Subcellular Fractionation of Mouse Brain Homogenates

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[Abstract] This subcellular fractionation protocol is used for separation of cellular organelles based on their density. We have designed and optimized the protocol for separation of subcellular compartments of brain homogenates with focus on the localization and trafficking of transmembrane proteins, but we have also successfully used this protocol for fractionation of other types of tissue. The protocol has two major steps 1) preparation of homogenate from dissected tissue and 2) separation of organelles by centrifugation of homogenates using a continuous sucrose gradient.

Materials and Reagents

1. Mice
2. PBS
3. Phosphatase inhibitor (*e.g.* PhosStop) (F. Hoffmann-La Roche, catalog number: 04906845001)
4. HEPES
5. EDTA
6. Distilled water
7. Glucose
8. Protease inhibitors (*e.g.* Complete) (F. Hoffmann-La Roche, catalog number: 11697498001)
9. Solution A (see Recipes)
10. 2.5 M sucrose stock (see Recipes)
11. Sucrose solution (0.8 M and 1.6 M) (see Recipes)

Equipment

1. A pair of scissor
2. A brain scooper
3. Falcon tubes (15 ml)
4. Centrifuge

5. Ultracentrifuge
6. Ultracentrifuge rotor (T70.1 Ti and Sw41Ti)
7. Ultracentrifuge tubes for T70.1 Ti rotor (Polycarbonate, 16 x 76 mm) (Beckman Coulter, catalog number: 355651)
8. Ultracentrifuge tubes for SW41 Ti rotor (14 x 89 mm) (Seton Open-Top Polyclear centrifuge tubes, catalog number: 7030)
9. Thomas® Teflon Pestle (A.H Thomas Co., model: 3431-E20)
10. 1 and 10 ml syringes
11. Needles (18 and 27 G)
12. Gradient Master 107 ip (BioComp, model: 107) with marker block and cannula
13. Refractometer

Procedure

1. Mouse is sacrificed by cervical dislocation; restrain the mouse on a hard, flat surface. Hold a strong stick or metal rod firmly against the base of the skull, and the tail firmly with the other hand. Pull the mouse body away from the head in one single quick motion. Verify the dislocation by feeling for a separation between cervical vertebras.
2. Cut the head off the mouse, and open the skin of the head with a pair of scissor.
3. Open the skull of the mouse using a pair of scissor, and gently lift out the exposed brain using a brain scooper and transfer to ice-cold PBS.
4. Brains are quickly rinsed in ice-cold PBS.
5. Brains are put in 10 ml solution A containing proteinase inhibitors and continue to point 9 when all brains have been isolated.
6. Repeat steps 1-7 for the remaining mice.
7. The brains are homogenized using a Thomas® Teflon Pestle.
8. Transfer the homogenized brain tissue to a 15 ml Falcon tube.
9. Centrifuge at 1,000 *x g* for 10 min at 4 °C.
10. Transfer the supernatant (there are typically several layers of supernatant – take them all) to a new 15 ml Falcon tube.
11. Centrifuge at 3,000 *x g* for 10 min at 4 °C.
12. Transfer the supernatant to an ultracentrifugation tube.
13. Centrifuge at 13,000 rpm for 10 min using an ultracentrifuge with a T70.1 Ti rotor.
14. Transfer the supernatant to a new ultracentrifugation tube and centrifuge at 50,000 rpm for 45 min at 4 °C (using the same rotor (T70.1 Ti rotor) as previous step). *Note: During this centrifugation step, one can prepare the sucrose gradient.*
15. Discard the supernatant.

16. Dissolve pellet in 600 µl solution A containing proteinase inhibitors. Use a 1 ml syringe with an 18 G needle first, thereafter a 27 G needle to dissolve pellet.
17. Carefully layer 500 µl of the homogenate (= dissolved pellet) on top of a continuous 0.8 to 1.6 M sucrose gradient and centrifuge at 25,000 rpm for 18 h/overnight, using a SW41 Ti swing bucket rotor.

    Notes for preparation of the gradient:

    a. *The gradient is made with a Gradient Master 107 ip.*
    b. *Insert a tube in the marker block and mark a line on the tube at the half-full mark for short, 4 mm cap.*
    c. *Add 5.5 ml 0.8 M sucrose solution to the tube.*
    d. *Add 1.6 M sucrose beneath the 0.8 M sucrose solution using a cannula (a cannula is received together with the Gradient Master) and a 10 ml syringe until the bottom of the 0.8 M sucrose solution reaches the recently marked line.*
    e. *Adjust the level of the Gradient Master before use, to ensure that the plate is in level.*
    f. *Prepare the 10-57% linear gradient, by choosing "SW41" in the" gradient menu" list of BioComp Gradient Master. This program will mix the gradient at 50 degrees for 10 min, followed by 1 min at 80 degrees.*
18. Next day the gradient is fractionated into 24 samples (500 µl/sample), using a 1 ml pipette. Alternatively, one can use a Piston Gradient Fractionator to collect the fractions.
19. Store the fractions at -20 °C until Western blot analysis.

Notes

1. Perform steps 1-9 as fast as possible.
2. Do as many steps as possible on ice.
3. Remember to tare the balance of the weight of the samples before performing ultracentrifugation. Use Solution A to adjust the weight.
4. Stored fractions should be thawed on ice and vortexed before preparation of samples for Western blot analysis.

Recipes

1. Solution A (200 ml)

   0.25 mM sucrose

   1 mM EDTA

   20 mM HEPES (pH 7.4)

   Mix 17 g sucrose with approximately 100 ml dH_2O

Add 400 µl EDTA (500 mM stock, to a final concentration of 1 mM)
Add 8 ml HEPES (500 mM stock, to a final concentration of 20 mM)
Add dH_2O to a final volume of 200 ml
pH 7.4
Store at 4 °C

2. 2.5 M sucrose stock (580 ml)
   500 g sucrose
   Add a little volume of distilled water at the time, while heating, until final volume is approximately 580 ml.
   Measure refractive index to ensure a concentration of approximately 2.5 M
   Store at 4 °C.
3. 0.8 M sucrose solution (50 ml) in 10 mM HEPES (pH 7.2)
   16 ml of 2.5 M sucrose stock
   1 ml HEPES (500 mM stock, to a final concentration of 10 mM) (pH 7.2)
   Add 33 ml dH_2O to a final volume of 50 ml
   Mix all ingredients
   Store at 4 °C if the solution is made shortly before experiment, otherwise the solution can be stored at -20 °C
4. 1.6 M Sucrose solution (50 ml) in 10 mM HEPES (pH 7.2)
   32 ml of 2.5 M sucrose stock
   1 ml HEPES (500 mM stock, to a final concentration of 10 mM) (pH 7.2)
   Add 17 ml dH_2O to a final volume of 50 ml
   Mix all ingredients
   Store at 4 °C if the solution is made shortly before experiment, otherwise the solution can be stored at -20 °C

References

1. Gustafsen, C., Glerup, S., Pallesen, L. T., Olsen, D., Andersen, O. M., Nykjaer, A., Madsen, P. and Petersen, C. M. (2013). Sortilin and SorLA display distinct roles in processing and trafficking of amyloid precursor protein. *J Neurosci* 33(1): 64-71.

