5% CO₂.

6. Remove medium from both the insert and lower culture well. Replace with 'Stimulating medium' from Intestinal Differentiation Media Pack. Repeat daily until infection.

Within the 'Stimulating medium' are factors, including butyric acid, that stimulate the formation of tight junctions and the differentiation of epithelial cells into a polarized monolayer.

7. Once cells become polarized, they can be infected with *C. trachomatis*. To maintain polarity, all infection steps must be performed in 'stimulating medium'.

To determine if cells become polarized, typically, one measures transepithelial resistance (TEER) using an ohm meter. This is effective for 6.5 mm inserts, but is inaccurate for inserts greater than 24mm in size. Measure the ohm values, using the 'chop stick' electrodes, which measures the resistance between the upper and lower wells. As cells become polarized the resistance increases. TEER values (Ωcm^2) are obtained by taking the raw ohm values and subtracting the ohm value of the blank insert (medium in upper and lower chambers with no cells), multiplied by the surface area of the insert (for 6.5 mm inserts, this value is 0.33 cm^2). Cells are considered weakly polarized (and suitable for infection) at $150\text{ }\Omega\text{cm}^2$ and fully polarized at $200\text{ }\Omega\text{cm}^2$ or higher. For more details on polarized cell models and chlamydial infection, please consult (Moore et al., 2008).

SUPPORT PROTOCOL 3. REVERSE TRANSFECTION OF SIRNA PRIOR TO LABELING CELLS WITH FLUORESCENT LIPID

Transfection is the method used to deliver recombinant DNA or RNA into cells and was typified by 'lipofection' or a lipid-mediated transfection protocol (Felgner et al., 1987). A 'forward' transfection refers to seeding cells first, then a day later (typically) adding the lipid-DNA (or lipid-RNA) complex to the cells. A 'reverse' transfection refers to adding the lipid mixture to the cells as they are being seeded. While both transfection methods are viable, the reverse transfection method saves time and may increase the efficiency of transfection in cells that are typically difficult to transfect. This protocol is efficient at delivering siRNA to eukaryotic cells. siRNA or *small-interfering RNA* targets a gene of interest and prevents the expression of the gene, essentially eliminating the protein product from the host cell. Several companies provide siRNA constructs; Dharmacon and Ambion (Invitrogen) are two such suppliers.

Materials

Trypsinized eukaryotic cells (consult Table 1)
 Tissue culture plate/ dish (consult Table 1)
 Antibiotic-free, complete tissue culture medium (consult Table 1)
 0.4% Trypan blue solution *or* Erythrocin B Stain
 0.5 ml microcentrifuge tube (does not have to be sterile)
 Hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA)
 Light Microscope with a 10X objective
 Autoclaved glass coverslips, circles, 12mm diameter, 0.12 mm thickness (required for live cell imaging only)

Experimental and control siRNA at 20 μ M stock concentration (Ambion)

Opti-Mem® + Glutamax® (Life Technologies)

Lipofectamine RNAiMAX® (Life Technologies)

Rocker or orbital shaker

1. Add indicated amount of Opti-Mem® to appropriate sized culture plate (consult Table 2).

As with standard transfection protocols, the use of Opti-Mem® increases the rate of transfection and ultimately the knockdown efficiency.

2. Dilute siRNA in Opti-Mem® from stock. The final concentration (*after* addition of cells) should be 10 nM (or concentration of siRNA that results in greater than 90% knockdown).

For example, if you are seeding a 6-well plate and the final concentration of siRNA desired is 10 nM, dilute the stock to 50 nM in 500 μ l of Opti-Mem®. Ultimately, you need to empirically determine the amount of siRNA required for efficient knockdown of your gene of interest.

3. Add appropriate amount of Lipofectamine RNAiMAX® (consult Table 2). Incubate plate or flask on a rocker or orbital shaker for 10 to 20 minutes at room temperature.
4. Count and dilute cells in antibiotic-free complete medium as indicated in Table 2.
Addition of antibiotics to the complete medium may decrease your transfection efficiency and subsequent knockdown.
5. Add cells to the plates or flasks and transfer cultures to an 37°C, 5%CO₂ incubator.
6. After an overnight incubation, remove transfection medium and replace with antibiotic-free complete tissue culture medium. Culture cells for an additional 48 to 96 hours for maximal knockdown prior to chlamydial infection.

As with the optimum amount of siRNA required for an efficient knockdown, you will need to empirically determine the optimal amount of time required to obtain an efficient knockdown. Also, you should determine how long the knockdown will last and if the knockdown will be stable during a chlamydial infection.

