Purification and Fluorescent Labeling of Exosomes

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[Abstract] Exosomes are small membrane vesicles of endocytic origin secreted into the extracellular environment from a variety of different cells, and are thought to play important roles in intercellular communications. Here, we provide a useful protocol to purify the exosomes released from cell lines using sucrose gradient centrifugation. In this protocol, we also applied a red-fluorescent lipophilic dye, DiI, which is incorporated in the outer membrane of exosomes. This fluorescently labeled exosomes allow us to visualize individual exosomes by a confocal laser scanning microscope.

Materials and Reagents

1. Burkitt's Lymphoma B cell lines (*e.g.* Mutu$^-$, Mutu I, Mutu III cell lines)
2. RPMI 1640 medium (Wako Chemicals USA, catalog number: 189-02025)
3. Sucrose (Sigma-Aldrich, catalog number: S7903)
4. Anti-CD63 monoclonal antibody (clone MEM-250) (Abnova, catalog number: MAB0931)
5. Bradford protein assay kit (Bio-Rad Laboratories, catalog number: 500-0006JA)
6. 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI) (Life Technologies, catalog number: D3911)
7. Fetal Bovine Serum (FBS) (Sigma-Aldrich, catalog number: F9423)
8. Tris
9. NaCl
10. EDTA
11. Exosome-depleted FBS (see Recipes)
12. TNE buffer (see Recipes)
13. 0.25-2.5 M sucrose gradient in TNE buffer (see Recipes)

Equipment

1. 10 cm dish

2. Centrifuge (Eppendorf, model: 5810R or that with equivalent equipment spec)
3. Ultracentrifuge (Beckman Coulter, model: Optima L-80 XP or that with equivalent equipment spec)
4. 37 °C, 5% CO_2 cell culture incubator
5. Autopipette
6. 50 ml polypropylene concal plastic tubes (BD Biosciences, Falcon®, catalog number: 352070 or that with equivalent spec)
7. SW28 rotor (Beckman Coulter, model: 342204)
8. SW40Ti rotor (Beckman Coulter, model: 331301)
9. Polyallomer centrifuge tubes 1 x 3½ in (25 x 89 mm) for SW28 rotor (Beckman Coulter, catalog number: 326823)
10. Polyallomer centrifuge tubes 9/16 x 3½ in (14 x 89 mm) for SW41Ti rotor (Beckman Coulter, catalog number: 331372)
11. Spectrometer
12. Fluorescent or confocal laser scanning microscope

Procedure

A. Purification of exosomes

1. Burkitt's Lymphoma cell lines are grown up from 1 x 10^7 (in one 10 cm dish) to 2 x 10^8 cells (in twenty 10 cm dishes) in 200 ml RPMI 1640 medium containing 10% exosome-depleted FBS in the 5% CO_2 incubator at 37 °C.
2. Culture medium containing exosomes are harvested and centrifuged in 50 ml conical tubes at 1,500 rpm for 10 min at room temperature to remove cells.
3. The supernatant is centrifuged in 50 ml conical tubes at 3,500 rpm for 15 min at room temperature to remove cell debris.
4. The supernatant is ultracentrifuged in polyallomer centrifuge tubes at 25,000 rpm for 1 h at 4 °C with an SW28 rotor.
5. The pelleted exosomes are resuspended in 100 µl TNE buffer over night at 4 °C.
6. The exosomes are fractionated by use of a 0.25-2.5 M sucrose gradient in TNE buffer in polyallomer centrifuge tubes at 25,000 rpm for 4 h at 4 °C with an SW40Ti rotor. After that, you will see a band derived from exosomes (if you collect 1 ml of each fraction from the top, exosome fraction usually locates around 6th fraction from the top).
7. The band is collected (about 1 ml) carefully with an autopipette.
8. Fractionated exosomes are ultracentrifuged at 25,000 rpm for 1 h at 4 °C with an SW40Ti rotor.
9. The pelleted exosomes is resuspended in 100 ~ 200 µl TNE buffer over night at 4 °C.

10. The total protein concentration in the fractions is determined by the Bradford protein assay.
11. The fraction containing exosomes (4 µg, each) is confirmed by western blot analysis with anti-CD63 monoclonal antibody (1:1,000 dilution) (Figure 1).

