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Monitoring sphingolipid trafficking in cells using fluorescence microscopy

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Abstract

Sphingolipids are structural components of organelle membranes that also participate in signal transduction pathways. Complex sphingolipids are trafficked from their site of synthesis in organelles of the early secretory pathway to the Golgi apparatus, the plasma membrane, and the endo-lysosomal system. We have developed fluorescence microscopy-based methods to monitor sphingolipid trafficking in coordination with secretory protein sorting. A sphingomyelin binding protein fused to a fluorescent protein, which we term “EQ-SM”, is implemented to monitor sphingomyelin trafficking from the Golgi apparatus to the plasma membrane via secretory vesicles. A protocol is provided to determine if a query protein of interest is secreted from the cell via vesicles enriched in EQ-SM, an indication that the vesicle membrane is enriched in sphingomyelin. A complementary protocol is described that implements a chemically modified form of sphingosine, a metabolic precursor to complex sphingolipids, to visualize ceramide and complex sphingolipids in fixed cells.

Keywords

sphingolipid; sphingomyelin; Golgi apparatus; secretion; equinatoxin; click chemistry; pacSphingosine; fluorescence microscopy

Introduction

Organelle membranes of the eukaryotic cell possess distinct, characteristic lipid compositions that are vital for the proper functioning of each organelle (van Meer, Voelker, & Feigenson, 2008). Most lipids are synthesized by enzymes that reside in organelles of the early secretory pathway and they are subsequently distributed to other organelles via vesicle-mediated and non-vesicle-mediated transport pathways. Elucidating how lipids are sorted and trafficked to the correct location within the cell, and how this is integrated with protein sorting, is key for understanding organelle biogenesis pathways.

The Golgi apparatus is the central hub of the intracellular trafficking network. Complex sphingolipid synthesis is completed in the Golgi apparatus, and in the final compartment of the Golgi apparatus, the *trans* Golgi network (TGN), proteins and sphingolipids are loaded

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into vesicular and tubular carriers to be trafficked to other organelles. Whereas a great deal has been learned about the selection of proteins to be included in transport carriers, very little is known regarding the lipid composition of transport carriers. Recent data from our laboratory indicates that a specialized arm of the secretory pathway mediates Golgi-to-plasma membrane (PM) trafficking of sphingomyelin, the most abundant sphingolipid of the cell (Deng, Rivera-Molina, Toomre, & Burd, 2016); we refer to this as the ‘SM trafficking pathway’.

Monitoring lipid trafficking in cells is complicated by the properties of lipids. They are not genetically encoded as proteins are, so it is not possible to tag them with fluorescent proteins to enable direct visualization within the cell by light microscopy. Early studies of lipid trafficking employed fluorescently labeled lipids, but the masses of the fluorescent moieties of these molecules are typically nearly as large as the lipid itself and often render the labelled lipid a poor metabolic substrate. Thus, the degree to which they report the trafficking of native lipid species is not firmly established. The identification of protein structural domains that recognize certain lipid headgroups has been enormously useful for establishing the intracellular locations of lipids (Maekawa & Fairn, 2014; van Meer & Holthuis, 2000), especially phosphoinositides, which are low abundance signaling lipids whose synthesis and turnover are tightly controlled by enzymes that localize to specific organelles throughout the cell. The use of lipid-binding protein probes has been implemented chiefly for investigating lipids that reside in the cytoplasmic leaflets of organelle membranes. By contrast, sphingolipids reside nearly entirely in the exofacial/luminal leaflets of organelle membranes, so this approach cannot be exploited in the same straightforward manner.

Sphingomyelin (SM), the most abundant sphingolipid in the cell, is synthesized primarily in the inner membrane leaflet of the TGN, but the PM harbors most of the SM, indicating that newly synthesized SM is trafficked to the cell surface (Holthuis & Menon, 2014). To understand the trafficking of native sphingomyelin species in the cell, we developed two protocols that allow visualization of endogenous SM and other sphingolipids. The first method uses a fluorescent protein probe that is derived from a natural SM-binding protein, Equinatoxin II, produced by a sea anemone, *Actinia equina*. Mutations that diminish Equinatoxin toxicity and that enhance the protein’s specificity for SM were introduced to engineer the final SM probe, termed ‘EQ-SM’ to indicate that it is derived from Equinatoxin II and has specificity for binding sphingomyelin (Deng et al., 2016). This method is ideal to study trafficking of SM from the TGN to the PM, and we describe its application in determining if a protein of interest is enriched in secretory vesicles containing the SM probe using total internal reflection microscopy (TIRFM). It is important to note that the SM probe is ill-suited to answer bimodal questions such as whether or not SM is localized to a specific region of the secretory pathway. Researchers interested in answering such questions should instead see the second method which uses a commercially available ‘clickable’ sphingosine that was originally developed by Haberkant et al; it contains a terminal alkyne chemical bond that can be chemically modified with fluorescent moieties to facilitate in situ detection by fluorescence microscopy (Haberkant et al., 2016). When applied to cells, this reagent serves as an enzymatic precursor to complex sphingolipids. In parallel, these two methods