SUPPORT PROTOCOL 4. SMALL SCALE CHLAMYDIAL PURIFICATION FOR LIPID EXTRACTION

The basis from this protocol is rooted in the standard large-scale purification of chlamydial organisms (Caldwell et al., 1981; Scidmore, 2005). However, when assaying chlamydial sphingomyelin acquisition in the context of an experimental parameter, such as siRNA knockdown, it is not fiscally feasible to perform a purification by standard methods. This purification protocol has been scaled down to accommodate a variety of experimental parameters, while obtaining a pure chlamydial fraction.

Materials

Seeded, labeled cells (refer to Table 1)

HBSS

SPG

4" Cannula

Large cell scraper (38 cm)

5–10 ml syringe

30%, 40%, 44%, 54% Renografin

Avanti J-30I high speed centrifuge (Beckman Coulter) or equivalent, see notes in protocol

Polycarbonate centrifuge bottles with caps, 29 × 104 mm (361693, Beckman Coulter)

JA-25.50 Fixed angle rotor (Beckman Coulter) or equivalent, see notes in protocol

Harvard Trip Balance

Ultra-clear centrifuge tubes, 14 × 89 mm tubes (344059, Beckman Coulter)

JS-24 rotor or equivalent, see notes in protocol

JS-24.15 swinging buckets or equivalent, see notes in protocol

1. Save 1 ml of back-exchange medium from the representative flask/condition. Store at 4 °C for lipid extraction (refer to Basic Protocol 3).

To control for proper metabolism of NBD-ceramide (for example), you need an aliquot of the back-exchange medium to demonstrate the production of the metabolites, NBD-glucosylceramide and NBD-sphingomyelin and trafficked appropriately to the plasma membrane. Omit this step if not using back-exchange medium.

2. Decant all medium.

The medium may be harboring infectious organisms, so decant into a 3% Roccal or 10% bleach solution.

3. Scrape cells into 10 ml cold HBSS. Transfer this same 10 ml to the other flask of the same condition and continue to scrape cells. Transfer to a centrifuge bottle on ice.
4. Wash the first flask with an additional 10 ml of HBSS. As before, transfer this 10 ml and wash the remainder of the flasks for each individual condition. There will be a total of 20 ml in each centrifuge bottle.
5. In the biosafety cabinet, sonicate each sample with ~100 watts, three times for 20 seconds.

It is highly important to perform this step in a biosafety cabinet, wearing appropriate PPE, including a mask. It is important to keep your samples cold to preserve the EBs. Therefore, it is helpful to sonicate the sample while holding the centrifuge bottle in a glass beaker containing ice.

6. Using the JA-25.50 rotor, pellet unbroken cells at 500 × *g* for 10 minutes and 4°C. Transfer the supernatant to a fresh centrifuge bottle.

Alternatively for this step, you can use the J2-21 M centrifuge with the same JA-25.50 rotor.

7. Resuspend the pellet in 7 ml HBSS with a cannula attached to a syringe. Repeat the sonication and centrifugation step. Remove the supernatant and combine with the above supernatant.

8. Using the JA-25.50 rotor, centrifuge the supernatants at 24,000 x *g* for 30 minutes at 4°C. Gently decant supernatant.
9. Resuspend the pellet in 5 ml ice-cold SPG and overlay onto 5 ml 30% renografin in an ultra-clear centrifuge tube. Wash the centrifuge bottle with an additional 2 ml of SPG and carefully layer on top of the first 5 ml.

This pellet will contain chlamydial organisms plus membranous fractions and organelles; therefore, you may wish to take an aliquot for Western blot purposes here to access knockdown or other protein levels. If you have knocked-down a cytosolic protein, then take an aliquot for Western blot from the supernatant from Step 6.

For the following steps using ultra-clear centrifuge tubes. If not using the Avanti J-30I centrifuge, you can also use an L8-55 ultracentrifuge (or equivalent) with an SW-41 rotor.

10. Load the centrifuge tubes into the JS-24.15 swinging buckets and balance the tubes using a Harvard Trip Balance.

For safety and sterility reasons, open and close the swinging buckets within the confines of the biosafety cabinet.

11. Load the swinging buckets on the JS-24 rotor and centrifuge at 55,000 x *g* for 30 minutes at 4°C.

12. Make the renografin gradient: 3 ml 54% renografin, 4 ml 44% renografin, 2 ml 40% renografin.

The easiest way to pour a gradient is to hold the centrifuge tube as close to a 90° angle as possible. You need to clearly see the boundaries between each renografin layer or the EBs will not purify appropriately. If you do not see clear boundaries, then pour out the gradient and try again.

13. Using a cannula, resuspend the pellet from step 11 with 3 ml SPG and wash the centrifuge tube with additional 2 ml SPG and carefully layer the entire 2.5 ml of the renografin gradient.

