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Association of Glycolipids and Growth Factor Receptors in Lipid Rafts

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Abstract

The traditional methods to study lipid rafts and their association with membrane proteins are based mainly on the isolation of a detergent-resistant membrane by biochemical fractionation. However, the use of detergents may induce lipid segregation and/or redistribution of membrane proteins during the process of sample preparation. Here, we describe a detergent-free method to study the glycolipid and growth factor receptor interaction and their association with lipid rafts. This method combines the biochemical and immunoblotting tools with confocal microscopic imaging, which allows for evaluation and verification of the membrane protein interaction and association with the lipid rafts components in a multifaceted manner.

Keywords

Lipid raft; Ganglioside; Neural stem cell; Density gradient; Growth factor receptor

1 Introduction

Lipid rafts are operationally defined as small cholesterol- and sphingolipid-enriched membrane microdomains that compartmentalize cellular processes [1, 2]. In the central nervous system (CNS), such specialized membranous structures play an important role in cellular signal transduction, neuronal cell adhesion, axon guidance, vesicular trafficking, and synaptic transmission during neuronal cell development and maturation [3, 4]. The selective enrichment of specific neurotrophic/growth factor receptors and neurotransmitter transporters within the raft structures of neuronal cells indicates a significant role of lipid rafts for the healthy development and function of the brain [5, 6]. Gangliosides, a class of sialic acid-containing glycosphingolipids (GSLs), are particularly abundant in the CNS. These GSLs can affect brain cell functions by modulating the signaling of cell membrane proteins, such as the growth factor receptors [7]. During brain development, maturation and aging, the quantity and species of gangliosides undergo dynamic changes in the mammalian brain [8]. To establish methods to evaluate ganglioside and growth factor interactions and their association with lipid rafts is crucial for understanding how changes of ganglioside composition can contribute to alterations in brain cell biological properties during neural development.

Ganglioside GD3 (NeuAc α 2-8NeuAc α 2-3Gal β 1-4Glc- β 1-1' Cer; CD60a) is a b-series disialoganglioside that has been recognized to be the predominant GSL species in

embryonic mammalian brains; however, its abundance in the brain is dramatically decreased soon after birth [8]. It has been demonstrated that ganglioside GD3 is the predominant ganglioside species (>80% of the total ganglioside) in cultured neural stem cells (NSCs) from rodent brains [9]. To address the questions of whether and how ganglioside GD3 regulates NSC proliferation, self-renewal and fate-determination, we established a series of methods to investigate the interaction of ganglioside GD3 with epidermal growth factor receptor (EGFR) in cultured NSCs. These methods combined biochemical and biophysical tools, which allow for a multifaceted evaluation of the interaction of gangliosides and growth factor receptors. NSCs isolated from GD3-synthase-knockout mice were used as a control to verify the specificity of immunostaining signals with antibody, and the potential biological functions of ganglioside GD3.

The first evidence supporting the lipid-raft model was the fractionation of a detergent-resistant membrane (DRM) fraction enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI) anchored proteins using nonionic detergent extraction [10]. Therefore, the extraction of DRMs using detergent became a method of choice for the isolation of lipid rafts and identification of the raft-associated proteins. However, several later studies have noted a number of shortcomings associated with the use of detergent in investigating the raft–protein association, including (1) the protein composition of DRMs may vary widely depending on the type and concentration of detergents used [11]; (2) the detergent may remove certain lipids and proteins from the rafts, resulting in variability of the raft structure during the process of isolation [12]; (3) detergent treatment may generate clusters of lipids and protein rafts that do not normally exist in the intact cell [13]. Therefore, we employed a modified detergent-free method based on the initial procedure developed by Linda Pike for lipid rafts isolation [14]. We also developed a detergent-free method to facilitate the detection of glycolipid localization using immunohistochemical technology [9].