provide new avenues for studying complex sphingolipid trafficking from the site of synthesis in the Golgi to the cell surface.

Strategic Planning

Because SM is largely restricted to the luminal/exofacial leaflets of cellular membranes, in order for EQ-SM to access SM, the probe is first directed to the entry point to the secretory pathway, the endoplasmic reticulum (ER), via an N-terminal signal sequence derived from human growth hormone (Figure 1). EQ-SM is trafficked from the ER to the Golgi apparatus and then exported from the Golgi apparatus and delivered to the PM (Figure 2). We have shown that depletion of the Golgi SM synthase (SMS1) by siRNA results in delayed export of EQ-SM from the Golgi, indicating that ongoing SM synthesis is required for efficient secretion of EQ-SM (Deng et al., 2016). Nevertheless, EQ-SM cannot by itself be used to infer the presence of SM in a particular compartment, including a secretory vesicle. Rather, at present it must be used in combination with other reporter molecules (lipids or proteins) to determine if EQ-SM and a given query molecule are co-sorted into the same transport carrier, either as they bud from the TGN or as they fuse with the plasma membrane during exocytosis. Furthermore, query protein sorting with EQ-SM should be compared to sorting with the related equinatoxin-derived probe (EQ-sol) that does not bind lipid (hence, it is soluble) and is secreted by default (Deng et al., 2016). An additional constraint on the use of the EQ-SM probe is that it must be used to analyze a population of events (vesicle budding or exocytosis) rather than a single event. The assay therefore implements population statistics to determine whether a protein of interest is preferentially co-packaged with EQ-SM compared to EQ-sol.

Design of the query protein

To study whether a protein of interest is co-sorted with EQ-SM, there are a few considerations to take into account when designing the query protein construct. In our assay, we tag the query protein with a pH-sensitive fluorophore, pHluorin (Miesenbock, De Angelis, & Rothman, 1998). The fluorescence intensity of pHluorin increases dramatically upon exposure of the fluorophore to the near neutral pH of the imaging media after exocytosis. This allows one to confidently distinguish fusion events from docked vesicles that are within the TIRF field of illumination. In order to take advantage of the pH-sensitivity of pHluorin, the construct must be designed so that the fluorophore is fused to the extracellular portion of the query protein. For some proteins, this means pHluorin will be located at the N-terminus of the query protein. If this is the case, pHluorin should be located after the endogenous signal sequence to ensure that the protein is still targeted to and correctly oriented at the ER. Before proceeding with a sorting experiment, it is worthwhile to assess the expression and trafficking of the query protein. We routinely conduct a titration experiment to determine the minimal amounts of plasmids necessary for sufficient expression to detect the equinatoxin-derived proteins and the query protein with fluorescence imaging, in order to minimize the possibility of saturating sorting mechanisms.

Visualizing sphingolipid in situ

The use of photoactivatable and clickable Sphingosine (pacSph) to visualize sphingolipids requires cells that are deficient in sphingosine 1-phosphate lyase activity. Pioneering studies used *SGPL1*^{-/-} mouse cells generated by a genetic knockout strategy (Colie et al., 2009; Haberkant et al., 2016) and more recently in cultured human cells using CRISPR/Cas9 gene editing approaches (Gerl et al., 2016). In our lab, we have used the CRISPR/Cas9-based method to generate SGPL1 null HeLa cells. To apply method 2 in other cells requires that the SGPL1 locus be modified to eliminate this enzymatic activity.

Basic Protocol 1: Detecting secretion of proteins in sphingomyelin-containing vesicles using an SM-binding protein

This protocol describes the use of SM-binding protein, EQ-SM, to test whether a query protein is secreted via vesicles enriched in SM. The assay described relies on comparison of query protein secretion with EQ-SM versus EQ-sol, which does not recognize SM. All plasmids are transiently transfected into cells and single exocytic events are recorded using TIRFM. Details of the assay will differ depending on the cell types that are used and the query proteins to be tested. We provide two protocols for transfection that we have found to be effective with our query proteins, but other protocols for transfection will also be compatible with the assay.