14. Load the centrifuge tubes into the JS-24.15 swinging buckets and balance the tubes using a Harvard Trip Balance.

Use extreme care when transferring these samples to the centrifuge. Too much jostling will disturb the gradient.

15. Centrifuge the samples at 55,000 x *g* for 80 minutes at 4°C.
16. Remove the elementary bodies from the 54%/44% renografin interface with a clean cannula.
17. Mix EB's with 12 ml SPG and place in ultra-clear tubes, balance the tubes in the JS-24.15 buckets and centrifuge at 55,000 x *g* for 30 minutes at 4°C.
18. With a cannula, resuspend the pelleted EB's in 1 ml HBSS.

BASIC PROTOCOL 2. LIVE CELL IMAGING OF FLUORESCENT (NBD-) LIPIDS

The ability to visualize the transfer of lipids from subcellular organelles, such as the Golgi, to the chlamydial inclusion is a powerful tool. The best way to visualize fluorescently labeled lipids is by live cell imaging. Fixing monolayers containing fluorescent lipid in

paraformaldehyde is possible, but fixative will cause degradation of fluorescent lipid. Therefore if quantifying lipid transfer by measuring pixels or using a fluorometer to quantify fluorescent emission, live cell imaging is the gold standard. While there is very minimal risk to aerosolizing organisms while performing this protocol, caution must be exercised while performing this procedure and all equipment must be adequately sanitized at the completion of this experiment.

Materials

Cells seeded on coverslips or in 6.5 mm inserts (refer to Table 1) and labeled with C₆-NBD-ceramide (refer to *BASIC PROTOCOL 1*)

18-gauge needle, 1.5" long

Curved forceps, serrated, 4.5"

HCMF (see recipe)

Kimwipes

Slides

Fluorescent microscope with 60X oil objective

Oil

Additional supplies for polarized cells:

Scalpel

Additional coverslips

For cells seeded directly on coverslips:

1. After 30 minutes to overnight after back-exchange, remove tissue culture plate from cell culture incubator and transfer to a biosafety cabinet closest to fluorescent microscope.

The timing of the back-exchange is linked to the scientific question that you are asking. Are you looking for a delay in chlamydial lipid acquisition? Only back-exchange for 30 minutes to an hour or two. Are you looking at long-term retention of fluorescent lipid? Then back-exchange overnight, before performing live cell imaging.

2. Carefully take the tip of the 18-gauge needle and coax an edge of the coverslip, and grab the edge with the curved forceps.
3. Dip coverslip twice in HCMF.
4. Blot the edge of the coverslip with the Kimwipe.

Be careful not to dislodge cells on the coverslip. You want the 'non-cell' side to be as dry as possible.

5. Place the coverslip cell-side down on the glass slide.
6. Quickly move slide to the microscope.

It is important to move as quickly as possible as these samples tend to dry out within 10 minutes or so of being mounted. You will want to mount one coverslip and visualize it, before mounting subsequent coverslips. Make sure you dispose of the sample properly, as there are live chlamydial organisms on the slide.

7. Focus using a 60X objective and brightfield; then, visualize the sample using a 488/FITC filter.

At this stage, you can collect data by snapping images with a camera attached to the microscope or collect 'lux' measurements (to measure the intensity of the retained lipid within the inclusion) using the parameters of the microscope. The C₆-NBD-fluorescence bleaches very quickly, which is why you focus on your sample first using brightfield.

For polarized cells:

1. After 30 minutes to overnight after back-exchange, remove the tissue culture plate from the cell culture incubator and transfer to a biosafety cabinet closest to the fluorescent microscope.
2. Decant the liquid from the insert and invert the insert either on a Kimwipe or in the lid of the tissue culture dish.
3. Use the scalpel to remove the filter from the insert housing.
4. Using the forceps, dip the filter twice in HCMF.
5. Place the filter cell-side up onto a glass slide and cover the filter with a coverslip.
6. Visualize the sample using a 488/FITC filter.

It is highly recommended that you use a fluorescent microscope (like a confocal microscope) in which you can control the intensity of the 488 wavelength that your sample is exposed to. Due to low resolution of samples seeded on fibrillar collagen filters, you will likely be unable to focus on a chlamydial inclusion using brightfield microscopy. To minimize bleaching of the sample, set the intensity for the fluorescent filter (or preferably a laser) on a low setting that will allow you to focus on the inclusions without significant bleaching of the sample.

BASIC PROTOCOL 3. LIPID EXTRACTION

The lipid extraction procedure described here is directly derived from the classical Bligh and Dyer method of lipid extraction (Bligh and Dyer, 1959). This method will efficiently extract most lipids from both eukaryotic and prokaryotic cells. Ultimately, the lipids extracted by this method can be resolved by thin layer chromatography, high performance liquid chromatography (HPLC) or by gas chromatography methods. The latter methods are highly specialized and require expensive equipment. In contrast, the requirements to perform TLC are relatively inexpensive and easily accessible. The TLC method in which you resolve these lipids will dictate which species of lipids you will be able to visualize (Fried and Sherma, 1999). The thin layer chromatography (**TLC**) method described here will efficiently separate/resolve the species of ceramide, glucosylceramide and sphingomyelin (Moore et al., 2008).