In this article, we describe the protocol for investigating the interaction of gangliosides and growth factor receptors and the association of such interaction in lipid rafts using a non-detergent-based three-step method. This method includes the following basic steps: (1) establishing colocalization of glycolipids and growth factor receptors using confocal microscopy; (2) fractionation of lipid rafts–associated growth factor receptors using a detergent-free density gradient; (3) demonstration of interaction of glycolipids with growth factor receptors using a coimmunoprecipitation (Co-IP) method. This protocol has been successfully employed in our study to isolate lipid rafts and to define the interaction of ganglioside GD3 with the EGFR in cultured NSCs [15]. With modifications, it can also be applied to the study of the interaction of gangliosides/glycolipids with other cell membrane proteins in other cells.

2 Materials

2.1 Buffers

2.1.1 Base Buffer

Reagent/final concentration	For 500 ml	For 250 ml
Tris-HCl (1 M stock, pH 7.8)/20 mM	10 ml	5 ml
Sucrose/250 mM	42.8 g	21.4 g
CaCl ₂ (1 M stock)/1 mM	500 µl	250 µl
MgCl ₂ (1 M stock)/1 mM	500 µl	250 µl
Add ddH ₂ O to	500 ml	250 ml

2.1.2 Cell Lysis Buffer—The cell lysis buffer is prepared from a base buffer containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₃VO₄, and a protease inhibitor cocktail (e.g., Roche, 05892970001).

2.1.3 6× Sample Buffer

Reagent/final concentration	For 50 ml	For 25 ml
Tris-HCl (1 M stock, pH 6.5)/375 mM	18.8 ml	9.4 ml
DTT/0.6 M	4.65 g	2.33 g
SDS/12.0% (w/v)	6 g	3 g
Glycerol/60%	30 ml	15 ml
Bromophenol blue/0.06% (w/v)	30 mg	15 mg
Add ddH ₂ O to	50 ml	25 ml

Aliquot at 1 ml/tube and store it at −20 °C freeze.

2.1.4 10× Tris-Glycine Buffer

Reagent/final concentration	For 1000 ml	For 2000 ml
Tris base/250 mM	30.3 g	60.6 g
Glycine/1.92 M	144 g	288 g
Add ddH ₂ O to	1000 ml	2000 ml

2.1.5 1× SDS Running Buffer

Reagent/final concentration	For 1000 ml	For 2000 ml
10× Tris-glycine buffer	100 ml	200 ml
10% SDS (w/v)	10 ml	20 ml
ddH ₂ O	890 ml	1780 ml

2.1.6 1× Transfer Buffer

Reagent/final concentration	For 1000 ml	For 2000 ml
10× Tris–glycine buffer	100 ml	200 ml
Methanol	200 ml	400 ml
ddH ₂ O	700 ml	1400 ml

2.2 Preparation of Coated Coverslips and Reagents for Confocal Microscopy

2.2.1 Reagents and Materials

1. Poly-L-ornithine (e.g., Sigma P3655).
2. Fibronectin (e.g., Sigma F0162).
3. Sterile 1× PBS (pH = 7.4).
4. Paraformaldehyde 16% solution (e.g., Electron Microscopy Sciences, Cat. # 15710 or equivalent).
5. Coverslips (e.g., Electron Microscopy Sciences, Cat. # 72229 or equivalent).
6. DAPI solution (1 mg/ml) (e.g., Thermo Fisher, Cat. # 62248 or equivalent).
7. 95% ethanol.
8. 24-well cell culture plate.

2.2.2 Reagent Preparation

1. *Poly-L-ornithine solution*: Dissolve poly-L-ornithine in sterile 1× PBS to make a 15 mg/ml stock solution (1000×). Aliquot and store it at –20 °C freezer and avoid repeated freeze–thaw cycles. Right before use, prepare the 1× solution (15 µg/ml) by diluting the 1000× poly-L-ornithine solution in sterile PBS, and gently mix it well by swirling (do not vortex).
2. *Fibronectin solution*: Dissolve fibronectin in sterile 1× PBS to make a 1 mg/ml stock solution (1000×). Aliquot and store at –20 °C freezer and avoid repeated freeze–thaw cycles. Right before use, prepare the 1× solution (1 µg/ml) by diluting the 1000× fibronectin solution in sterile PBS, and gently mix it well by swirling (do not vortex).
3. *4% or 2% paraformaldehyde fixative solution*: Mix 10 ml of paraformaldehyde 16% solution with 32 ml of 1× PBS to make a 4% paraformaldehyde fixative solution, and dilute the 4% paraformaldehyde fixative solution with an equivalent volume of 1× PBS to make the 2% paraformaldehyde fixative solution.
4. *Blocking buffer*: 10% normal donkey serum in 1× PBS.
5. *Antibody dilution buffer*: 1% normal donkey serum and 0.01% sodium azide in 1× PBS.