Materials

Dulbecco's Modified Eagle Medium (DMEM)
DMEM + 10% Fetal Bovine Serum (FBS)
Opti-MEM
HeLa cells
Tissue culture hood
Tissue culture incubator
Lipofectamine 2000 or Fugene HD
Glass Bottom Culture Dishes (MatTeK, P35GC-1.5-14-C)
EQ-SM-mKate2 plasmid, available from the authors
EQ-sol-mKate2 plasmid, available from the authors
Plasmid that directs expression of the secreted cargo protein (query protein) as a fusion to pHluorin (Ii-str_SS-SBP-pHluorin-GPI is used in the example)
Live Cell Imaging Solution (Molecular Probes) with 10 mM glucose
1x PBS
TIRF microscope equipped with an environmental chamber (temperature is controlled at 37 °C) and sCMOS camera.

Image J
Excel
D-Biotin (Sigma)

Sample preparation for TIRF microscopy: transfection of plasmids encoding pHluorin-tagged query protein with EQ-SM-mKate2 or EQ-sol-mKate2

- 1 Plate 0.5×10^5 HeLa cells on MatTek imaging dishes one day before transfection.
- 2 On the day of transfection, in a fresh tube add 0.5 μ L lipofectamine 2000 to 100 μ L opti-MEM.

For transfection, both Lipofectamine and Fugene HD are used by our lab. When transfecting up to two plasmids, we prefer Fugene HD because it allows for a high transfection efficiency with relatively low expression levels in transfected cells (see Alternate Protocol 1). For three or more plasmids, lipofectamine results in a higher rate of co-transfection compared to Fugene HD

- 3 Add 0.5 μ g Ii-str_SBP-pHluorin-GPI plasmid and 0.2 μ g EQ-SM-mKate2 or EQ-sol-mKate2 plasmid to 100 μ L opti-MEM.

When working with a new cargo plasmid, the amount of transfected DNA should be titrated so that the expression levels of cargo protein and EQ-SM are similar. It is advisable to determine the minimum expression level that still yields a strong enough signal to observe fusion events to minimize the possibility of saturating the sorting pathway of your query protein.

- 4 Combine lipofectamine and DNA solutions and incubate at room temperature for 5 min.
- 5 Remove medium from the imaging dish and add 1 mL of fresh DMEM + 10% FBS. If you are transfecting a RUSH plasmid, add 1.5 μ L Avidin (1mg/mL) to medium as well.

For abundant query proteins that localize to the PM, it may be difficult to image single exocytic events due to the magnitude of the fluorescence of the pre-existing PM pool of the protein. If this is the case, we recommend using an experimental approach in which the query protein is retained in the ER (Figure 3) and released by the investigator with the addition of a small molecule to the medium. We have found the retention using selective hooks (RUSH) system to be ideal for this purpose (Boncompain et al., 2012; Boncompain & Perez, 2012). When using a RUSH construct, the addition of Avidin to the transfection medium improves protein retention in the ER by binding any biotin that is present in the cell culture medium.

When designing a new RUSH cargo construct, you will want to confirm that your cargo protein is initially retained in the ER and then released following the addition of biotin. We find that different hooks (Ii or KDEL) are better at retaining different cargo proteins. Additionally, if your construct is not well-retained in the ER, it may be useful to add a linker between the streptavidin binding protein (SBP) sequence and your cargo protein sequence to ensure that the SBP is accessible for interaction with the streptavidin hook in the ER lumen.

- 6 Add Lipofectamine/DNA mixture to cells dropwise with gentle swirling to mix.

When EQ-SM or EQ-sol are expressed by transient transfection in HeLa cells, we do not observe cell death associated with their expression. However, due to differences in the lipidomes of other cell types, this should be tested. If cell death is an issue, we recommend reducing the amount of lipofectamine or of EQ-SM plasmid that is used.

- 7 Incubate cells in a 5% CO₂ incubator at 37°C for overnight or 16 hours.

We always image cells 16–24 hours after transfection as a longer transfection time may result in a strong EQ-SM signal on the cell surface. This elevated background signal can obscure visualization of newly delivered vesicles.

