Separation of the Inner and Outer Mitochondrial Membrane in HeLa Cells

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[Abstract] Mitochondria are organelles that have important functions in oxidative phosphorylation, fatty acid oxidation and apoptosis signaling. They have two distinct membranes, outer membrane (OM) and inner membrane (IM). IM contains respiratory chain complexes that produce ATP. IM is rich in cardiolipin, a specific phospholipid reportedly having a critical role for organizing super-complex formation of respiratory chain complexes. IM abundant in cardiolipin exhibits resistance to extraction by digitonin (a non-ionic detergent), whereas the detergent easily lyses OM. Therefore, digitonin is useful to separate mitoplast (IM plus matrix) and OM from mitochondria. Here, we describe a method to isolate mitochondria from HeLa cells, and a method to isolate mitochondrial outer membrane proteins and inner membrane proteins by using digitonin. This method is applicable also to other types of cultured cells such as COS-7.

Materials and Reagents

1. HeLa cells (80-100% confluent) pre-cultured in 8 to 12 pieces of dishes (diameter 10-cm)
2. PBS (-) (without Ca^{2+} and Mg^{2+})
3. EDTA
4. Mannitol
5. Sucrose
6. HEPES
7. EGTA
8. Complete Mini EDTA-free (Roche Diagnostics, catalog number: 11 836 170 001)
9. Digitonin (Wako Pure Chemical Industries, catalog number: 040-02123)
10. Trichloroacetic acid (optional)
11. Mitochondria isolation buffer (MTiso-buffer) (see Recipes)
12. Digitonin stock solution (see Recipes)
13. Digitonin buffer (see Recipes)

Equipment

1. Culture dishes
2. Aspirator
3. Cell scraper (*e.g.* Rubber policeman)
4. Dounce homogenizer (Glass 7 ml Dounce Tissue Grinder) (WHEATON, catalog number: 357542)
5. 50 ml-tube
6. Phase-contrast microscope (upright-type, x100 ~ x400) (for confirming disruption efficiency of the cells)
7. Centrifuges [for centrifugation at 500 *x g* (swing rotor) and 10,000 *x g* (angle rotor)]
8. Micro tube mixer (*e.g.* TOMY MT-400, Digital Biology, catalog number: MT-400) or vortex mixer
9. Ultracentrifuge (*e.g.* Hitachi, catalog number: CS100) (for centrifugation at 100,000 *x g* (angle rotor)

Procedure

1. Remove medium from the culture dishes (harboring 80-100% confluent cells) by aspiration, and then add 3-4 ml of PBS (-) containing 1 mM EDTA into each dish.
2. After 5 min, scrape the cells by using a cell scraper (or by gentle pipetting), collect the cells into a 50 ml-tube, and centrifuge at 500 *x g* (5 min, 4 °C). Then, remove the supernatant by aspiration.
3. Re-suspend the pelleted cells with 50 ml of PBS (-) by inverting the tube, and then centrifuge it at 500 *x g* (5 min, 4 °C), followed by removing the supernatant by aspiration.
4. Suspend the pelleted cells with 5 ml of MTiso-buffer by inverting the tube, and then transfer the suspension into Dounce homogenizer pre-cooled on ice.
5. Homogenize the cell suspension by dounce homogenization [about 50 strokes (up and down)]. If necessary, verify the disruption efficiency of the cells under microscope.
6. Pile up the homogenate on an equal volume of 340 mM sucrose in a 50 ml-tube, and centrifuge it at 500 *x g* (10 min, 4 °C) to remove nuclei and unbroken cells as pellet.
7. Collect the supernatant in another tube (*e.g.* 5 ml-tube or some pieces of 1.5 ml-tubes), and then centrifuge it at 10,000 *x g* (10 min, 4 °C) to isolate mitochondria as pellet.
8. Re-suspend the isolated mitochondria with 1 ml of digitonin buffer containing appropriate concentration of digitonin (usually 0.15-5 mg/ml, see Recipes) (digitonin extraction).
9. Mix the suspension intensely for 15 min by using micro tube mixer (at max speed) or vortex mixer. If necessary, add an equal volume of MTiso-buffer to stop digitonin

extraction.

10. Centrifuge the suspension at 10,000 *x g* (10 min, 4 °C) to isolate the pellet containing mitoplast (IM plus matrix) and the supernatant containing solubilized OM and inter-membrane space (IMS) proteins.
11. To separate IM fraction and matrix fraction, re-suspend the pellet (mitoplast) in small amount of MTiso-buffer (usually 0.1-0.5 ml), gently sonicate it to disrupt mitoplast in the water-bath-type sonicator filled with ice-cold water, and then centrifuge it at 100,000 *x g* (30 min, 4 °C), giving IM fraction as pellet and matrix fraction as supernatant.
12. (Optional) If it is necessary to concentrate soluble proteins, please perform TCA-precipitation method using trichloroacetic acid.

Recipes

1. Mitochondria isolation buffer (MTiso-buffer)
   3 mM HEPES-KOH (pH 7.4)
   210 mM mannitol
   70 mM sucrose
   0.2 mM EGTA
   Complete Mini EDTA-free (protease inhibitor cocktail)
2. Digitonin stock solution (freshly prepared)
   4% digitonin in water (heating at 60 °C will improve the insolubility of digitonin)
3. Digitonin buffer (MTiso-buffer containing 0.15-5 mg/ml digitonin)
   Prepare the buffer by mixing MTiso-buffer and Digitonin stock solution
   Note: The efficiency of digitonin extraction largely depends on the quality of the digitonin reagent (e.g. manufacture and lot number). It is recommended to test the efficiency at several different concentration of digitonin. Digitonin extraction at appropriate concentration will clearly separate OM and mitoplast, although the treatment at higher concentration will solubilize not only OM but also IM.

References

1. Nishimura, N., Gotoh, T., Oike, Y. and Yano, M. (2014). TMEM65 is a mitochondrial inner-membrane protein. *PeerJ* 2: e349.
2. Pallotti, F. and Lenaz, G. (2007). Isolation and subfractionation of mitochondria from animal cells and tissue culture lines. *Methods Cell Biol* 80: 3-44.
3. Schnaitman, C. and Greenawalt, J. W. (1968). Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J Cell Biol* 38(1): 158-175.

4. Yano, M., Hoogenraad, N., Terada, K. and Mori, M. (2000). Identification and functional analysis of human Tom22 for protein import into mitochondria. *Mol Cell Biol* 20(19): 7205-7213.
5. Yano, M., Kanazawa, M., Terada, K., Namchai, C., Yamaizumi, M., Hanson, B., Hoogenraad, N. and Mori, M. (1997). Visualization of mitochondrial protein import in cultured mammalian cells with green fluorescent protein and effects of overexpression of the human import receptor Tom20. *J Biol Chem* 272(13): 8459-8465.