A Novel Role for Nutrition in the Alteration of Functional Microdomains on the Cell Surface

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Summary

Membrane rafts are ordered microdomains of the plasma membrane consisting of cholesterol, sphingolipids, and saturated fatty acids which appear to regulate many cellular signaling pathways. One such type of membrane raft is caveolae, which are cave-like invaginations of the plasma membrane. Interestingly, changes in the acyl composition of cellular membranes have been shown to alter the specific localization of membrane raft associated proteins and their function. This is noteworthy because modification of membrane acyl composition is readily accomplished through changes in dietary fat composition. Here we describe a common approach used to fractionate cell membranes to obtain an enriched preparation of caveolae and gas chromatographic techniques to determine fatty acyl composition. In addition, methods used to visualize and quantify lipid rafts using a fluorescent probe Laurdan in living cells will also be described.

Keywords

Caveolae; Fatty acids; Laurdan; Lipid rafts; Membrane rafts; Nutrition

1. Introduction

Alteration of cell membrane structure, specifically the acyl fatty acids of phospholipids, is readily achieved through simple changes in dietary fat composition. This alteration is very rapid and can be readily detected within hours in cell culture or days in humans. The influence and impact of dietary fat composition on membrane structure has gained increasing interest due to advancements in techniques and refinements in our understanding of membrane rafts (1). Membrane rafts include structures known as caveolae, which are cave-like structures enriched in cholesterol, sphingolipids, and phospholipids containing saturated fatty acids. These membrane rafts are now implicated in a diversity of biological functions which impact on human health and disease (2–4). Studies have shown that alterations in dietary fat composition to include omega-3 fatty acids from marine oils can impact on the structure and function of colonocyte, immune, and cancer cell membranes (2,5–7). Collectively, this body of work demonstrates that simple nutritional interventions can have significant impact on cell signaling in part mediated through membrane rafts.

Concentrated caveolae can be isolated using a nondetergent approach following the methodology developed by Smart et al. (2,8,9). This protocol works well with isolated cells from cell culture and single cell populations from tissue. The procedure below describes the isolation of caveolae from cell culture. Depending upon the cell type and expression of caveolae, preliminary work is required to determine the optimum number of cells required to

obtain sufficient caveolae. Start with 100×10^6 cells, which may be the equivalent of several T-175 flasks. An overview of the procedure and resultant fractions are shown in Fig. 1.

2. Materials

2.1. Equipment

1. Ultracentrifuge at 4°C.

2. Benchtop centrifuge.

3. Water bath at 37°C.

4. Gradient maker.

5. Stirrer plate.

2.2. Reagents

1. Optiprep (60% v/v solution of iodixanol).

2. Percoll.

3. Sucrose.

4. Na$_4$EDTA (monohydrate, 380.2 MW).

5. Tricine.

3. Methods

3.1. Fatty Acid Treatment Protocol

1. Adherent cells are first grown for 2 days in basal medium to acclimate cells (*see* **Note** [1]).

2. On day 3, media are replaced with media containing supplemental fatty acids.

3. Stock nonesterified fatty acids (Nu-Chek Prep, Elysian Falls, MN, USA) are conveniently stored in ethanol at 1–10 mg/mL.

4. Prepare media containing 0–100 µM fatty acids. Note: concentrations exceeding 200 µM are toxic. Stock fatty acids are transferred to a 15-mL sterile Falcon tube and ethanol is evaporated under a gentle stream of nitrogen.

5. Fatty acids are bound to bovine serum albumin (fraction V) (0.1%w/v) by incubating in fetal bovine serum and basal medium in a shaking water bath at 37°C for 30 min.

6. Media is replaced.

7. Cells are grown until ~90% confluency. Media should be replaced every 3 days.

3.2. Caveolae Isolation

3.2.1. Solutions to be Prepared in Advance—*1× PBS*. 500 mL warm (37°C), 500 mL cold (4°C)

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[1]Isolation of caveolae is best accomplished using a single cell type from cell culture. In addition, this method is also suitable for purified cells isolated from tissue. Caveolae are found in most cell types, however their function is also cell specific. Therefore, if whole tissue is used, it must be recognized that results represent an aggregate from different cell types.