Imaging of sphingomyelin probe and query protein exocytosis by total internal reflection fluorescence microscopy (TIRFM)

- 8 For live cell imaging wash the cells twice with PBS. Then add Imaging Solution (supplemented with 40 μM D-biotin to release RUSH cargo proteins).
- 9 Add a drop of immersion oil to the 100X microscope objective. Place cells on microscope.
- 10 Using widefield illumination, search the field of view for cells that have a clear Golgi signal of both the query protein and EQ-SM or EQ-sol (for pHluorin-GPI in the RUSH system, the protein accumulates in the Golgi about 20–30 minutes after biotin addition).

While using the RUSH system, we have observed that the amount of time for a cargo protein to be released from the ER can vary for different cargo proteins, so the release time needs to be empirically determined. We find that most protein cargoes release from the ER and accumulate in the Golgi within 15–30 min of D-biotin addition. To capture fusion events, most RUSH constructs are imaged between 30 and 60 minutes post-biotin treatment. Within an hour, we typically image between 1 and 4 cells for an individual plate.

- 11 After identifying a cell, switch the illumination to the TIRF zone and wait several minutes to see if there are fusion events for your query protein. Cells

with a strong Golgi signal do not always have active secretion (see the note below). If pre-existing EQ-SM-mKate2 signal on the cell surface prevents you from observing new vesicle delivery, bleach the EQ-SM-mKate2 signal by increasing the laser power for 1 to 2 minutes until you can see individual vesicles move below the cell surface or fuse with the PM.

We have observed substantial variation in both the level of protein expression for transfected plasmids and the amount of secretory activity in a given cell. It is best to select cells with an easily visible Golgi-localized signal of both cargo and EQ-SM, but this does not ensure an optimal rate of secretion. Before acquiring a movie, it is important to make sure that both proteins are secreted, keeping in mind that the imaging dish must stabilize at 37 °C to see maximal secretory activity.

- 12 Obtain dual-color, high-frequency images (2Hz). We usually acquire 1200 frames within 3–4 minutes.

An individual cell should not be imaged for more than 10 minutes because photobleaching will substantially weaken the red signal.

Image analysis

- 13 Open two-color TIRF movie in ImageJ (Schindelin et al., 2012).
- 14 Split the channels and look only at the pHluorin signal to identify vesicle fusion sites by bright fusion flashes at the PM by eye.
- 15 Create a small circular region of interest around the fusion event and add it to the ROI manager to generate a list of fusion events in that cell. If a cell does not have at least 10 fusion events, do not include it in the analysis.
- 16 For each fusion event, the region of interest is examined in the red channel to see if the vesicle contains EQ-SM. It may be helpful to plot the fluorescence intensity of the region of interest in each channel to determine if EQ-SM signal increases with that of the pHluorin-tagged cargo (Figure 4).

To ensure that enough data are collected for a statistically valid analysis, you should consider the criteria for determining the total number of cells to include and the parameters for a given cell to be included in your dataset. The empirically determined criteria that we follow are: each cell included in the analysis must have at least 10 fusion events during time lapse image acquisition (no more than 10 minutes). Aggregate data from at least 10 cells, from two or more different transfections, are included in the analysis. We routinely distinguish a statistically significant ($p < 0.05$) difference between sorting of a query protein with EQ-SM or EQ-sol using these criteria.

Alternate Protocol 1: Cell transfection with Fugene HD

1. Plate 0.5×10^5 HeLa cells on MatTek imaging dishes one day before transfection.
2. Add 0.5 μ g Ii-str_SBP-pHluorin-GPI plasmid and 0.2 μ g EQ-SM-mKate2 or EQ-sol-mKate2 plasmid to 100 μ L opti-MEM.
3. Add 4 μ L Fugene HD to the DNA solution and incubate at room temperature for 20 min.
4. Remove medium from the imaging dish and add 1 mL of fresh DMEM + 10% FBS. If you are transfecting a RUSH plasmid, add 1.5 μ L Avidin (1mg/mL) to medium as well.
5. Add Fugene/DNA mixture to cells dropwise with gentle swirling to mix.
6. Incubate cells in a 5% CO₂ incubator at 37°C for overnight or 16 hours.