2.2.3 Preparation of Poly-L-Ornithine- and Fibronectin-Coated Coverslips

1. Sterilize coverslips with 70% ethanol and flame, and place one coverslip into each 24-well plate.
2. Add 0.5 ml of 1× PBS to each well, and gently sink the floating coverslips with a sterile pipette tip.
3. Aspirate the PBS completely, and add 0.5 ml of 1× poly-L-ornithine solution to each well, incubate overnight at 37 °C.
4. Aspirate the poly-L-ornithine solution completely, wash each well with 0.8 ml of 1× PBS for 3 times.
5. Add 0.5 ml of 1× fibronectin solution to each well, incubate at 37 °C for 3–24 h, and wash each well with 0.8 ml of 1× PBS for 3 times.
6. Dry the plate in a cell culture hood. Extra plates can be wrapped with foil paper, and stored in a refrigerator (4–8 °C) for up to 1 week.

2.3 Cell Culture

NSCs collected from ganglionic eminence of embryonic (E14 embryonic day) mouse or postnatal subventricular zones (SVZs) are grown in Neurobasal-A medium (e.g., Gibco, 10888022) supplemented with B-27TM minus vitamin A (e.g., Gibco, 2587010), 2 mM GlutaMAXTM (e.g., Gibco, 35050079), basic fibroblast growth factor (e.g., bFGF, 20 ng/ml; PeproTech, Inc.), and EGF (e.g., 20 ng/ml; PeproTech, Inc.). Neurospheres formed after 5–7 days are collected for passage. Neurospheres grown for 5 days at passage 2 or 3 are used for co-immunoprecipitation in lipid rafts fractionation. For confocal microscopy, neurospheres at passage 2 are dissociated into single cells, followed by seeding onto poly-L-ornithine and fibronectin-coated coverslips.

2.4 Density Gradient Preparation

An OptiPrepTM density gradient medium with a 60% (w/v) solution of iodixanol in water is purchased from Sigma (D1556) or an equivalent supplier.

Density gradient (v/v, 10 ml)	OptiPrep TM	BS
50% OptiPrep TM	5 ml	5 ml
20% OptiPrep TM	2 ml	8 ml
15% OptiPrep TM	1.5 ml	8.5 ml
10% OptiPrep TM	1 ml	9 ml
5% OptiPrep TM	0.5 ml	9.5 ml
0% OptiPrep TM	0 ml	10 ml

2.4.1 Equipment and Supplies

1. 22-G needle and 1 ml syringe.
2. 50 ml and 15 ml centrifuge tube.

3. 1.5 ml microcentrifuge tube.
4. Precooled 4 °C centrifuge and microcentrifuge.
5. Beckman ultracentrifuge.
6. SW55Ti rotor (Beckman, 342196) and 13 × 51 mm, 5 ml ultra-clear centrifuge tube (Beckman, 344057).
7. Zeiss LSM 510/710 META confocal microscope.

3 Methods

3.1 Colocalization of Glycolipid and Growth Factor Receptor Using Confocal Microscopy

Confocal microscopy is one of the most powerful tools for visualizing the localization and investigating the cellular components in studying the functions of proteins and other molecules. Compared to the epifluorescence microscopy, which lacks the intrinsic ability to distinguish between in-focus and out-of-focus light sources, it can examine very thin optical sections and produce high-quality images [16]. Colocalization, the presence of two or more different molecules residing at the same physical location in a specimen, can be examined by the sample stained for interested markers with different fluorescent probes, and further analyzed with overlapped pixels of two probes [17].

