Chapter 6

Isolation of Giant Plasma Membrane Vesicles for Evaluation of Plasma Membrane Structure and Protein Partitioning

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Abstract

Although investigation into the structure of eukaryotic cell membranes has been an intense focus of cell biology for the past two decades, definitive insights have been limited by the lack of coherent methods for the isolation of specific organelle membranes and the identification of membrane subdomains. Here we describe a method for the isolation of mammalian cell plasma membranes as Giant Plasma Membrane Vesicles (GPMVs) and strategies for imaging membrane lateral structure and quantification of protein partitioning between coexisting domains by fluorescence microscopy.

Key words Lipid rafts, Plasma membrane, GPMV, Phase separation

1 Introduction

The lipid raft hypothesis [1] posits nanoscale organization of mammalian cell membranes driven by preferential interactions between specific lipids and proteins that give rise to functional lateral membrane domains. The physicochemical basis for this organization is the cholesterol-dependent formation of a liquid-ordered membrane phase that can coexist with a liquid-disordered phase rich in unsaturated lipids and depleted of saturated lipids/cholesterol/glycosphingolipids [2]. Although these interactions and their resultant long-range organization have been extensively characterized in purified lipid model membranes [2, 3], the complexity of live cells, lack of robust experimental methods, and inability to directly image lipid rafts led to significant controversy regarding their functions [4] and even existence [5]. However, recent advances in plasma membrane isolation [6–9], nanoscale spectroscopy [10, 11], super-resolution [12] and electron microscopy [13], and lipidomics [14, 15] have led to definitive observations of lateral domains in biological membranes. Such structures

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can be generated either by lipid-driven phase separation (i.e., membrane rafts) or by cholesterol-independent protein–protein and protein–lipid interactions, likely with significant overlap and cooperation between these mechanisms [16]. In all cases, the result of domain formation is the functional segregation of membrane components.

In live cells, lipid-driven membrane rafts are rarely observable by light microscopy because of the length and time scales involved—domains are estimated to be tens of nanometers in size and are dynamic on millisecond timescales. Thus, advanced techniques are required for the measurement of domain compositions and properties. Giant plasma membrane vesicles (GPMVs) are one such method for efficient isolation of intact plasma membranes (PMs) maintaining the full diversity of native membrane components [17]. The coexistence of two liquid phases with distinct physical properties [18, 19] and compositions [20, 21] in these natural membranes provides compelling evidence for the central tenet of the lipid raft hypothesis. More importantly, GPMVs comprise an intermediate biological membrane model system, combining the compositional complexity and protein content of live cell membranes with the macroscopic phase separation and experimental malleability of synthetic vesicles. The most important advantage of this model system is that it allows quantitative measurement of protein partitioning [18, 20] and (potentially) function in large, stable, well-resolved domains. This capability has allowed investigation of the structural determinants of raft partitioning [20–22] and is likely to yield more insights about raft-dependent protein function in the coming years.

Here, we describe methods to isolate plasma membranes of mammalian cells as GPMVs, and observe their lateral compositional heterogeneity. The entirety of the experiment, comprising fluorescent labeling of live cell membranes, GPMV isolation, and microscopic visualization can be achieved in 2–3 h.

2 Materials

Prepare all solutions using ultrapure water (at least 18 MΩ) and analytical grade reagents. Reagent preparation can be done at room temperature, but buffers are stored at 4 °C. Some reagents used are toxic and proper regulations must be followed for their handling and disposal.

2.1 Reagents

1. Cell culture medium (varies depending on cell type).
2. Paraformaldehyde (PFA) *Caution: PFA is a suspected carcinogen. Use gloves and avoid contact.*
3. Dithiothreitol (DTT) *Caution: DTT is toxic. Use gloves and avoid contact.*

