Isolation and Culture of Neurospheres for the Study of Pathogenesis of Prion Disease

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[Abstract] Neurosphere contains neural stem cells that are capable of self-renewal and multilineage differentiation including neurons, astrocytes, and oligodendrocytes (Gage, 2000). Cell culture model using differentiated neurosphere cultures are suggested to be a valuable tool for studying the pathogenesis of prion disease at the cellular level (Iwamaru *et al.*, 2013). This protocol describes the procedure for a culture of whole brain-derived neurospheres from newborn mouse brains. Neurosphere formation steadily occurs within a week from the cultures of neonatal whole brains and these cells have stem cell properties.

Materials and Reagents

1. Newborn mice (age at one day after birth)
   Present culture method can be applied to any mouse strains, regardless of sex. We used prion protein overexpressing transgenic mouse and prion protein-deficient mouse for our present study (Iwamaru *et al.*, 2013).
2. Dulbecco's phosphate buffered saline without Ca and Mg (D-PBS) (Nacalai Tesque, catalog number: 14249-95)
3. Di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA) (Nacalai Tesque, catalog number: 151-11)
4. Hank's balanced salt solution (HBSS) (Sigma-Aldrich, catalog number: H8264)
5. Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12 Ham) (Sigma-Aldrich, catalog number: D8437)
6. N-2 supplement (Life Technologies, catalog number: 17502-048)
7. Penicillin-Streptomycin (Sigma-Aldrich, catalog number: P0781)
8. Fetal bovine serum (FBS) (Hyclone, catalog number: SH30070.03)
9. Accutase (Innovative Cell Technologies, catalog number: AT104)
10. Trypsin (Sigma-Aldrich, catalog number: T8003) (see Recipes)
11. Deoxyribonuclease I (DNase I) (Worthington Biochemical Corporation, catalog number:

LS002139) (see Recipes)

12. Epidermal growth factor human (EGF) (Sigma-Aldrich, catalog number: E9644) (see Recipes)
13. Fibroblast growth factor-basic human (bFGF) (Sigma-Aldrich, catalog number: F0291) (see Recipes)
14. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9576) (see Recipes)
15. Neurosphere basal medium (see Recipes)

Equipment

1. Falcon 15 ml conical centrifuge tube (Corning, catalog number: 352096)
2. Falcon 100 mm cell culture dish (Corning, catalog number: 353003)
3. Nunc 60 mm dish with HydroCell surface (Thermo Fisher Scientific, catalog number: 174912)
4. Disposable micro homogenizer (TaKaRa Bio, TaKaRa BioMasher Standard, catalog number: 9791A)
5. Aerosol resistant tip (ART 1000 REACH tips) (Thermo Fisher Scientific, model: 2079)
6. Membrane filter (EMD Millipore, Millex-GV 0.22um, 33mm)
7. Dissecting microscope (Olympus, model: SZH10)
8. Centrifuge (KUBOTA, model: 5220)
9. CO_2 incubator (set at 37 °C and 5% CO_2-95% air)
10. Reciprocal shaker (TAITEC, model: NR-1)
11. Laminar flow hood (W800 x H1050 x D500 mm) (Panasonic Corporation, SANYO, model: MCV-710ATS)
12. Scissors (NAPOX B-5H, 154 mm; NAPOX B-1112H, 110 mm), fine forceps (NAPOX A-5, 110 mm; NAPOX MA-45, 110 mm), spatula (Laboran 9-891-03, 180 mm)
13. Ice bucket

Procedure

1. Dissect the brain from newborn mice under Laminar flow hood (Figure 1). Spray 70% ethanol upon newborn mice and cut the head (Figure 2A). Make small incisions (Figure 2B, arrows) and remove the skin. Then, carefully remove the skull with forceps and expose the brain (Figure 2C).