3.1.1 Preparation of Poly-L-Ornithine and Fibronectin-Coated Coverslip—

See Subheading 2.2.3.

3.1.2 Preparation of NSCs Treated with Different Concentrations of EGF

1. Dissociate NSCs at passage 2 or 3 into single cells, resuspend the cells with NSC culture medium (5 ng/ml EGF, 20 ng/ml bFGF) and adjust the cell density to 10,000 cells/ml.
2. Plate 0.5 ml cell suspension into each well of a 24-well plate with poly-L-ornithine- and fibronectin-coated coverslips (about 5000 cells/well), incubate the plate at 37 °C overnight.
3. Aspirate the cell culture medium in each well completely, and add 0.5 ml of fresh medium with 5 ng/ml or 20 ng/ml EGF and 20 ng/ml FGF into each well, and incubate at 37 °C for 24 h.

3.1.3 Preparation and Fixation of Cells on Coverslips

1. After culturing with desired EGF concentration in a 0.5 ml medium for 24 h, prefix the cells by adding 0.5 ml of 4% formaldehyde fixative solution into each well. (The prefixation step can avoid any change of the growth factor receptor and glycolipid distribution on the cell surface caused by complete removal of cell culture medium.)
2. After 2 min, replace the prefix medium with 0.5 ml 2% formaldehyde fixative solution and incubate for 20 min at room temperature.

3. Wash each well with 0.5 ml of PBS 3 times, and add 0.8 ml of PBS into each well to cover the coverslips. The plate with cells on coverslips can be stored at 4 °C for up to 1 week.

3.1.4 Fluorescent Immunocytochemistry Staining of NSCs on Coverslips—

(Make sure all dilution buffers and wash buffers used for the following procedure are detergent-free).

1. Wash the fixed cells on the coverslips 3 times with 0.5 ml PBS.
2. Block nonspecific staining by adding 0.5 ml of blocking buffer into each well, and incubate for 60 min at room temperature.
3. Dilute the primary antibody in an antibody dilution buffer in an optimized dilution or according to the manufacturer's instructions.
4. Aspirate the blocking buffer, incubate the cells with 0.5 ml of primary antibodies in the dilution solution at 4 °C overnight.
5. Wash the cells on the coverslips 3 times with 0.5 ml 1× PBS.
6. Dilute the secondary antibodies in the dilution buffer according to the manufacturer's instructions.
7. Add 0.5 ml of secondary antibody dilution solution into each well, and incubate at room temperature for 60 min in the dark. (Samples should be protected from light for the following steps.)
8. Aspirate the secondary antibody, wash cells in each well with 0.5 ml of 1×PBS 3 times.
9. Counterstain nuclei by adding 0.5 ml of diluted DAPI solution to each well, and incubate at room temperature for 5 min.
10. Rinse the coverslips once with PBS and once with distilled water.
11. Dispense one drop of antifade mounting medium (avoid to use any mounting medium that contains organic solvents, such as xylene) for each coverslip onto a microscope slide. Usually, one microscope can hold up to three coverslips.
12. Carefully pick up the coverslips in the wells with a fine forceps and remove any extra water by gently patting it on a piece of Kimwipes.
13. Mount the coverslip with the cells facing toward the microscope slide.
14. Remove any extra mounting medium surrounding the coverslips and seal the coverslip on the microscope slide with nail polish.

3.1.5 Confocal Microscopy: Expected Results and Data Analysis (See Note 1)

1. Take images of cells under a 63× Plan-Apochromat objective of a Zeiss LSM 510/710 META confocal microscope.
2. Analyze the weighted colocalization coefficient using the Zeiss LSM-enhanced co-localization software, which is based on calculating the sum of overlapped

pixel intensity relative to the sum of the total pixel intensity of growth factor receptor after background correction.