4. *N*-ethyl maleimide (NEM) *Caution*: *NEM is toxic. Use gloves and avoid contact.*
5. 3,3′-dilinoleyloxacarbocyanine perchlorate (FAST-DiO; Invitrogen, cat.no. D3898).
6. 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate (FAST-DiI; Invotrogen, cat. no. D7756).
7. Cholera Toxin B subunit with desired fluorescent tag (CTxB; Invitrogen, cat. no. C-34775, C-34776, C-34777, C-34778).
8. Sodium Chloride (NaCl).
9. Potassium Chloride (KCl).
10. Calcium Chloride ($CaCl_2$).
11. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES).
12. Potassium phosphate monobasic (KH_2PO_4).
13. Sodium phosphate dibasic (Na_2HPO_4).
14. Hydrochloric Acid (HCl).
15. Sodium Hydroxide (NaOH).
16. Bovine Serum Albumin (BSA).
17. Distilled water.
18. Vaseline® (Sigma-Aldrich, cat. no. 16415)—or other water-repellent sealant/lubricant.
19. PBS (*see* Reagent Setup).
20. GPMV buffer (*see* Reagent Setup).

2.2 Solutions to Prepare

1. *Phosphate-buffered Saline* (*PBS*): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4. For 1 L solution, dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, and 0.27 g KH_2PO_4 in distilled water. Bring to 1 L. Adjust the pH with HCl or NaOH to 7.4.
2. *GPMV buffer*: 10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4. For 1 L solution, dissolve 8.75 g NaCl, 0.22 g $CaCl_2$ and 2.38 g HEPES in distilled water and bring to 1 L. Adjust the pH with HCl or NaOH to 7.4. Store at 4 °C.
3. *FAST DiO solution*: Dissolve dye in ethanol to make stock solutions of 0.5 mg/mL. Store at −20 °C in lightproof vials.
4. *4 % PFA*: D*i*ssolve 4 g of PFA in 50 mL PBS. Heat to approximately 60 °C, and add a few drops of 1 M NaOH until the solution becomes clear. pH to 7.4 with HCl or NaOH, and bring final volume to 100 mL with PBS. Store in aliquots at −20 °C.
5. *1 M DTT*: Dissolve 1.54 g DTT in distilled H2O and bring to 10 mL. Aliquot and store at −20 °C.
6. *1 M NEM*: Dissolve 1.25 g NEM in 100 % ethanol and bring to 10 mL. Aliquot and store at −20 °C.
7. *1 mg/mL Bovine Serum Albumin* (*BSA*): Dissolve 50 mg BSA in distilled water and bring to 50 mL. Store at 4 °C.

2.3 Equipment

1. 37 °C Incubator.
2. Tissue culture dishes.
3. Microcentrifuge tubes.
4. Microcentrifuge.
5. #1.5 coverslips.
6. Inverted epifluorescence microscope.
7. Temperature controller (Warner Instruments; cat. no. 64-0352).
8. Thermal insert (Warner Instruments; cat. no. 64-1636, 64-1646).
9. 40× air objective.
10. Circulating water bath with hoses.
11. Dehumidified air or nitrogen (N_2).
12. Tally Counter (with two positions).

3 Methods

3.1 Preparation of GPMVs

1. Seed the cells in a 35 mm dish (or other appropriate chamber—*see* **Note 1**).
2. Incubate the culture vessels in appropriate conditions until cells reach ~70 % confluency. Check American Type Culture Collection (ATCC) database for the appropriate conditions and media. Optional: Manipulate the cell culture (e.g., transfection, drug-treatment) depending on the experiment (*see* **Note 2**).
3. Label the cell membranes:
   (a) Wash cells 2× with 1 mL PBS.
   (b) Label cells with fluorescent dye. Add dye at appropriate concentration to 0.5 mL of PBS and carefully pipet onto to cells (*see* **Note 3**).
   (c) Incubate at 4 °C for 10 min to allow dye incorporation into membranes.
   (d) Aspirate dye solution, wash 5× with PBS to remove unincorporated dye, and proceed to GPMV isolation.
4. Wash cells 2× with 1 mL of GPMV buffer.
5. Add vesiculation agents to GPMV buffer and apply to cells (*see* **Note 4**):
   (a) For PFA/DTT (25 mM PFA/2 mM DTT): 18 μL of 4 % PFA solution and 2 μL of 1 M DTT solution to 1 mL of GPMV buffer.
   (b) For NEM (2 mM): 2 μL of 1 M NEM to 1 mL of GPMV buffer.

