In vitro Lipid Transfer Assay

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[Abstract] This is a protocol to detect lipid transfer activity of NRF-5, a member of the LPS binding/lipid transfer protein family. The lipid transfer activity is examined by using isotope-labeled cholesterol and liposomes, and tested in two directions (Figure 1): from proteins to liposomes and from liposomes to proteins.

Materials and Reagents

1. PC (Avanti-Polar Lipids)
2. PE (Avanti-Polar Lipids)
3. Cholesterol (Avanti-Polar Lipids, catalog number: 700000)
4. 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) (Avanti, catalog number: 850725)
5. 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC) (Avanti, catalog number: 770375)
6. 1 mCi (37 MBq) (PerkinElmer, catalog number: NET139001MC)
7. Protein of interest tagged with Flag
8. Tris-HCl (pH 7.4)
9. NaCl
10. Anti-Flag M2 agarose beads (Sigma-Aldrich, catalog number: A2220)
11. Flag peptide (Sigma-Aldrich, catalog number: F3290)
12. Chloroform
13. Wash buffer (see Recipes)
14. Elution buffer (see Recipes)

Equipment

1. Avanti Mini-Extruder (Avanti, catalog number: 610023)
2. Vortexer
3. Centrifuges
4. Rotator

5. Branson tip-sonicator (Cole-parmer Cp750)
6. Scintillation counter (Wallac MicroBeta TriLux, catalog number: 1450-023)

Procedure

A. Preparation of liposomes (room temperature)
   1. Liposome in the absence of cholesterol is made by Avanti Mini-Extruder at room temperature. The dried 1.25 mg mix of PC (75%) and PE (25%) is hydrated in 1 ml buffer (50 mM Tris-Cl, 150 mM NaCl). 100 nm unilamellar vesicles are obtained by extrusion as described: (http://www.avantilipids.com/index.php?option=com_content&view=article&id=185&Itemid=193)
   2. Liposomes containing [^{3}H]cholesterol are generated by using a standard sonication procedure.
      a. Dissolve dried 2.5 mg mix of PC (75%) and PE (25%) in 200 µl chloroform by vortex.
      b. Take 8 µl of above chloroform dissolved lipid mix and add 0.001 mg [^{3}H]cholesterol (~2% molar mass).
      c. The chloroform is evaporated and dried under a stream of nitrogen. Longer drying time (4-12 h) can be used to remove any trace of organic solvent.
      d. The dry lipid film is hydrated by adding 0.5 ml of buffer (50 mM Tris-Cl, 150 mM NaCl). After vortex at room temperature for 20 min, the large multilamellar vesicle suspension is disrupted with a Branson tip-sonicator until the suspension clear. For sonication, the samples are placed on ice and sonicated for 10 min with cycles including 9 sec sonication, 9 seconds interval and 35% input.
      e. Metal particles from the sonicator tip and undisrupted lipid aggregates are removed by centrifugation at 100,000 *x g* for 30 min at 4 °C. The resulting hazy supernatant, composed primarily of small unilamellar vesicles, is stored at 4 °C. The liposomes can be stored at this condition for one week.

B. Examine [^{3}H]cholesterol transfer from liposomes to proteins (Figure 1)

   Reactions are performed on ice.
   1. In the [^{3}H]cholesterol/liposome to protein transfer assay, each reaction contains, in a final volume of 200 µl buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4), 4 µg of PC: PE: [^{3}H]cholesterol liposomes, and different amounts of the acceptor protein EGFP-FLAG and EGFP::NRF-5-FLAG(15 and 30 µg) purified from 293T cells (Zhang *et al.*, 2012). EGFP-FLAG is used as the negative control.

2. After incubation for 30 min at 4 °C, each mixture is diluted with 600 µl 50 mM Tris-Cl, 150 mM NaCl, followed by adding 70 µl 50% Flag beads.
3. After incubation for about 1 h at 4 °C on shaker, the Flag beads are washed 4-5 times in 1,000 µl wash buffer on shaker, and bounded [^{3}H]cholesterol is quantified by scintillation counting.