3. Using a detergent-free (without Triton X-100 treatment) immunocytochemical method, the expression of GD3 can be detected only on the cell membrane surface (Fig. 1a *left* as shown by costaining with f-actin). The cell surface signal of GD3 can be washed off by detergent (by 0.3% Triton X-100 for 15 min) (Fig. 1a *right*). NSCs isolated from GD3S-KO (GD3S^{-/-}) embryos do not have any signal for GD3 staining (Fig. 1b. *lower panel*).
4. A control with detergent treatment can be applied in parallel to the detergent-free immunohistochemistry to verify that the cell surface immunostaining signals are glycolipids or associated with glycolipids (*see Note 2*).
5. As shown in Fig. 1, partial colocalization of EGFR and GD3 can be observed on the NSC surface membrane at a low-EGF concentration (5 ng/ml) in the WT (GD3S^{+/+}) cells. At a higher EGF concentration (20 ng/ml), EGFR and GD3 co-localization in the GD3S^{+/+} NSC surface membrane is enhanced. In GD3S^{-/-} cells, no GD3 staining is detected, and EGFR shows a scattered punctate expression pattern.

3.2 Fractionation of Lipid Raft-Associated Growth Factor Receptors Using a Detergent-Free Density Gradient Method

So far, biochemical fractionation remains the most widely used technique to study lipid rafts and their associated proteins [18]. Below, we describe a protocol for the rapid isolation and fractionation of lipid rafts by OptiPrep™ density gradient ultracentrifugation, which is modified from a method previously reported by Linda Pike et al. [19]. This assay retains the principle of using a density gradient for floatation of the low-density lipid rafts and eliminates the disadvantages of using a detergent.

3.2.1 Cell Membrane Preparation from NSCs for Isolation of Lipid Rafts (See Note 3)—All steps are performed on ice or at 4 °C except step 1.

1. Neurospheres at passage 2 or 3 are grown in 2 × 150 mm petri dish (e.g., Corning, Cat. SB93102) for 5 days.
2. Collect the neurospheres into a 50 ml tube and centrifuge at 250 × *g* for 2 min.
3. Discharge the supernatant, wash the cells once with ice-cold phosphate-buffered saline (PBS).
4. Centrifuge at 250 × *g* for 2 min, and completely remove the PBS with aspiration.
5. Add 0.5 ml cell lysis buffer to resuspend the cells, transfer the cell suspension to a 1.5 ml microcentrifuge tube.
6. Lyse the cells by passing through a 22-G needle connected to a 1 ml syringe 20 times.
7. Centrifuge at 1000 × *g* for 10 min.

8. Collect and transfer the resulting postnuclear supernatant to a separate 15 ml centrifuge tube.
9. Add 0.5 ml cell lysis buffer to the remaining pellet, and lyse the pellet again by sheering 20 times through a 22-G needle and syringe.
10. Centrifuge at $1000 \times g$ for 10 min, and combine the second postnuclear supernatant with the first.
11. Add an equal volume (1 ml) of base buffer containing 50% OptiPrep™ to the combined postnuclear supernatant, mix well by pipette several times (do not vortex).

3.2.2 Preparation of OptiPrep Density Gradients and Fractionation of a Gradient

1. Transfer a 2-ml cell membrane lysate and OptiPrep™ (which is now 25%) mixture to the bottom of a 5-ml ultra-clear centrifuge tube.
2. Subsequently, overlay ~0.6 ml of 20%, 15%, 10%, 5% and 0% OptiPrep™ medium (from high to low density) on top of the lysate, which adds up to a total of 3 ml gradient (Fig. 2) (see Note 4).
3. Centrifuge the gradient for 90 min at 25,000 rpm using an SW-55 rotor in a Beckman ultracentrifuge.
4. Collect the ten fractions (0.5 ml each) into prelabeled 1.5 ml microcentrifuge tubes by gently suction of the medium from the top, and avoid mixing layers formed during centrifugation.

3.2.3 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

1. Mix 100–200 μ l of each fraction with 6 $\times$ sample buffer, followed by denaturation at 95 °C for 5 min.
2. Eighty microliters of each fraction can be analyzed by SDS-PAGE using a Bio-Rad Protein Gel system.
3. Western blotting for the proteins of interest (as shown in Fig. 3 for EGFR and β 1-integrin) can be achieved.