6. Incubate the cells at 37 °C for 1 h.
7. Remove chambers from incubator and check for presence of vesicles. These should be readily observable at 20× magnification as dark free-floating spheres just above the plane of the cells (Fig. 1d).
8. Transfer the GPMV-rich cellular supernatant into a microcentrifuge tube. After isolation, GPMVs can be stored at 4 °C for 1–2 days without visible degradation.
9. Concentrate GPMV suspension:
   (a) For biochemical or spectroscopic experiments where purity of plasma membrane is more important than vesicle morphology, centrifuge GPMV suspension at 100 rcf for 10 min to pellet cell debris. Then, centrifuge the supernatant at 20,000 rcf for 1 h to pellet GPMV membranes. Pellet can be stored at −20 °C, or resuspend in appropriate buffer for subsequent assays (protein biochemistry, lipid mass spectrometry, etc.).
   (b) For imaging experiments where purity is less important than GPMV integrity, leave GPMV suspension in microcentrifuge tube for 20–30 min—GPMVs will concentrate at the bottom of the tube.

3.2 Analysis of Isolated GPMVs: Imaging of GPMVs and Quantification of Partitioning

1. Concentrate vesicles without centrifugation as in **step 9b** in Subheading 3.1.
2. Prepare BSA-coated coverslips:
   (a) The bottom coverslip of the imaging chambers should be coated with BSA to avoid nonspecific sticking/bursting of the GPMVs on the glass. Incubate coverslips in 1 mg/mL BSA solution for at least 1 h. Prior to use, rinse with distilled H_2O and dry using a paper wipe (e.g., Kimwipes®). For LabTek chambers, add 250 μL of 1 mg/mL BSA solution into the wells and incubate at least 1 h. Wash 2× with PBS.
3. Prepare the cooling system:
   (a) Connect the temperature controlled stage and/or objective cooler to a circulating, temperature-controlled water bath with rubber hoses, sealed at the connection points. Set the water bath temperature to approximately the desired chamber/sample temperature. If using a Peltier-element temperature controller, water circulation should begin prior to temperature regulation, to remove the heat produced in the device. If the cooling setup in Fig. 1 is used with an air immersion objective, cooling the objective is unnecessary. However, condensation can form on the sample surface, hindering imaging. To avoid this, a stream of dehumidified air or N_2 can be blown directly onto the