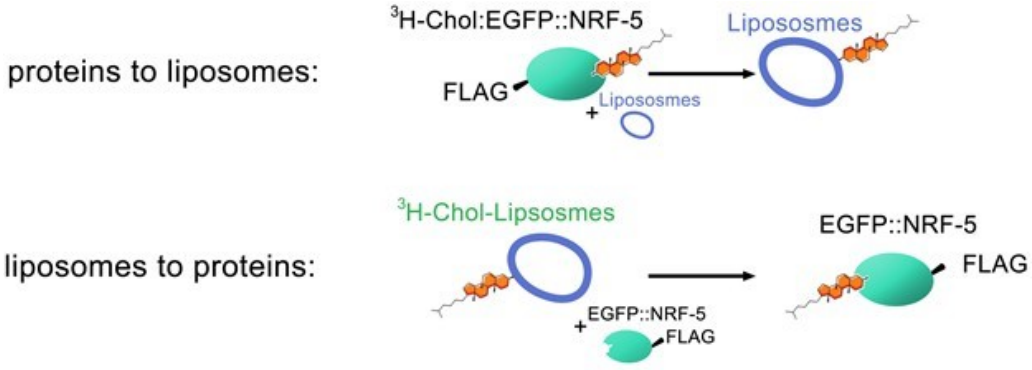

Figure 1. Schematic diagrams of the lipid transfer assay in two directions

C. Examine [^{3}H]cholesterol transfer from proteins to liposomes (Figure 1)
1. Protein-[^{3}H]cholesterol complex is obtained by incubating EGFP-FLAG or EGFP::NRF-5-FLAG (400 pmol) with [^{3}H]cholesterol (100 pmol) in a final volume of 300 µl buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4) for 3 h at 4 °C. The protein-[^{3}H]cholesterol complex is pulled down by incubating with 100 µl 50% Flag beads for 2 h at 4 °C, washing 6 times as above and eluted with 100 µl Flag peptide (100 mg/ml) for two times.
2. In the protein-to-liposome transfer assay, each reaction contains, in a final volume of 200 µl buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4), [^{3}H]cholesterol complexed to either EGFP or EGFP-NRF-5 (40 µl), and different amounts of acceptor PC liposomes (50 and 100 ng).
3. After incubation for 30 min at 4 °C, each mixture is diluted with 600 µl of buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4).
4. Liposomes are separated by centrifuging at 10,000 *x g* (hard to detect weight at this stage) for 30 min at 4 °C. The liposososmes can be seen as a small white patch at the bottom of the tube.
5. Wash the liposomes 4-5 times in the buffer (50 mM Tris-HCl, 150 mM NaCl), with 500 µl buffer used in each tube at each time. The liposomes are collected by centrifuge (10,000 *x g*) after each wash. The amount of [^{3}H]cholesterol transferred to liposomes is determined by scintillation counting.

Recipes

1. Wash buffer
   50 mM Tris-Cl, 150 mM NaCl (pH 7.4)
2. Elution buffer
   50 mM Tris-Cl, 150 mM NaCl (pH 7.4), Flag peptide

Acknowledgments

This protocol is adapted from Zhang *et al*. (2012) and Infante *et al*. (2008).

References

1. Infante, R. E., Wang, M. L., Radhakrishnan, A., Kwon, H. J., Brown, M. S. and Goldstein, J. L. (2008). NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. *Proc Natl Acad Sci U S A* 105(40): 15287-15292.
2. Zhang, Y., Wang, H., Kage-Nakadai, E., Mitani, S. and Wang, X. (2012). *C. elegans* secreted lipid-binding protein NRF-5 mediates PS appearance on phagocytes for cell corpse engulfment. *Curr Biol* 22(14): 1276-1284.