Materials

Nitrile gloves, at least 8 mil (essentially, chemically-resistant PPE)
 Fume Hood
 Chemically resistant glass vials with lids, 4 ml, 12 ml, 20 ml
 Chloroform, thin layer chromatography grade
 Methanol, thin layer chromatography grade

dH₂O, greater than 15 mΩ-cm resistivity, autoclaved

Glass serological pipettes

Vortex with large/ampule tube attachment (optional)

Glass Pasteur pipettes and bulb

Thermo Reacti-Therm and Reacti-Vap manifold (This is an apparatus that gently heats the glass vials and allows nitrogen gas to dry the lipid extracts)

Thin Layer Chromatography Plates, Silica Gel 60 Å, 20 × 20 cm

Ruler

Pencil

Glass scoring pencil with tungsten carbonide tip

Oven

Hamilton syringe

Drummond microcaps

Standards (eg. NBD-sphingomyelin, Matreya, Inc., Pleasant Gap, PA)

TLC developing chamber (many of these chambers exist, a 'Latch Lid' model, such as provided by Sigma or Camag will limit the evaporation of solvents used to develop TLC plates)

1. Harvest samples to be extracted into an appropriate glass vial. All following steps are performed in a fume hood.

The capacity of the glass vial should be determined by the final volume of the lipid extraction. For example, for a 2 ml sample, you will need the capacity to hold a final volume of at least 11 ml. If you would like to 'normalize' your samples, at this step add 100 ng/ml of NBD-lactosylceramide per sample. If you perform densitometry after step 16, you can use the NBD-lactosylceramide as a 'loading control'.

2. Using a glass serological pipette, add equal amount of volume, of the original sample, of chloroform. Add twice the original volume of methanol.

For a 2 ml sample, you will add 2ml chloroform and 4 ml methanol.

3. Invert or vortex for 2 minutes.
4. Add the same amount of volume of chloroform as in step 2. Vortex for 30 seconds.
5. Add half the original volume of dH₂O and vortex for 30 seconds.

Adding water at this step helps to precipitate any protein in the sample, which will help you visualize the boundary between the chloroform and methanol phases, as it occurs between the chloroform and methanol phases.

6. Transfer to 4°C, to allow the layers to separate.

As the layers separate the white protein precipitate will become a distinct band separating the methanol (upper) phase from the chloroform (lower) phase. Both the methanol and chloroform phases will be 'clear' and non-viscous. Occasionally, the protein precipitate will form a 'web' like

pattern around the glass in the chloroform phase, this will not prevent sample extraction.

7. Using a Pasteur pipette, remove the lower chloroform layer and transfer to a fresh 4 ml glass vial.

If your chloroform phase is greater than 4 ml, dry down the chloroform phase in stages to concentrate the final sample. It is very important to not remove any of the methanol layer at this stage. To err on the side of caution, you can leave a small amount of the chloroform stage within the glass vial.

8. Using the Reacti-Vap and Reacti-Therm manifold, dry the samples under a stream of nitrogen and moderate heat.
9. Using the glass scoring pencil, and a ruler, cut the 20 × 20 cm plate into 4 squares.
10. On one plate, with a pencil, make a line one inch from the bottom of the plate. And also mark where you will be placing samples. Do not place samples any closer than 0.5 cm.
11. Dry the TLC plate in a 65°C oven for at least 30 minutes. Allow the plate to cool.

This step is particularly important in areas of high humidity. Any moisture on the plate will hinder the migration of the resolving solution up the TLC plate.

12. With the Hamilton syringe, resuspend the dried lipid extraction samples with a 2:1 chloroform:methanol solution.

Using the above starting volumes, one can resuspend in 20–40 µl of 2:1 chloroform:methanol.

13. Using a microcap, load the sample onto the TLC plate. Be consistent in how you load the samples. Also load standards, such as NBD-ceramide, NBD-glucosylceramide and NBD-sphingomyelin.

Typically, you load 10–20 µl of sample and 1–2 µl of 20 µg/ml of standard.

14. In the latch-lid TLC chromatography chamber combine, 32.5 ml chloroform, 12 ml methanol, and 2 ml dH₂O. Gently swirl tank to combine the liquids. Be careful not to splash the lid.
15. Load TLC plate and allow the solvent to resolve the plate up to two inches from the top.