Basic Protocol 2: Visualization of sphingolipids in fixed cells using pacSphingosine and ‘click chemistry’

This protocol describes a procedure to directly visualize sphingolipids in cultured cells using a commercially available metabolic precursor to complex sphingolipids called pacSphingosine (trade name: PhotoClick Sphingosine), which was developed by Haberkant et al (2016). Using this reagent, we modified a pulse-chase labeling regimen based on that described by Haberkant et al (2016) to better visualize key aspects of the complex sphingolipid synthesis and trafficking pathways in which pacSphingosine is metabolized to ceramide, which is transported to the Golgi apparatus and then trafficked to the PM. In the time frame used for the analysis, Haberkant et al (2016) have shown that ceramide and SM are the major labeled species. After fixation and permeabilization of the cells, an alkyne group at the terminus of pacSphingosine is derivatized with a fluorescent moiety using ‘click chemistry’, thus enabling in situ visualization by fluorescence microscopy. This protocol was developed using HeLa cells in which the gene encoding sphingosine-1-phosphate lyase (*SGPL1*) has been inactivated (by gene editing) which is necessary to restrict pacSphingosine metabolism to the sphingolipid pathway (Haberkant et al., 2016). A methodology for using CRISPR/Cas9 gene editing technology to generate *Sgpl1* null cells is described in Gerl et al (Gerl et al., 2016).

Materials

SGPL1-KO cells – see Gerl et al (Gerl et al., 2016) for a gene editing strategy.

PhotoClick Sphingosine (pacSphingine, Avanti, 900600)

Click-iT™ Cell Reaction Buffer Kit (Thermofisher, C10269)

Alexa Fluor™ 647 Azide (Thermofisher, A10277)

Fetal Bovine Serum, charcoal stripped (delipidated FBS, Thermofisher, A3382101)

Glass Bottom Culture Dishes (MatTeK, P35GC-1.5-14-C)

Electron microscopy-grade paraformaldehyde (Electron Microscopy Sciences)

Saponin

Water bath sonicator

Label live cells with Photoactivatable and Clickable Sphingosine

- 1 Plate 0.5×10^5 HeLa cells on MatTek dishes one day before labeling.
- 2 Wash the cells twice with DMEM/delipidated FBS.
- 3 Incubate the cells with 0.5 μ M PhotoClick Sphingosine (pacSph) in DMEM/delipidated FBS (pre-warmed to 37°C) for 30 minutes at 37°C.

PhotoClick Sphingosine is dissolved in ethanol to a stock concentration of 6 mM. To prepare a working solution of PhotoClick Sphingosine, add 1 μ L of stock PhotoClick Sphingosine to 12 mL DMEM/delipidated FBS. Incubate the working solution at 37°C for 5 minutes, sonicate for 5 minutes, and incubate at 37°C for an additional 5 minutes. This step ensures that the lipid mixes homogenously in the medium.
- 4 Wash the cells three times with DMEM/delipidated FBS.
- 5 Incubate the cells with normal culture medium DMEM/FBS for 1 hour at 37°C.

Fix and label cells

- 6 Wash the cells with PBS three times
- 7 Fix the cells with 4% paraformaldehyde in PBS for 20 minutes.
- 8 Wash the cells three times with PBS.
- 9 Incubate the cells with permeabilization and blocking Buffer (0.1% saponin with 5% serum in PBS) for 1 hour. Wash with PBS.

Fixation and permeabilization methods must be chosen to minimize impact on the lipids. Methanol should not be used to fix cells as this may dissolve the lipids. Similarly, Triton detergents should be avoided as a permeabilization reagent.
- 10 Prepare Click-iT™ Cell Reaction Buffer following the kit instructions. Briefly, add water, 1X Click-iT® cell reaction buffer, CuSO₄, and Click-iT® cell buffer additive in order, mixing well after each component is added. 1 μ M Alexa Fluor™ 647 Azide is added last. This reaction mixture should be added to the cells immediately.

When preparing the Click-iT™ Cell Reaction Buffer, it is very important to mix well after the addition of each new component. Alexa Fluor™ 647 Azide is very sticky if not totally thawed, making it difficult to pipette.

- 11 Incubate cells in Click-iT™ Cell Reaction Buffer for 30 minutes at room temperature.

We strongly recommend performing the click reaction last, after completing all immunofluorescence labeling. There should be no saponin in buffers added to the cells after clicking the fluorophores to the lipids.
- 12 Wash cells more than 5 times with PBS for 1 hour.
- 13 Image cells immediately using a fluorescence microscope or store up to 24 hours at 4 degrees.