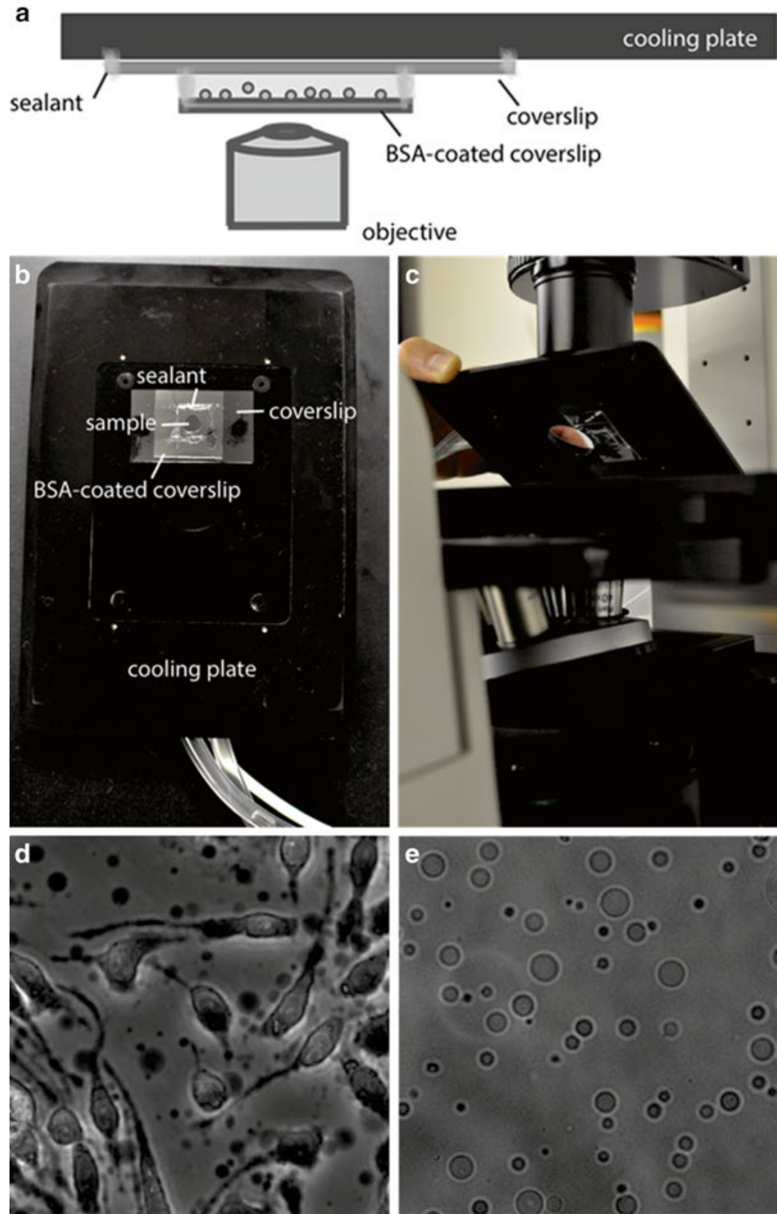

Fig. 1 Experimental setup and expected results. (**a**) Graphical representation of the microscopic setup comprising the GPMV suspension between two coverslips mounted on a temperature-controlled microscope stage. (**b**) Photograph of sample mounted on the cooling plate and (**c**) then on the microscope stage. (**d**) Free-floating and cell-attached GPMVs above adherent cells imaged at 20× using DIC microscopy after **step 7**. (**e**) Isolated GPMVs imaged at 40× after **step 9**

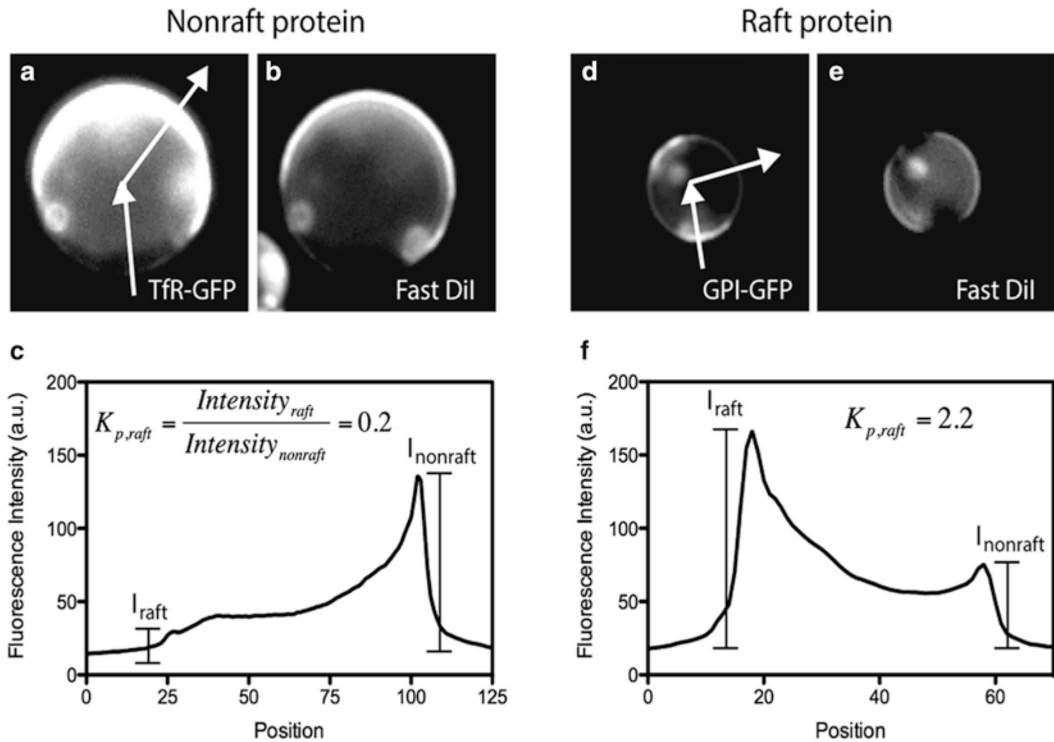

Fig. 2 Quantification of raft partitioning. (**a**) A non-raft, GFP-tagged protein (Transferrin Receptor—TfR-GFP) is imaged in the same vesicles with a non-raft marker lipid (FAST DiI, **b**). (**c**) $K_{p,raft}$ is quantified by the ratio of the background-subtracted fluorescence intensities in the two domains. (**d**–**f**) Analogous images and quantification for a raft-enriched protein (GPI-GFP)