It is important that the solvent migrates in a straight horizontal line up the TLC plate, in order to resolve the samples at the same rate. If solvent is running in a 'crooked' or haphazard manner, remove the TLC plate and repeat step 11. Placing the plate, loaded with samples, in the oven will not compromise the samples.

16. The TLC plate can be visualized by exposure to UV light.

You can use a common 'gel doc' or Typhoon imager to capture the image of the TLC plate in order to do densitometry of the samples. To visualize endogenous or radiolabeled lipids, TLC plates can be run as described above, but, in the fume hood, you will need to spray the TLC plate with Molybdenum spray (Sigma). Then char the plate on a hot plate in the fume hood.

17. You can store the plate long-term, by wrapping the plate in aluminum foil and placing in an -20°C freezer.

REAGENTS AND SOLUTIONS

EMEM+ 0.7% dfBSA

100 ml EMEM

0.7 g de-fatted BSA (Sigma)

Mix until BSA goes into solution

Filter sterilize

Store in dark at 4°C for up to 1 month

EMEM + 0.035% dfBSA

2.5 ml filter-sterilized EMEM + 0.7% dfBSA

47.5 ml EMEM

Mix well

Filter sterilize if necessary

Store in dark at 4°C for up to 1 month

HCMEF, 10 mM HEPES-buffered calcium and magnesium-free Puck's saline

1.8 ml 3M KCL

11 ml 100 mM KH_2PO_4

8.01 g NaCl

10.8 ml 100mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

2.38 g HEPES

H_2O to 1 L

Adjust pH to 7.4

Autoclave 20 min

Store indefinitely at RT out of direct sunlight

C_6 -NBD-ceramide stock solution, 5 mM

Dissolve 1mg C_6 -NBD-ceramide (Molecular Probes, N-1154) in 340 μl ethanol.

Vortex vigorously

Store at -20°C for up to 6 months.

SPG

Refer to *UNIT 11A.1* (Scidmore, 2005).

COMMENTARY

Background Information

The principles of examining lipid trafficking using fluorescent lipid metabolites—Given the obligate intracellular nature of *C. trachomatis*, advances in chlamydial biology mirror the advances in cell biology. In the early 1980's, Naomi Lipsky

and Richard Pagano began using C₆-NBD-ceramide to examine lipid trafficking dynamics in cultured fibroblasts (Lipsky and Pagano, 1983, 1985a, b). In painstaking characterization of C₆-NBD-ceramide trafficking, they found that it is metabolized in equivalent quantities of fluorescent sphingomyelin and glucosylceramide (Lipsky and Pagano, 1983). Initial studies demonstrated that at 2°C, C₆-NBD-ceramide indiscriminately labels intracellular membranes, such as the endoplasmic reticulum, nuclear membrane and mitochondrial membrane. However, at 37°C, C₆-NBD-ceramide, labels the Golgi apparatus, where it undergoes metabolism into the aforementioned products (Lipsky and Pagano, 1985a, b). An additional feature of these fluorescently labeled lipids, is ability to harvest or 'back-exchange' the products once they reach the plasma membrane. To back-exchange NBD-labeled lipids, there needs to be an appropriate acceptor available in the culture medium, such as unlabeled dioleoyl phosphatidyl choline (DOPC) vesicles (Lipsky and Pagano, 1985a) or dfBSA (Hackstadt et al., 1995). Therefore, one can label cells with a defined substrate and track its metabolites throughout the cell.

In the mid 1990's, Hackstadt and colleagues labeled chlamydial infected cells with C₆-NBD-ceramide and made the following fundamental observations: ¹. fluorescent lipid was transferred from the Golgi to the chlamydial inclusion; ². chlamydial infected cells retained greater amounts of fluorescent lipid than uninfected cells; ³. the fluorescent lipid was being incorporated into the cell wall of chlamydial organisms; ⁴. the fluorescent lipid is sphingomyelin, not ceramide or glucosylceramide (Hackstadt et al., 1995). This seminal work began a new era for chlamydial biology and the principles and methods established in this publication have been applied to identify key mechanisms in chlamydial sphingolipid acquisition.

Current understanding of sphingolipid delivery to the chlamydial inclusion—

While it is difficult to study chlamydial growth and development in cells devoid of sphingolipid metabolism due to the poor viability and growth of sphingolipid-free cells, several studies have convincingly demonstrated that chlamydial organisms require host-derived sphingolipids for survival (Robertson et al., 2009; vanOoij et al., 2000). Sphingomyelin acquisition by the chlamydial inclusion is temperature dependent, requires energy and chlamydial protein synthesis, is partially brefeldin-A sensitive, and is conserved across chlamydial species (Hackstadt et al., 1996; Rockey et al., 1996; Wolf and Hackstadt, 2001). The chlamydial inclusion acquires sphingomyelin by intercepting Golgi-derived exocytic vesicles (Hackstadt et al., 1996) or interacting with multivesicular bodies (Beatty, 2006).