Commentary

Background

With an increased understanding of the importance of lipid environment in regulating protein localization and activity, there is a greater need for methods to study lipid trafficking and localization in the cell. Structural protein domains that recognize the head groups of lipids present in the cytoplasmic leaflets of organelle membranes, such as phosphoinositides (Maekawa & Fairn, 2014) and phosphatidylserine (Yeung et al., 2008) have proven enormously useful for investigating spatio-temporal aspects of lipid synthesis, turnover, and trafficking. Lipids that reside in the luminal/exofacial leaflets of cellular membranes, such as SM, have predominantly been studied by monitoring the movement of exogenously added, fluorescent lipid species, whose trafficking may not accurately reflect the movement of the endogenous lipid. To study endogenous SM, we engineered the natural SM-binding protein Equinatoxin II for use as a nontoxic, SM probe. The method we developed using this SM probe enabled us to identify an SM-enriched branch of the secretory pathway and GPI-anchored proteins as cargoes of this pathway. Our assay can be broadly applied to determine if a protein of interest is sorted into an SM-rich lipid environment prior to its delivery to the cell surface. However, an important limitation of the approach that we have developed is that it cannot be used to quantify, even crudely, the amount of SM within a particular compartment, such as a transport carrier. Rather, the method is suited for comparing the distributions of query proteins and EQ-SM within a population of carriers.

Researchers interested in directly visualizing sphingolipids in a particular cellular compartment should follow our second method using pacSphingosine. Cells incubated with a pulse of pacSphingosine will incorporate the clickable lipid into native sphingolipids. Only after fixation is click chemistry used to attach a fluorophore to the lipid, eliminating concerns that the fluorescent moiety will alter trafficking of the lipid. We have used our method in colocalization studies of SM with proteins of interest at the TGN. In addition to its use for imaging sphingolipids, pacSphingosine also contains a diazirine ring that can be used to photocrosslink the lipid to vicinal proteins (Haberkant et al.), which facilitates biochemical studies of protein-sphingolipid interactions (Gerl et al., 2016; Haberkant et al., 2016).

Critical parameters and Troubleshooting

See Table 1 and Table 2 for troubleshooting Basic Protocols 1 and 2 respectively.

Basic Protocol 1: Construct design is critical for optimal use of query proteins in sorting experiments with EQ-SM and EQ-sol. Identification of fusion events relies on the flash of signal produced by a pHluorin-tagged cargo when pHluorin is exposed to the near neutral pH of the imaging media following vesicle fusion. Care should be taken to ensure that the pHluorin is fused to the extracellular terminus of the query protein. The trafficking of new query proteins should also be evaluated to test whether the tag interferes with trafficking and/or proper localization of the tagged protein. A common problem may be that tag placement prevents use of a native signal sequence in the protein, in which case you may need to add a signal sequence to the N-terminus of the construct.

Prior to testing the sorting of a query protein with the sphingomyelin probe, we recommend that you familiarize yourself with the fusion profiles of EQ-SM and EQ-sol by imaging each construct alone. Upon fusion, approximately half of the secreted EQ-SM remains bound to the membrane at the site of fusion, allowing one to infer the presence of SM in the newly fused vesicle (Figure 2) (Deng et al., 2016). In contrast, EQ-sol diffuses away from the cell surface immediately upon vesicle fusion. Familiarity with the fusion profile of each probe will be helpful when analyzing the TIRFM data for co-sorting.

Given that our assay provides a tool to identify proteins in a sorting pathway, it is important to optimize plasmid expression levels to prevent saturation of sorting pathways. It is best to identify the lowest expression level for a query protein that still produces a visible Golgi signal under wide-field illumination and that allows for observation of fusion events that are clearly discernible above background signal by TIRFM. Not all cells will receive a similar amount of DNA, so within a dish it is important to image only cells with a distinct Golgi signal for both proteins.

Basic Protocol 2: Although we have found that 0.5 μ M PhotoClick Sphingosine works well in our protocol, the amount of pacSphingosine used should be tested in new cell types because the ideal concentration may vary. The addition of too much sphingosine may cause cell toxicity and produce a high background for the click fluorophore. The addition of too little sphingosine will make it difficult to distinguish the signal from background. The chase time required for a strong Golgi accumulation of the lipid also needs to be determined for different cell types. In HeLa cells, we observe some Golgi signal after the initial 30-minute pulse, but the background in the ER starts to clear only after 1–2 hours of washout. After 4 hours of washout, the sphingosine signal becomes more diffuse, perhaps due to localization to other organelles.