sample or in the space between the objective and sample. If immersion objectives are used to image GPMVs, objective cooling may be necessary to avoid heat flow to the sample. In this case, care must be taken to thermally isolate the objective from the rest of the microscope to avoid condensation inside the microscope box.

4. Construct imaging chamber as shown in Fig. 1a–c and let temperature equilibrate for at least 10 min. This time will also allow the vesicles to sink to the bottom of the chamber.
5. Decrease the temperature below 15 °C for GPMVs derived with PFA/DTT and below 10 °C for those derived with NEM (*see* **Notes 4–7**).
6. One channel should be used to image a well-characterized marker of either the raft or non-raft phase (e.g., unsaturated lipids for non-raft; fluorescently labeled cholera toxin for raft) while imaging the component of interest in the other fluorescent channel (as in Fig. 2; *see* **Note 8**). Image each channel sequentially at the same focal position.

7. Draw a segmented line scan starting from outside of the raft phase to the middle of the GPMV, continuing through the middle of the nonraft phase. Raft partitioning can be quantified by calculating $K_{p,raft}$ from the line scans as shown in Fig. 2 (*see* **Note 9**).

3.3 Analysis of Isolated GPMVs: Imaging of GPMVs and Quantification of Miscibility Temperature

1. Concentrate vesicles without centrifugation as in **step 9b** in Subheading 3.1.
2. Construct imaging chamber as shown in Fig. 1a–c and let temperature equilibrate for at least 10 min. This time will also allow the vesicles to sink to the bottom of the chamber.
3. Increase the temperature to 25 °C.
4. Using a tally counter, count the number of vesicles that are and are not phase-separated (*see* Fig 3b, c for examples of each).
5. If the percentage of phase-separated vesicles is more than 10 %, increase the temperature to 30 °C and count again. If the percentage of phase-separated vesicles is less than 10 %, decrease the temperature by 4 °C, and count again. Repeat this process of decreasing the temperature and counting the percentage of phase-separated vesicles until the vesicles are 95–100 % phase-separated.
6. Plot the percent of phase-separated GPMVs versus the temperature and fit to a sigmoidal function (e.g., Eq. 1) as shown in Fig. 3d to derive the miscibility transition temperature at which 50 % of vesicles would be expected to be phase separated.

   (a)

$$\%\,\text{phase separated} = 100 \times \left(A - \frac{1}{1 - \mathrm{e}^{-B \times \text{Temp}}} \right) \tag{1}$$

A and B are fit parameters.

4 Notes

1. Protocol is optimized for cells seeded in a 35-mm cell culture dish. For microscopy experiments, a 70 % confluent 35-mm dish should be sufficient for a number of individual samples. For biochemical assays, it is often necessary to start with a 10-cm dish. Seed the cells on #1.5 glass slide (or a glass-bottom chamber slide) if imaging or spectroscopy of cell-attached GPMVs is needed.
2. Some common transfection agents (e.g., Lipofectamine™) use cationic lipids to form complexes with DNA for transmembrane delivery. If possible, these reagents should be avoided