Because of the available cell biology tools (like C₆-NBD-ceramide), Golgi-derived sphingomyelin is the best studied pathway regarding chlamydial sphingomyelin acquisition, and acquisition of sphingomyelin via this pathway is initiated a few hours after chlamydial entry into host cells (Hackstadt et al., 1996). Use of a polarized cell model demonstrated that Golgi-derived sphingomyelin is acquired from the basolaterally targeted exocytic pathway (Moore et al., 2008), indicating a specificity to the interactions with the chlamydial inclusion and the exocytic pathway. Through interrogation of specific Golgi-associated Rab proteins relative to *C. trachomatis* infection, several Rabs have been found to play a role in chlamydial sphingomyelin acquisition: Rab 14 (Capmany and Damiani, 2010), Rab 6 and Rab 11 (Lipinski et al., 2009). Interestingly, the localization of Rab proteins to the chlamydial inclusion is not conserved across chlamydial species (Moorhead et al., 2007; Rzomp et al., 2006; Rzomp et al., 2003), suggesting that recruitment and chlamydial manipulation of Rab proteins is not a conserved mechanism associated with sphingomyelin acquisition.

Recent studies have indicated that *C. trachomatis*-induced fragmentation of the Golgi via cleavage of golgin-84 may also contribute positively to chlamydial acquisition of sphingomyelin (Heuer et al., 2009); however, cleavage of golgi-84 and subsequent Golgi fragmentation is not noted until 24 hours post-infection (Heuer et al., 2009). These data suggest that Golgi fragmentation could have a role in sustaining lipid acquisition, as opposed, to initiating it. While previous mechanisms have been limited to studies examining *C. trachomatis*, the SRC family kinase Fyn, likely impacts sphingolipid lipid acquisition of multiple chlamydial species. siRNA knockdown of Fyn, decreased sphingomyelin trafficking to *C. trachomatis* and *C. caviae* (Mital and Hackstadt, 2011). Studies interrogating non-brefeldin-A sensitive sphingomyelin trafficking pathways found that CERT, an ER-associated ceramide transfer protein, is recruited to the chlamydial inclusion and involved in chlamydial sphingomyelin acquisition (Derre et al., 2011; Elwell et al., 2011). Combined these studies highlight the necessity of chlamydial sphingolipid acquisition as multiple cellular mechanisms contribute to the overall process.

While there are multiple redundant mechanisms controlling sphingolipid trafficking to the inclusion, as this is a process necessary for chlamydial survival, it is unknown exactly the process that allows the organisms to obtain the lipid once the lipid is delivered to the chlamydial inclusion membrane. In addition to sphingomyelin, the presence of host-derived phosphatidylcholine, phosphatidylinositol and cholesterol have been found within the chlamydial cell wall (Carabeo et al., 2003; Hatch and McClarty, 1998; Newhall, 1988; Wylie et al., 1997). Additionally, the reason behind chlamydial acquisition of host lipids is unknown, although it likely contributes to the ability of the organism to avoid host immune defenses and efficiently enter eukaryotic host cells (AbdelRahman and Belland, 2005; Simonetti et al., 2009). And while, this Unit focuses on chlamydial acquisition of Golgi-derived host lipids, the inclusion membrane also requires host-derived lipids to create the specialized niche which supports chlamydial growth and development. Hence, there are many important questions that remain to be answered and will positively contribute the understanding of chlamydial biology.

Critical Parameters

Handling organisms—If you are unfamiliar to working with chlamydiae, it is important to consult *UNIT 11A.1* (Scidmore, 2005) prior to performing any experiments. Additionally, when considering experiments involving *Chlamydia*, one must think about their developmental cycle. Chlamydiae have evolved a unique biphasic developmental cycle. To initiate an infection, the infectious metabolically dormant form, termed the elementary body (**EB**), is endocytosed by the host cell and remains within a vesicle termed the inclusion. Within the inclusion, the EBs differentiate into metabolically active, noninfectious reticulate bodies (**RBs**). The chlamydial developmental cycle, with the exception of the last step, occurs exclusively within the membrane-bound chlamydial inclusion. Within the inclusion, *Chlamydia* rapidly multiply and redifferentiate from RBs to EBs to perpetuate the infectious cycle (reviewed in (AbdelRahman and Belland, 2005)). Towards the end of the developmental cycle, the inclusion consumes the entirety of the host cell cytoplasm. Therefore specific experimental questions must be put in the context of the chlamydial developmental cycle. For example, if performing live cell imaging in *C. trachomatis* serovar L2 infected cells, 16 to 18 hours post-infection is the perfect time frame to be able to visualize the chlamydial inclusion while most host cell structures remain intact. In contrast, when examining trafficking patterns or quantifying chlamydial lipid acquisition waiting towards the end of the development cycle (~42 hours or greater for *C. trachomatis* serovar L2) will maximize the EB yield.