Regarding permeabilization of the cells in preparation for labeling of the lipid, the choice of detergent is very important. Saponin and digitonin both work to permeabilize the PM of cells without bulk lipid extraction. It is critical to avoid use of any detergent in cell wash buffers after click chemistry has been used to attach the fluorophore to the lipid, as detergents might wash out the labeled lipid. When testing immune-labeling of desired

proteins in pacSphingosine labelled cells, it is important to remember that the use of some antibodies may not be compatible with saponin permeabilization.

Anticipated results

Method 1 is designed to determine if sorting of a query protein is biased in favor of the SM pathway by determining the proportion of transport carriers that contain the query protein and EQ-SM, compared to EQ-sol. The protocol generates a time lapse movie of the PM of cells in which exocytic events are definitively identified by the characteristic flash of fluorescence elicited by the pHluorin-tagged protein (Figure 4). Figures 2 and 4 show that EQ-SM remains bound on the cell surface at the site of vesicle fusion while EQ-sol diffuses away from the cell surface. For cargo that is sorted with EQ-SM, we typically observe that 70–90% of transport vesicles contain the query protein and EQ-SM. In comparison, we observe that the same query protein is present in only 40–50% of vesicles that also contain EQ-sol. Both of these requirements must be met in order for a protein to be classified as a cargo of the SM pathway. For cargoes that are not enriched in vesicles containing EQ-SM, we have observed a variety of different results. In some cases, the query protein is present in vesicles containing EQ-SM or EQ-sol in high (>60% of vesicles), but equal proportions. In these cases, we consider such proteins to not be sorted in the SM pathway because there is no sorting differential between EQ-SM and EQ-sol. Such results demonstrate that it is critical to always conduct sorting experiments with both the SM probe and the EQ-sol control. If cargoes are co-sorted with both EQ-SM and EQ-sol in low proportion (<30% of vesicles), it is important to make sure that EQ-SM and EQ-sol are expressed at a similar level to the query protein. If there is no problem with expression of EQ-SM and EQ-sol, then the result is again counted as a lack of sorting in the SM pathway.

Method 2 was optimized to incorporate pacSphingosine into the native sphingolipid synthesis and trafficking pathways. A variety of results are possible, depending the rates of vesicular and non-vesicular inter-organelle trafficking pathways and the distribution of sphingolipids between different organelles of the cell, which may differ between cell types. Using this method, we anticipate that the signal should first become enriched in membranes of the Golgi apparatus within one hour of chase, and then it will be depleted at the Golgi over time as the lipid is transported to other organelles. In HeLa cells we observe a prominent accumulation of labelled pacSphingosine in the Golgi within 30–60 minutes after initiation of the chase period, and by 22 hours there is a very prominent cell surface signal.

Time considerations

The most time-consuming steps for Method 1 are the design and testing of new query protein constructs. This process may take several weeks, especially if the protein must be expressed using the RUSH system. Once constructs are optimized, a TIRF sorting experiment can be completed in about 3 days. In our protocol, we suggest plating cells on day 1, transfecting plasmids on day 2, and imaging sorting at the TIRF microscope on day 3. Each dish of cells takes about 1 hour to image. For many constructs, one dish will yield at least 5 cells for analysis. For proteins expressed using the RUSH system, however, it is not unusual to image only 1–2 cells per dish. For RUSH constructs, we often image 4 replicate dishes on a given day to ensure that fusion events in at least 5 cells are collected. This may

take up to 4 hours. Analyzing the data may take several days. The most time-consuming step in Method 2 is the generation of the SGPL1 KO cell line. After generating these cells, a pacSphingosine experiment can be completed in 2 days.

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Abbreviations

DMEM	Dulbecco's Modified Eagle Medium
ER	endoplasmic reticulum
FBS	fetal bovine serum
GFP	green fluorescent protein
PBS	phosphate buffered saline
PM	plasma membrane
RUSH	retention using selective hooks
sCMOS	scientific complementary metal oxide camera
SM	sphingomyelin
TGN	<i>trans</i> Golgi network
TIRFM	total internal reflection fluorescence microscopy

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Significance statement

Sphingolipids are principal components of cellular membranes that are synthesized predominantly in organelles of the early secretory pathway and then trafficked to other organelles. Understanding how sphingolipids are sorted to different organelles, and how sphingolipid sorting is integrated with protein sorting and trafficking, is fundamental to understanding organelle biogenesis pathways. Complementary experimental approaches for monitoring sphingolipid and protein sorting in the secretory pathway are presented.