3.2.4 Expected Results and Data Interpretation

1. After a successful fractionation, a high abundance of resident raft protein markers (e.g., caveolin, flotillin, and GPI-anchored proteins) should be detected in the low-density fractions (F1-F7). Nonraft protein markers, such as the transferrin receptor (TfR) should appear in the high-density fractions (F7-F10). (Fig. 3).
2. Following SDS-PAGE and Western blotting for the interested proteins in each gradient fraction, Western blots are scanned followed by densitometric quantification performed using the software, Image J. The percentage of a

protein in each fraction is calculated by its density dividing by the total density of that protein in all ten fractions.

3.3 Interaction of Glycolipids with Growth Factor Receptors Using Coimmunoprecipitation (Co-IP)

Co-IP is a widely used and straightforward method to study protein-protein interaction in vivo and in vitro. Here, we modify the procedure to identify physiologically relevant ganglioside–protein interaction in cultured NSCs. By using a specific antibody against a ganglioside, it is possible to pull down the entire ganglio-side–protein complex out of the cell lysate (detergent-free), thereby identify the protein components of the complex.

3.3.1 Cell Lysates Preparation and Immunocomplex Formation—All procedures are performed on ice or at 4 °C except **step 1**.

1. Neurospheres at passage 2 or 3 are grown in 1 × 100 mm dish for 5 days.
2. Collect the cells from the culture dish, and transfer to a 15-ml centrifuge tube.
3. Centrifuge 250 × *g* for 2 min at 4 °C and discharge the supernatant.
4. Wash twice with ice-cold PBS and centrifuge at 250 × *g* for 2 min, and remove the supernatant completely.
5. Resuspend the cells in 1-ml cell lysis buffer (base buffer supplemented with Complete Protease Inhibitor Mixture), and transfer the cell suspension to a 1.5-ml microcentrifuge tube.
6. Lyse the cells by passing through a 22-G needle connected to a 1-ml syringe 20 times.
7. Centrifuge at 1000 × *g* for 10 min to pellet the cell debris.
8. Collect and transfer the resulting postnuclear supernatant (about 900 µl) to a separate 1.5 ml microcentrifuge tube. Place the samples on ice for later use or at –80 °C for long-term storage.
9. Add the primary anti-ganglioside antibody or IgG (as control) to the cell lysate (400 µl each), and incubate overnight at 4 °C with gentle agitation (*see* Note 5).

3.3.2 Magnetic Beads Prewash

1. Resuspend the Protein G magnetic beads in its original tube by pipetting up and down several times.
2. Transfer 40 µl of the suspended beads for each sample into a separate 1.5 ml micro-centrifuge tube.
3. Place the tube into the magnetic stand for seconds to allow the beads to migrate to the magnet, then carefully remove the clear supernatant with a pipette.
4. Wash the beads with 500 µl of the base buffer, and pipette up and down for several times.

5. Place the tubes back in the magnetic stand for seconds, and remove the clear supernatant completely.

3.3.3 Immunoprecipitation

1. Add the preformed immunocomplex (from **step 9** of Subheading 3.3.1) to the prewashed beads.
2. Incubate at room temperature with continuous mixing for 1 h.
3. Place the tube into the magnetic stand for seconds and remove the sample solution with a pipette.
4. Wash the beads 3 times with 500 μ l of the base buffer.
5. After the last wash, remove the tube from the magnetic stand, add 60 μ l of 1 $\times$ SDS sample buffer, and mix to resuspend the beads.
6. Heat at 90 $^{\circ}$ C for 10 min.
7. Place the tube into the magnetic stand, and immediately transfer the denatured sample solution into a new tube with a pipette.

3.3.4 SDS-PAGE, Western Blotting and Expected Results

1. Mix 100 μ l of remaining cell lysate with 6 $\times$ sample buffer, and denature by heating 95 $^{\circ}$ C for 5 min as an input control.
2. Load 60 μ l of input control, IgG-IP, and ganglioside antibody-IP samples on one SDS-PAGE Gel.
3. Analyzed the ganglioside-associated protein by SDS-PAGE using a Bio-Rad Protein gel system.
4. Western blotting for the target proteins (as shown in Fig. 4). IgG is blotted as control.
5. After a successful Co-IP, the ganglioside-associated proteins (e.g., GD3 associated with EGFR but not β 1-integrin, Fig. 4) can be detected in the lanes of ganglioside antibody-IP samples.
6. The abundance of the ganglioside-associated proteins can be estimated by densitometric analysis of the band intensity of the ganglioside antibody-IP sample, and compare it to that of the input control sample (input $\sim$ 1/8 of the IP sample).