Cell type—The prototypical cell line used in most chlamydial studies is the HeLa cells. These cells are readily infected by chlamydial organisms and are incredibly easy to culture. However, HeLa cells do not express many proteins involved in polarized cell trafficking pathways (Moore et al., 2008; Moore et al., 2011); therefore depending on which experimental questions you are asking, it might be necessary to evaluate the eukaryotic cell line used.

Experimental design—Consult Table 1 when thinking about the type of experimental question you would like to answer.

Troubleshooting

For troubleshooting issues regarding the culture of *C. trachomatis*, please consult *UNIT 11A.1* (Scidmore, 2005). Table 3 covers many of the common problems encountered with the methods, including their possible causes and solutions.

Anticipated Results

Keep in mind that there are many redundant pathways in chlamydial sphingolipid acquisition. It will be virtually impossible to obtain a complete loss of chlamydial sphingolipid acquisition. Any change in chlamydial sphingomyelin should be noted. The mechanisms thought to play a significant role in chlamydial sphingolipid acquisition, generally, yield a 60% or greater decrease in sphingomyelin acquisition.

Time Considerations

When considering the time frame in which these experiments can be completed, several factors must be considered: ¹. how will you set up the cells; ². at what time point post-infection will you be analyzing the chlamydial infected cells; ³. which end assay will you be choosing? It is important to think through all of these steps before setting up cells for an experiment.

Seeding subconfluent cells will take between 24–48 hours prior to infection. Polarization of cells will take 4 days to several weeks, depending on the cell line used. siRNA knockdown of cells will have to be determined empirically, but typically require 48–72 hours for efficient knockdown.

Live cell imaging (typically performed on cells ~18 hours post-infection), including labeling, will take 3 to 4 hours. The small scale chlamydial purification will take around 6 to 7 hours from start to finish. Lipid extraction of cell extracts or purified EBs (usually performed ~36–48 hours post-infection) will take 1 to 2 hours, followed by thin layer chromatography, which takes about an hour from start to finish.

Acknowledgments

I would also like to thank Emily McDowell, Andrea Lucas and Maryam Salehi for critical evaluation of this Unit. E. Moore is funded by NIH NIAID K22AI089856 and by The University of South Dakota Division of Basic Biomedical Sciences start-up funds.

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Table 1

Eukaryotic cell seeding-conditions by application

Assay	Cell Type	Tissue Culture Plate/Dish Style	Culture Medium	Cell density	Total volume	Applicable protocols
Live Cell Imaging	HeLa	24-well plate w/coverslips	RPMI, 10% FBS	1×10^5 cells/ml	1 ml	Basic Protocol 1 Support Protocol 1 Basic Protocol 2
	C2BBel-unpolarized	24-well plate w/ coverslips	DMEM, 10% FBS, 4mM L-glutamine, 0.01 mg/ml human transferrin (DMEM complete)	2×10^5 cells/ml	1 ml	Basic Protocol 1 Support Protocol 1 Basic Protocol 2
	C2BBel-siRNA KD	24-well plate w/coverslips	DMEM complete	8×10^4 cells/well	600 μ l	Basic Protocol 1 Support Protocol 3 Basic Protocol 2
	C2BBel-polarized	6.5 mm fibrillar collagen transwells	DMEM complete	0.4×10^6 cells/ml	600 μ l in insert	Basic Protocol 1 Support Protocol 2 Basic Protocol 2
Lipid Extraction of Cell Extracts	HeLa	6-well plate	RPMI + 10% FBS	1×10^5 cells/ml	3.0 ml	Basic Protocol 1 Support Protocol 1 Basic Protocol 3
	C2BBel-unpolarized	6-well plate	DMEM complete	2×10^5 cells/ml	3.0 ml	Basic Protocol 1 Support Protocol 1 Basic Protocol 3
	C2BBel-siRNA KD	6-well plate	DMEM complete	4×10^5 cells/well	2.5 ml	Basic Protocol 1 Support Protocol 3 Basic Protocol 3
	C2BBel-polarized	24 mm fibrillar collagen transwells	DMEM complete	0.4×10^6 cells/ml	1.1 ml in insert	Basic Protocol 1 Support Protocol 2 Basic Protocol 3
Lipid Extraction of purified EBs	HeLa	4 T-150 flasks	RPMI + 10% FBS	1×10^5 cells/ml	30 ml	Basic Protocol 1 Alternative Protocol 1 Support Protocol 1 & 4 Basic Protocol 3
	C2BBel-unpolarized	4 T-150 flasks	DMEM complete	2×10^5 cells/ml	25 ml	Basic Protocol 1 Alternative Protocol 1 Support Protocol 1 & 4 Basic Protocol 3
	C2BBel-siRNA KD	4 T-150 flasks	DMEM complete	80×10^5 cells/flask	20 ml	Basic Protocol 1 Alternative Protocol 1 Support Protocol 3 & 4 Basic Protocol 3
	C2BBel-polarized	(4) 75 mm fibrillar collagen transwells	DMEM complete	0.8×10^6 cells/ml	10 ml in insert	Basic Protocol 1 Alternative Protocol 1 Support Protocol 2 & 4 Basic Protocol 3