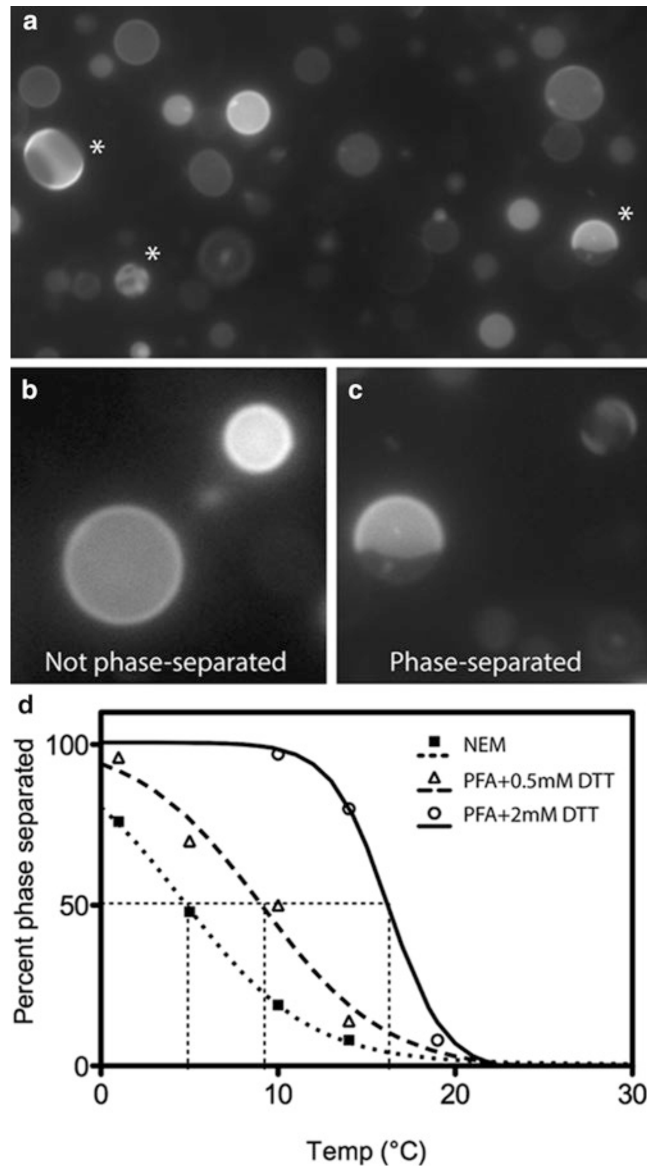

Fig. 3 Quantification of phase separation temperature. Exemplary images of GPMVs isolated from cells prestained with FAST DiO. (**a**) A mixed population of phase separated and non-phase separated GPMVs (*asterisks* mark phase separated GPMVs); (**b**) not phase separated GPMVs; and (**c**) phase separated GPMVs. Dozens of GPMVs can be quickly manually scored for phase separation and the fraction of phase-separated vesicles can be quantified for various temperatures. (**d**) Sigmoidal fits to this data yield the miscibility transition temperature, where 50 % of vesicles would be expected to show phase separation

Table 1
Fluorescent lipid dyes used in labeling GPMVs

Dye	Excitation (nm)	Emission (nm)	Phase partitioning	Working concentration (μg/mL)
FAST DiO	484	501	Nonraft/disordered	0.5–5
FAST DiI	549	565	Nonraft/disordered	0.5–5
Fluorescently labeled Cholera Toxin B	[a]	[a]	Raft/ordered	1–10
NBD-DSPE	470	530	Raft/ordered	5–20

[a]Excitation and emission is dependent on choice of fluorescent label for Cholera Toxin B

due to unknown effects of exogenous lipids on membrane composition and properties. This is particularly relevant for biophysical experiments on the phase behavior of isolated membranes where lipid contaminants could cause major artifacts. Nucleofection and calcium phosphate transfection are efficient alternatives.

3. Concentrations and incorporation conditions should be optimized for all dyes, although the conditions listed in Table 1 have been optimized for the dialkylcarbocyanine (i.e., FAST-DiO, FAST-DiI, etc.) dyes used here and can be used as a reasonable starting point for untested dyes. FAST DiO/DiI are recommended both because of their good fluorescence qualities and because their unsaturated acyl chains impart a high preference for the disordered/non-raft phase (compared to DiO/DiI which have saturated acyl chains and thus a low preference for either phase). High dye concentrations can affect the physical and biological properties of membranes, and these should always be kept to the minimal adequate concentrations (no more than 0.5 mol.%). For natural membranes (like GPMVs), mol fractions are not quantifiable, but can be estimated by comparing the brightness of incorporated dye to a synthetic membrane with well-defined dye concentration.