4 Notes

1. In order to obtain the best quality of images of glycolipids on the cell surface, cells should be imaged as soon as possible under confocal microscopy.
2. To verify that the cell surface immunostaining signals are glycolipids or associated with glycolipids, a control with detergent treatment can be applied in parallel to the detergent-free immunohistochemistry. Following washing the

fixed cells on the coverslips 3 times with 0.5 ml PBS (**step 1** in Subheading 3.1.4), add 0.5 ml of 0.5% Triton X-100 in PBS to the control wells, and incubate at room temperature for 15 min. Then wash two times with 0.5 ml PBS, and continue with immuno-blocking as described in **step 2** in Subheading 3.1.4. In the detergent-treated samples, cell surface signals for glycolipids (e.g., ganglioside GD3) and glycolipid-associated growth factor receptors are expected to be washed off.

3. The method of lipid raft fractionation using detergent-free density gradient can also be used to prepare lipid raft from adherent cells, such as neuroepithelial cells in culture. In this case, cells are allowed to grow in culture media to reach 90–95% confluency. Cells from 2 × 150 mm dishes are washed with cold PBS and scraped into the base buffer, then followed by steps in Subheading 3.2.4 to complete the procedures.
4. If a more concentrated raft fraction is desired, a 5% to 20% OptiPrep gradient can be overlaid on top than the lysate, and the 0% gradient can be omitted. This change will result in more raft markers concentrated in fraction 1 but not affect the separation of the raft-associated proteins in the later fractions.
5. This protocol is for using an anti-ganglioside antibody to precipitate the ganglioside-associated proteins, and analyze the precipitated proteins by SDS-PAGE and Western blotting. Therefore, an anti-ganglioside antibody must be added to the detergent-free cell lysate to form immunocomplex in **step 9** of Subheading 3.3.1.

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Fig. 1. Colocalization of EGFR and GD3 on the NSC surface membrane. **(a)** Representative images of NSCs costained for GD3 (red) and f-actin (green) with (Right) or without (Left) detergent treatment. **(b)** Representative images of NSCs co-stained for GD3 (red) and EGFR (green) growing in different concentrations of EGF as indicated. **(c)** Co-localization coefficient (Mander's overlap coefficient) for GD3 and EGFR was quantified on **(b)**. Values are expressed as means $\pm$ SEM; $n = 3$ biological independent experiments. $**p < 0.01$. Scale bar, 5 μ m. *ND* not detected

Fig. 2.

Flowchart demonstrating the process of fractionation lipid raft-associated membrane proteins by density gradient ultracentrifuge. Total membrane was isolated from neurospheres and then loaded at the bottom of the centrifuge tube, and then the OptiPre™ gradients were overlaid from high to low density on top of the sample. After centrifuging at 25,000 rpm for 90 min, the gradient was fractionated by gentle aspiration from the top. Proteins in each fraction were then analyzed by SDS-PAGE and Western blotting

Fig. 3. Representative Western blotting for lipid raft-associated proteins and raft/nonraft protein markers after detergent free fractionation. Distribution of indicated proteins from E14 passage 2 GD3S^{+/+} (a) and GD3S^{-/-} (b) neurosphere lysates (60 μl) in each gradient fractions prepared by a detergent-free method. Densitometric analysis of EGFR (c) and β1-integrin (d) distribution in each fraction to compare between the GD3S^{+/+} and GD3S^{-/-} NSCs

Fig. 4.

Interaction of GD3 with EGFR detected by co-IP. (a) Co-IP neurosphere lysate with anti-GD3 monoclonal antibody R24, and blotted with EGFR, β 1-integrin, and IgG