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equinatoxin-based sphingomyelin reporter

GPI example query protein (RUSH system)

Figure 1. Schematic diagrams of fusion proteins used for a typical TIRFM-based exocytosis sorting experiment

The plasmids encoding EQ-SM or EQ-sol contain an N-terminal signal sequence (SS) to direct the probe to the ER for trafficking to the Golgi. A C-terminal fluorescent protein (mKate2 in the example experiment shown in Figure 4) is used for visualization. The protein domains for an example RUSH query fusion protein are indicated. This example contains an N-terminal signal sequence, followed by the streptavidin binding peptide (SBP) to facilitate ER retention, a fluorescent protein (pHluorin in the example experiment shown in Figure 4), and a C-terminal GPI anchor.

Figure 2. Localization of EQ-SM and EQ-sol probes in HeLa cells

Plasmids encoding the indicated proteins were transfected into HeLa cells 16 hours prior to imaging by wide field deconvolution fluorescence microscopy. A variant of GFP engineered for use in the secretory pathway, oxGFP (Costantini et al., 2015), was used. Arrowheads indicate cytoplasmic and cell surface puncta that contain EQ-SM. The scale bar is 10 μ m.

Figure 3. Timeline for imaging RUSH constructs in a typical TIRFM-based exocytosis sorting experiment

Initially, oxGFP-GPI in the RUSH system is retained in the ER and then released by the addition of D-biotin to the growth medium. oxGFP-GPI is observed to begin to populate the Golgi approximately 10 minutes after addition of D-biotin. Protein export from the TGN and delivery to the cell surface can be visualized between 20 and 60 minutes. Single focal planes from a Z stack of wide field deconvolved fluorescence images are shown. The scale bar is 10 μm .

Figure 4. Examples of exocytosis captured by TIRFM in a sorting experiment

A) Representative TIRFM frames show an example exocytic event where pHluorin-GPI and EQ-SM-mKate2 are secreted from the same vesicle. A plot of the fluorescence intensities of both proteins highlights the characteristic flash of pHluorin signal upon vesicle fusion (at time 0), followed by a decrease in fluorescence intensity due to diffusion of pHluorin-GPI from the site of vesicle fusion. Note that EQ-SM-mKate2 remains at the site of vesicle fusion. B) Representative TIRFM frames show an example exocytic event where pHluorin-GPI and EQ-sol-mKate2 are secreted together. The fluorescence intensity plots for this fusion event shows that pHluorin-GPI and EQ-sol diffuse away from the site of vesicle fusion. C) Summary of pHluorin-GPI, EQ-SM, and EQ-sol exocytosis. Each point in the plot represents the mean proportion of exocytic events (a minimum of 10 events per point), identified first by pHluorin-GPI, in which vesicles also contained either EQ-SM or EQ-sol in an individual cell. At least 10 cells are plotted per condition. The mean and standard deviation are indicated.

Figure 5. *In situ* localization of pacSphingosine labeled lipids during a time course
Gene edited *Sgpl1* null HeLa cells were incubated with pacSphingosine (0.5 μ M) for 30 minutes, washed, and then incubated in pacSphingosine-free medium for the indicated chase periods. Click chemistry was used to derivatize pacSphingosine with Alexa FluorTM 647 Azide. Single focal planes from a Z stack of wide field deconvolved fluorescence images are shown. The scale bar is 10 μ m.

Table 1

Roadblock	Reason	Resolution
There is high cell death after transfection	The transfection reagent or the high expression of transfected proteins is toxic to the cells	Reduce the amount of plasmid and the amount of transfection reagent used for transfection
It is difficult to see query protein fusion events	Query protein signal is almost all on the cell surface 16–24 hours after transfection	Express the query protein in the RUSH system, making sure to add avidin to the media during transfection
There is no bright flash of fluorescence signal when the query protein is secreted	The pHluorin tag is not positioned on the luminal/extracellular portion of the protein	Redesign the query protein construct to have the pHluorin tag properly positioned
Frequency of EQ-SM or EQ-sol co-sorting is very low (<30%)	Plasmid expression levels are not balanced; query protein expression level is too high compared to EQ-SM/EQ-sol	Test a titration of query protein expression and consider using a higher concentration of EQ-SM/EQ-sol if their signal at the Golgi is very weak

Table 2

Roadblock	Reason	Resolution
There is high cell toxicity and a high background signal for pacSph	The concentration of pacSph pulsed into cells was too high	Test a titration of pacSph to find an appropriate concentration for your cell type
There is high background signal for pacSph with no clear Golgi signal	Labelled pacSph was washed out of cells	Make sure there is no detergent present in buffers after the click reaction

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