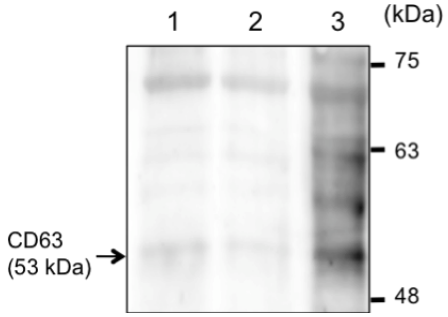

Figure 1. Purified exosomes derived from Burkitt's lymphoma Mutu cell lines. Exosomes were purified from culture medium of Mutu$^-$ (1st lane), Mutu I (2nd lane), and Mutu III (3rd lane) cells. 4 µg of exosomes were analyzed by western blot with anti-CD63. The arrow indicates the bands that correspond to CD63.

B. Fluorecent labeling of exosomes
1. 1 ml of fractionated exosomes (100 ng/ml) is incubated with 6 µl of 10 µM stock solution of 1, 1'-Dioctadecyl-3, 3, 3', 3'-Tetramethylindocarbocyanine Perchlorate (DiI) in methanol for 1 h in the dark at room temperature with gentle agitation.
2. Confirm the efficiency of labeling with small amount of exosomes under fluorescent or confocal laser scanning microscope.
3. Aliquot, stored at -80 °C.

Recipes

1. Exosome-depleted FBS
   Ultracentrifuge FBS at 25,000 rpm for 4 h at 4 °C
   Collect supernatant and stored at 4 °C
2. TNE buffer
   10 mM Tris-HCl (pH 7.6)
   100 mM NaCl
   1 mM EDTA
   Stored at room temperature
3. 0.25-2.5 M sucrose gradient in TNE buffer
   Prepare 0.25 M and 2.5 M sucrose in TNE buffer (Figure 2)

a. Fill 2.5 M sucrose solution up to the half the height of polyallomer centrifuge tubes (approximately 6 ml)
b. Add 0.25 M sucrose solution in layers up to the top of tubes (approximately 6 ml) and plug the tubes with rubber plugs
c. Lay down the tubes with the top slightly higher than bottom (use thin tube floater as a pillow) for al leaset 1.5 h at room temperature
d. Stand the tubes slowly and keep at 4 ˚C until just before use (storable up to 2 days)

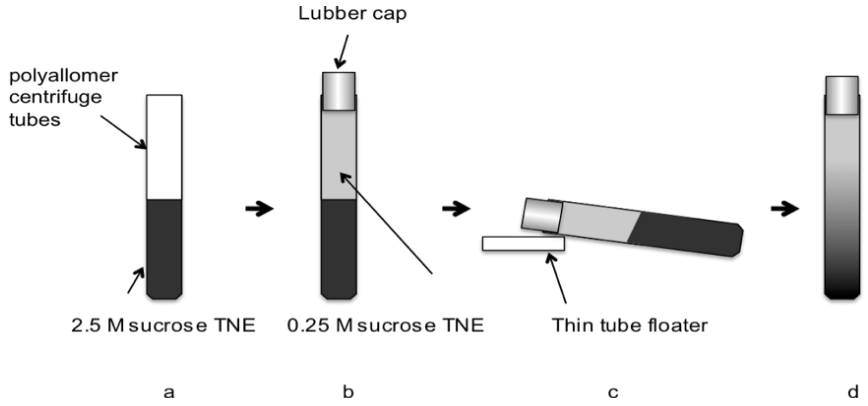

Figure 2. Preparation of 0.25-2.5 M sucrose gradient in TNE buffer (a) Fill 2.5 M sucrose solution up to the half the height of polyallomer centrifuge tubes (approximately 6 ml). (b) Add 0.25 M sucrose solution in layers up to the top of tubes (approximately 6 ml) and plug the tubes with rubber plugs. (c) Lay down the tubes with the top slightly higher than bottom (use thin tube floater as a pillow) for at least 1.5 h at room temperature. (d) Stand the tubes slowly and keep at 4 ˚C until just before use (storable up to 2 days).

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