4. The most common preparation for GPMVs uses 25 mM formaldehyde and 2 mM DTT as the chemical agents that induce vesiculation, largely because it prevents detachment of adherent cells and therefore yields a cleaner and more efficient vesicle isolation. However, this preparation includes a number of undesired artifacts: (a) cross-linking of proteins by the aldehyde, which interferes with many biochemical analyses (e.g., PAGE); (b) cleavage of protein disulfides and thioesters [20]; (c) coupling of specific lipid headgroups (phosphatidylethanolamines) to proteins [18]. These effects (especially c) combine

to significantly affect phase behavior/properties of GPMVs, progressively increasing the phase coexistence temperature by 10–15 °C (*see* Fig. 3). The requirement for vesiculation appears to be treatment with a chemical that crosses the plasma membrane and covalently blocks sulfhydryls, e.g., *N*-ethyl maleimide (NEM) (for a non-exhaustive list, *see* ref. 23). Non-cross-linking, non-reducing vesiculants are suggested to avoid the artifacts listed above. To observe extensive, microscopic phase coexistence in these preparations, it is often necessary to cool these samples below 10 °C (Fig. 3; *see* **Note 10**).

5. GPMV isolation conditions need to be optimized for different cell types. Although the common preparations (25 mM PFA + 2 mM DTT or 2 mM NEM) will yield GPMVs for most common cell types, vesiculant concentrations, chemistries, and incubation times/temperatures can be varied to achieve optimal GPMV yields and purities. Temperatures as low as 4 °C can be used, though incubation times up to 24 h are necessary to produce efficient vesicle yields.
6. Although the GPMV isolation protocol described here yields vesicles large enough for most microscopic applications (radius ~1–5 μm), it is possible to increase vesicle size by reducing the osmolarity of the GPMV isolation buffer (*see* Subheading 2.2). Reducing [NaCl] down to 50 mM will yield larger vesicles at little cost to yield.
7. A significant experimental hurdle for observing phase separation is the requirement for cooling the microscopic sample. A simple way to increase the phase separation temperature is to treat isolated vesicles with sub-mM concentrations of deoxycholate [24]; ~0.5 mM will often allow observation of phase separation at room temperature. This is only recommended for preliminary experiments, to ensure that phase separation can be observed. It must be stressed that the effects of these biological detergents on PM structure, domain formation, and component partitioning are not characterized, and thus great care must be taken in generalizing results derived with such treatments.
8. For microscopic evaluation of component partitioning (as in Fig. 2), it is important to be aware of fluorescent signal bleeding between channels. This is particularly important if GFP-labeled proteins are being observed in the presence of bright, red fluorescent dyes (e.g., FAST-DiI). Using standard microscope/filter settings, the intensity of DiI in the green channel (excitation ~480–500 nm; emission ~510–550 nm) can be up to 20 % of that in the red channel (excitation ~520–550 nm ; emission ~560–600 nm).
9. The $K_{p,raft}$ calculation shown in Fig. 2 is a very reasonable approximation of the thermodynamic partition coefficient for

fluorescent chimeras of transmembrane proteins because the fluorophores are far removed from the membrane and are thus unlikely to be affected by the differing physical properties of the coexisting membrane phases. This is not true for fluorescent lipids, whose fluorophores are typically embedded in the membrane. Additionally, a more rigorous calculation for component partitioning than the simple $K_{p,raft}$ ratio shown in Fig. is the logarithm of $K_{p,raft}$ (i.e., log$P$).

10. It must be emphasized that while the GPMV preparation is a useful model of the cell plasma membrane, and clearly more compositionally representative of natural membranes than synthetic membranes, it is certainly not completely representative of the PM of live cells. These are several PM changes that are known to occur during GPMV formation, including ATP depletion, PIP2 hydrolysis, detachment of the cytoskeleton, and exposure of phosphatidylserine coupled to the loss of strict transbilayer asymmetry. Additionally, GPMVs represent the PM at equilibrium, a state that is incompatible with life. On top of these are the myriad unknown (and likely unknowable) changes that cells likely undergo during the hour-long incubation in the presence of reactive chemicals. These artifacts must be considered when GPMV results are extrapolated to live cells physiology.

Acknowledgments

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