Buffer A: (stable for 1 week stored at 4°C)	Final Conc:
20 mL 2.5 M Sucrose	0.25 M
0.076 g Na$_4$EDTA (monohydrate, 380.2 MW)	1 mM
0.716 g tricine	20 mM

Combine with 170 mL water, pH to 7.8, bring to final volume of 200 mL

2XA Buffer: (stable for 1 week stored at 4°C)

13.32 mL 2.5 M Sucrose	0.5 M
0.05 g Na$_4$EDTA	2 mM
0.48 g tricine	40 mM

Combine with 40 mL of water, pH to 7.8, bring to final volume of 66.6 mL

OptiPrep Diluent: (stable for 1 week stored at 4°C)

1 mL 2.5 M Sucrose	0.25 M
0.022 g Na$_4$EDTA	6 mM
0.216 g tricine	120 mM

Combine with 7 mL water, pH to 7.8, bring to final volume of 10 mL

3.2.2. Solutions to be Prepared on Day of Experiment

30% Percoll:

30 mL Percoll

50 mL 2XA

20 mL water

50% OptiPrep:

36 mL OptiPrep

7.2 mL OptiPrep diluent

OptiPrep (60% v/v solution of iodixanol) Solutions:

5% – 0.4mL 50% OptiPrep + 3.6mL Buffer A

10% – 2.94mL 50% OptiPrep + 11.74mL Buffer A

15% – 1.5mL 50% OptiPrep + 3.5mL Buffer A

20% – 5.86mL 50 OptiPrep + 8.8mL Buffer A

1. Harvest cells at 90% confluency then centrifuge at $100 \times g$ for 3 min. After isolation, keep cells at 4°C or on ice.

2. Resuspend cells in 50 mL of cold 1× PBS and centrifuge at $100 \times g$ for 3 min.

3. Discard supernatant and resuspend pellet in 30 mL of Buffer A and centrifuge at $100 \times g$ for 3 min.

4. Discard supernatant and suspend pellet in 2 mL of Buffer A with protease inhibitor cocktail (Sigma) (40 µL/mL) and phosphatase inhibitor (orthovanadate) (10 µL/mL).

5. Homogenize 20 strokes on ice using Teflon /glass dounce homogenizer. Save aliquot (50 µL).

6. Spin at $1,000 \times g$, 10 min, 4°C.

Meanwhile precool ultracentrifuge and make 30% Percoll.

7. Collect supernatant (postnuclear) and keep on ice.

8. Resuspend pellet in 1.5 mL of Buffer A, therefore final volume is 3.5 mL. Combine supernatants, save an aliquot (50 µL).

9. The combined PNS (3.5 mL) was overlaid on 9 mL of 34.86% (v/v) Percoll solution in 13.2 mL Ultra-Clear Beckman Centrifuge Tubes. The 34.86% Percoll solution is made by diluting Percoll with 46.53% (v/v) 2XA Buffer.

10. Centrifuge in Beckman SW41 Ti rotor at $84,000 \times g$ for 30 min.

Meanwhile make remaining OptiPrep Solutions

11. Collect 2 mL (up to 3 mL) of plasma membrane fraction (visible band 6 cm from bottom of tube). Place in 15 mL Beckman tubes.

12. The PM fraction is then sonicated on ice, three times for 2 min each, with 2 min intervals between each sonication (*see* **Note** [2])

13. Pour Opti gradient by adding 2.3 mL of 50% OptiPrep to the samples and adjusting the final volume to 5.0 mL using Buffer A as a diluent.

14. A linear 20–10% OptiPrep gradient (3 mL each) is overlaid on the new 23% OptiPrep solution using a gradient maker connected to a peristaltic pump set at a flow rate of 1 mL/min

15. Centrifuge at $52,000 \times g$ (17,000 rpm) 90 min, 4°C in SW41 Ti rotar

16. Collect the top 5 mL into new tube, then prepare ~23% OptiPrep solution by adding 4 mL of 50% OptiPrep to sample and mix.

