Enrichment of Golgi Membranes from HeLa Cells by Sucrose Gradient Ultracentrifugation

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[Abstract] This is a protocol to extract intact Golgi Membranes from HeLa cells using sucrose gradient centrifugation. This extraction is very useful for several applications including pull-down of Golgi membrane proteins, electron microscopy and reconstitution of protein transport into an isolated system. Protocol adapted from Balch *et al*. (1984).

Materials and Reagents

1. HeLa cells (ATTC, Wesel, Germany)
2. PBS
3. 1 M Tris pH 7.4
4. 100 mM EDTA
5. Trypan Blue
6. Protease inhibitor cocktail tablets (Roche, catalog number: 11836153001)
7. Breaking buffer (BB) (see Recipes)
8. 29% (w/w) sucrose (see Recipes)
9. 35% (w/w) sucrose (see Recipes)
10. 62% (w/w) sucrose (see Recipes)

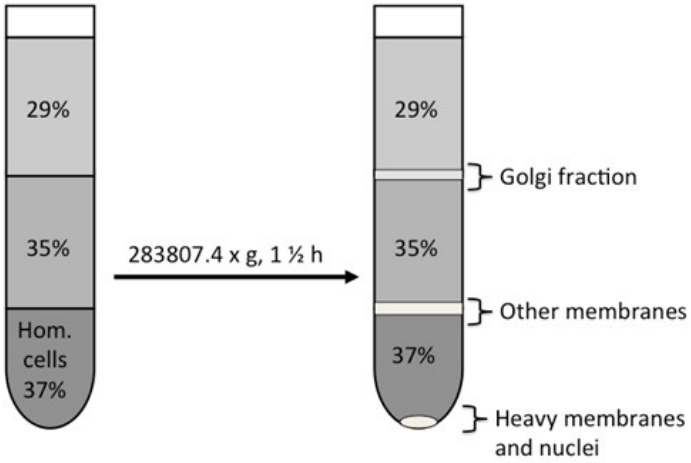
Equipment

1. Cell scrapers
2. Cell homogenizer (EMBL cell cracker) (EMBLEM Technology Transfer, Heidelberg)
3. Cell culture microscope
4. Ultracentrifuge (Beckman Coulter, model: Optima L-100K or equivalent)
5. Refractometer
6. SW40Ti rotor
7. Centrifuge tubes
8. 1 ml syringe with 20/21 G needle

Procedure

1. Remove medium and wash cells 3x with PBS and 1x with Breaking buffer (BB).
2. Harvest the cells by scraping and pellet the cells (for instance at 300 *x g*, 5 min).
3. Wash pellet 2x in PBS centrifuge cells at 300 *x g*, 5 min.
4. Wash 1x in ice-cold BB.
5. Dilute the pellet 1:5 in ice-cold BB.
6. Homogenize pellet with an EMBL cell cracker 20x on ice.
   Note: Make sure there are no air bubbles during the homogenization.
7. Mix a few µl of homogenate with a trypan blue solution on a glass slide and cover it with a coverslip. Check homogenization by microscope.
   Note: Plasma membrane should not be intact anymore. Cell nuclei should stain blue with Trypan Blue. There should be a lot of membrane fragments and particles in the homogenate, but the nucleus should stay intact.
8. Mix the homogenate with 62% sucrose
   a. 2 ml homogenate
   b. 1.83 ml of 62% ice-cold sucrose
   c. 41.7 µl of 100 mM EDTA (pH 7.4)

   Check the sucrose concentration to 37% +/- 0.5% with a refractometer.
   Sucrose gradients: solutions are w/w%.
   Check pH of the solutions after dissolving the sucrose.
9. Run gradient
   a. 4 ml homogenate (in 37% sucrose)
   b. 4.5 ml 35% sucrose
   c. 3.5 ml 29% sucrose (to the top)
   Note: Homogenate at the bottom, then add 35% sucrose, then add 29% sucrose.
10. Centrifugation: SW 40 Ti Rotor, centrifuge for 1.5 h at max speed (*x g*) at 4 °C.



11. Pull the Golgi band in 0.4 ml using a 1 ml syringe with 20/21 G needle (the Golgi band is located at the 35%/29% sucrose interphase).
12. Measure protein concentration and the functional Golgi membranes can now be snap frozen in liquid N_2 and stored at -80 °C.

Notes

1. Avoid salts/ions in the homogenate as it may aggregate the organelles.
2. Addition of high amount of sucrose affects the pH.
3. Don't homogenize too much in step 6 as organelles can break
   a. Proteases can leak out of the lysosomes.
   b. Broken organelles can reseal with other broken organelles.
   c. DNA can be released from nuclei which makes the sample sticky.
4. The isolated Golgi membranes are in a buffer containing about 30% of sucrose. Therefore, if Golgi membranes need to be pelleted for further analysis, the sucrose needs to be diluted out by addition of 3 volumes of an appropriate buffer such as PBS.

Recipes

1. Breaking buffer (BB)
   250 mM Sucrose
   10 mM Tris (pH 7.4)
   Add protease inhibitor cocktail tablets
2. 29% (w/w) sucrose
   65.08 g sucrose/200 ml
   10 mM Tris (pH 7.4)

3. 35% (w/w) sucrose
   80.60 g sucrose/200 ml
   10 mM Tris (pH 7.4)
4. 62% (w/w) sucrose
   161 g sucrose/200 ml
   10 mM Tris (pH 7.4)
   Note: Check all sucrose solutions with refractometer index and % of sucrose.

Acknowledgments

The protocol was adapted from the original version published by Balch *et al.* (1984).

References

1. Balch, W. E., Dunphy, W. G., Braell, W. A. and Rothman, J. E. (1984). Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* 39(2): 405-416.
2. von Blume, J., Alleaume, A.-M., Kienzle, C., Carreras-Sureda, A., Valverde, M. and Malhotra, V. (2012). Cab45 is required for Ca^{2+}-dependent secretory cargo sorting at the trans-Golgi network. *J Cell Biol* 199(7): 1057-1066.