Table 2

Standard conditions for reverse transfection of mammalian cells.

Plate size	Amount of Opti-Mem	Amount of Lipofectamine	Concentration of cells/well	Final total volume
24-well	100 μ l	1 μ l	9.6×10^4 cells/500 μ l/well	600 μ l
12-well	250 μ l	3 μ l	10×10^4 cells/0.75 ml/well	1 ml
6-well	500 μ l	5 μ l	4.8×10^5 cells/2ml/well	2.5 ml
T-150 flask	4 ml	40 μ l	96×10^5 cells/16 ml/flask	20 ml

These culture conditions were adapted from the manufacturer protocols provided by Life Technologies and Ambion and optimized for use in C2BBel cells. If you are using a different cell line, you may want to consult the companies' websites and optimize these conditions to your cell line.

Table 3

Troubleshooting Guide for Sphingolipid Trafficking and Purification in *Chlamydia trachomatis*-infected cells

Problem	Possible Cause	Solution
Inefficient labeling of cells	C ₆ -NBD-ceramide came out of solution	Vortex vigorously, until almost all of the C ₆ -NBD-ceramide is in solution
	C ₆ -NBD-ceramide is old	Make fresh stock solution
Inefficient back-exchange of C ₆ -NBD-ceramide	The EMEM-BSA mix is old	Make fresh stock solution
Inefficient labeling of cells with <i>N</i> -1-[¹⁴ C]oleoy-D-sphingosine	Used normal growth medium instead of medium supplemented with dfFBS	Make fresh medium with dfFBS.
	Radiolabel may be old	Using a scintillation counter verify that the stock of <i>N</i> -1-[¹⁴ C]oleoy-D-sphingosine has measurable counts
Debris on glass coverslips	Coverslips were not purchased pre-washed	Acid-wash the coverslips, then autoclave
Epithelial cells will not polarize	Tissue culture inserts may be old	If inserts appear to have build-up, then they are likely old or defective. Buy new ones
	Cells have been overly passaged or incorrectly cultured	Cells to be polarized are highly particular in how they are cultured and passaged. For example, C2BBE1 cells will not polarize beyond 22 passages from a frozen stock. Wake up a fresh stock of cells.
	Additives in the medium have gone bad	Exposure to light and heat will decrease the efficacy of additives in medium that supports polarization of cells. Aliquot original stock or make fresh weekly. Don't warm medium in a 37°C waterbath.
High amounts of cell death after siRNA transfection	Too much transfection reagent	Empirically determine the amount of transfection reagent to use for your cell line.
	Did not change medium	Cells should not be left in transfection medium longer than 24 hours. (<i>Unless using an Accell (Dharmacon) transfection protocol.</i>)
	siRNA is toxic to the cell	Empirically determine the appropriate concentration of siRNA to use for each knockdown.
	Cells have been overly passaged	Same principle described for difficulty in getting cells to polarize. Wake up new cells.
No pellet in the final step of small scale EB purification	Low EB yield	This is quite common and not indicative of a failed experiment; continue with experiment as planned
Low EB yield from small scale EB purification	Gradient not poured correctly	In step 12, it is important that you see a division between all concentrations of renografin. If you cannot see the division, pour the gradient out and start again.
	Did not harvest at correct time in the developmental cycle	Always harvest towards the end of the developmental cycle to maximize the EBs remaining in the monolayer; this will vary depending on the serovar used in the study. Consult <i>UNIT 11A.1</i> (Scidmore, 2005) for more details
Cannot tell chloroform layer from methanol layer in lipid extractions	There is not enough protein in the sample.	Add a small amount of water containing dissolved defatted BSA and remix; allow the layers to separate.
There are 'oily bubbles' on top of the chloroform layer to be dried down	It is contaminated with the methanol layer.	Repeat extraction or pull off 'oily bubbles' with Pasteur pipette
The solvent is running haphazardly or crooked on the TLC plate.	The TLC was not properly dried or completely dried prior to loading samples	Place TLC plate back in 65°C oven for at least ~15 minutes