17. Overlay with a discontinuous gradient with 1 mL of 15% OptiPrep followed by 0.5 mL of 5% OptiPrep

18. Centrifuge at $52,000 \times g$ (17,000 rpm) 90 min, 4°C.

19. Collect top white band (500–700 µL) within 5% OptiPrep layer, just above interface.

3.3. Fast GC Analysis

1. 100 µL aliquots of caveolae are transferred to a 15 mL glass screw cap tube with teflon cap.

2. Lipids are extracted in chloroform:methanol (2:1), 4 mL and 1 mL of KCl (0.88%w/ v), vortex.

3. Centrifuge at $200 \times g$ to separate phases.

4. Carefully transfer bottom chloroform fraction to a clean glass tube, with Teflon cap.

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[2]The sonication step may introduce variability in the procedure dependent on the type of sonicator, power, and duration of cell disruption. Furthermore, interindividual variability contributes to differences in overall yield of caveolae.

5. Add 2 mL of chloroform to top phase and repeat extraction.

6. Combine chloroform extracts and dry down under a gentle stream of nitrogen.

7. Saponify lipids by adding 2 mL of KOH–MeOH, then incubate at 100°C (water bath or oven) for 1 h.

8. Cool for 5 min in fume hood, then add 2 mL of 14% BF_3–MeOH and 2 mL of hexane, then incubate at 100°C (water bath or oven) for 1 h.

9. Cool for 5 min in fume hood and then add 2 mL of double distilled water.

10. Centrifuge at $200 \times g$ to separate phases.

11. Transfer upper hexane phase to clean 2 mL gas chromatography vial.

12. Dry down hexane under gentle stream of nitrogen.

13. FAME are quantified on an Agilent 7890 flame-ionization detector gas chromatograph (Palo Alto, CA, USA) separated on a DB-FFAP fused silica column, 15 m × 0.10mmID × 0.10 µm (Agilent). Samples were injected in split mode, 200:1. The injector and detector ports should be set at 250°C. FAME are eluted using a temperature program set initially at 150°C for 0.25 min, increased at 35°C/min and held at 170°C for 3 min, increased at 9°C/min and held at 225°C for 0.5 min, increased at 80°C and held at 245°C for 2.2 min. Total run time is 12.9 min. The carrier gas, hydrogen, is set to a 0.5 mL/min constant flow rate. The makeup gas is nitrogen. Fatty acids are identified using authentic standards obtained from Nu-Chek Prep (#GLC-463) (*see* **Note** [3]) (Fig. 2)

3.4. Protocol for Visualization of Lipid Rafts Using the Fluorescence Probe Laurdan

3.4.1. Poly-l-lysine Precoating on Coverglass

1. Dilute 0.1% (w/v) poly-l-lysine solution (Sigma) to 0.01% with sterile water.

2. Fill the chambered coverglass (Nunc) wells with 0.01% polyl-L-lysine solution (1 mL).

3. Allow solution to sit for 30 min.

4. Aspirate excess solution and allow the coverglass wells to air dry.

5. Sterilize the coverglass slides under UV light for 1 h.

3.4.2. Preparation of 10 mM Laurdan (Invitrogen, MW 353.55) in Ethanol

1. Measure 10 mg of Laurdan in a glass tube covered with aluminum foil (Laurdan is light sensitive).

2. Dissolve in 2.83 mL of ethanol to make 10 mM of Laurdan stock solution.

3. Vortex vigorously (~2 min) to dissolve Laurdan.

4. Keep at 4°C and prewarm at RT prior to use.

3.4.3. Cell Labeling of Laurdan

1. Cell concentration might differ by type of cells.

2. Start with 1×10^6 cells/mL, which has been shown to be optimal for CD4^{+} T cells (10).

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[3]Although a fast-GC analysis approach is described, any fatty acid methyl ester method will be suitable.

3. Transfer 2 mL of cell suspension into a 15 mL-conical tube.

4. Add 2 µL of (10 mM) Laurdan solution into 2 mL of cell suspension to dilute to 10 nM of Laurdan (*see* **Note** [4]).

5. Vortex for 2 s.

6. Incubate cells at 37°C for 10 min.

7. Centrifuge cell suspension at 300× gravity for 5 min.

8. Resuspend cell pellets in 2 mL of Leibovitz's medium.

9. Transfer 1 mL of cell suspension into a poly-l-lysine-precoated chambered glass.

10. Incubate cells in an appropriate condition, if necessary.

3.4.4. GP-Value Calculation

1. Capture Laurdan-labeled cell images using 2-photon microscopy. (Zeiss LSM 510 META NLO, 40× objective 1.3 NA oil. The coherent Chameleon femtosecond pulsed Ti:Sapphire laser should be set at an excitation wavelength of 770 nm)

2. Save the image files as 8-bit TIFF format.

3. Open the image file in an image analysis software package, such as Adobe Photoshop® CS (v.8.0).

(The following protocol is using Adobe Photoshop®)

4. Using a marquee tool, make an oval to select a region of interest (Fig. 3b, c)

5. In "Histogram" panel, choose each channel of RGB.

6. Once blue channel is selected, the panel displays "mean intensity" of marquee-selected region.

7. A histogram will also show the pixels chosen by marquee tool.

8. If you change the channel to green, mean intensity is changed, while pixel is not.

9. Record green and blue intensities to calculate GP-values by formula

$$GP=(I_{Blue} - I_{Green})/(I_{Blue}+I_{Green}).$$

3.5. Results

3.5.1. Visualization of Lipid Rafts—There have been several methodologies developed to identify and visualize lipid rafts. For instance, flotation of DRM fractions and immunofluorescence microscopy by antibody patching have been used to identify putative raft association and raft proteins (11,12). In addition, immunoelectron microscopy and single fluorophore tracking microscopy have been utilized to track the location of raft components (13). Fluorescence resonance energy transfer (FRET) has also been applied to determine whether two raft components are spatially impacted (14). However, due to the specificity of probes used, the visualization of heterogeneous lipid rafts have been difficult to determine.

Recently, Gaus et al. demonstrated that the fluorescent probe Laurdan can align itself parallel with the hydrophobic tails of phospholipids in membranes (15–17). Owing to its ability to emit two different wavelengths according to the fluidity of the microenvironment, Laurdan can be

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[4]Laurdan concentration and duration of labeling should be optimized for each cell type used.

used to measure and visualize membrane fluidity in living cells by two-photon microscopy (16). Generalized polarized values (GP-values) derived by the formula GP = $(I_{400-460} - I_{470-530})/(I_{400-460} + I_{470-530})$, where I stands for intensities of the blue and green channels, respectively, indicates the fluidity of cell membranes. Given that lipid rafts are highly enriched in cholesterol and sphingolipids, the liquid ordered state of the membrane detected by Laurdan labeling is assumed to be heterogeneous lipid rafts. As an example, representative images of Laurdan-labeled CD4^{+} T cells co-cultured with anti-CD3 mAb expressing hybridoma cells are presented in Fig. 3a.

Acknowledgments

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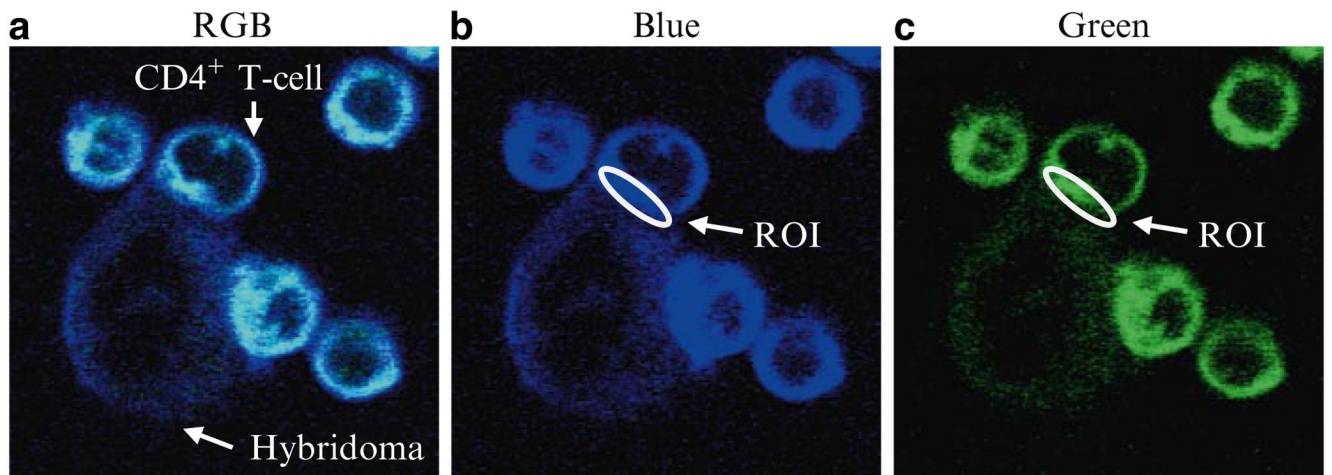
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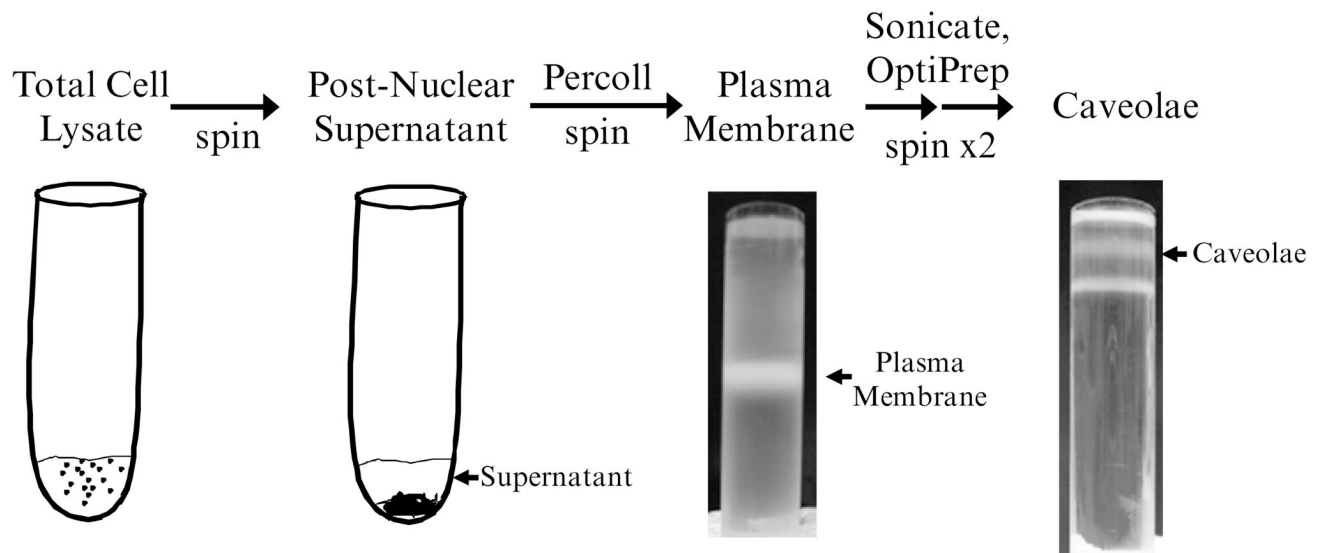
Fig. 1.
Shown in this figure are the bands obtained during the caveolae isolation procedure from cultured cells.

Fig. 2.
Shown in this figure is a representative fast gas chromatographic separation of fatty acid methyl esters.

Fig. 3.
Representative (**a**) RGB images of Laurdan-labeled CD4$^+$ T cells. Purified CD4$^+$ T cells were labeled with 10 µM Laurdan and stimulated by anti-CD3 mAb expressing hybridoma cells at 37°C for 30 min. GP-values were calculated from values of fluorescence intensity identified using Adobe Photoshop® to draw regions of interest (ROI) in each (**b**) blue or (**c**) green channel at the immunological synapse.