Figure 1. Dissecting equipments arranged in a Laminar flow hood

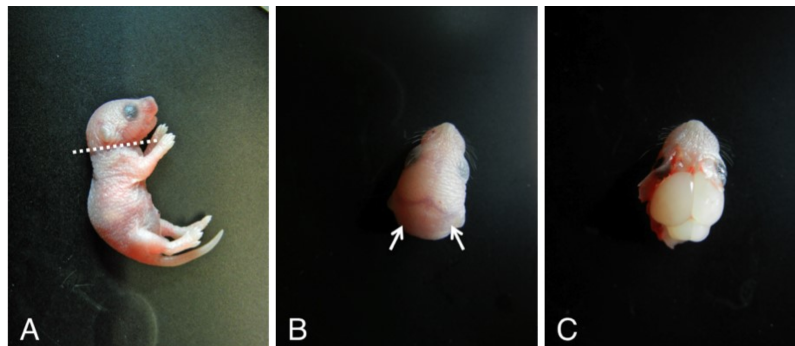

Figure 2. Dissection procedures to obtain neonatal mouse brain

2. Scoop the brain by a small spatula and place them in ice cold HBSS (10ml in 100mm dish, Figure 3A). By using fine forceps, remove meninges under dissecting microscope (Figure 3B-C).

Figure 3. Removal of meninges (B: arrow) from neonatal mouse brain under dissecting microscope. After removal of meninges, the brain becomes whitish color (C).

3. Place cortices in sterilized disposable micro homogenizer and centrifuge at 5000 *x g* for 30 sec at 4 °C.

4. Pipet the minced tissue with 2 ml of D-PBS containing 1 mM EDTA, 100 µg/ml trypsin, and 400 µg/ml DNase I until evenly homogenized and place them in a 15 ml Conical Centrifuge Tube.
5. Incubate for 15 min at 37 °C in an incubator with constant agitation using a reciprocal shaker at 50 rpm. Add 1 ml of FBS, then gently pipet up and down to help the dissociation by using aerosol resistant tip to avoid contamination. Stand tubes for 2 min at 25 °C and collect supernatant by pipetting. Avoid to suck any visible tissue fragments.
6. Centrifuge cell suspensions at 200 × *g* for 5 min at 25 °C and resuspend the pellet in Neurosphere basal medium (10 ml) by pipetting.
7. Centrifuge the cell suspension at 200 *x g* for 5 min at 25 °C. Discard the supernatant, resuspend in 5 ml of Neurosphere basal medium at 25 °C and transfer it into Nunc 60mm dishes with HydroCell Surface. Culture the cells at 37 °C in humidified CO_2 incubator (5% CO_2-95% air).
8. Refill 0.5 ml of Neurosphere basal medium everyday.
9. After 5-7 days, neurospheres with variable sizes (50-100 um in diameter) are formed in the culture (Figure 4). Centrifuge neurosphere suspensions at 200 × *g* for 5 min at 25 °C, discard the supernatant, and resuspend in 2 ml of Accutase at 37 °C for 10 min.

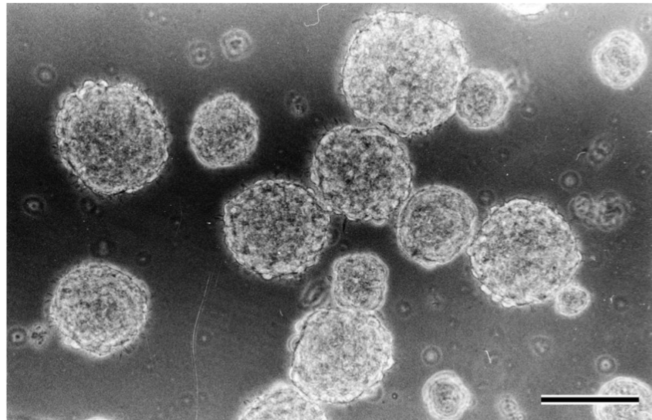

Figure 4. Neurosphere formation in Neurosphere basal medium after 5 days of culture. Scale bar = 100 um

10. Gently pipet up and down (about ten times) using aerosol resistant tip to dissociate into single cells. Check the cells under microscope for successful dissociation. Add 12 ml of HBSS and centrifuge at 200 *x g* for 5 min at 25 °C. Resuspend the pellet in 10 ml of Neurosphere basal medium at 25 °C and subculture into new Nunc 60mm dishes with HydroCell Surface at 1:4 split ratio (*i.e.* 25% of the harvested cells are seeded into the new dish with same size). Refill 0.5 ml of Neurosphere basal medium everyday. The

neurospheres are subcultured at 4-6 day intervals.

Recipes

1. Deoxyribonuclease I (DNase I)
   Dissolved at 4 mg /ml in D-PBS
   Sterile filtered (0.22 μm)
   Aliquoted (4 ml) and stored at -20 °C
   Use within 6 months
2. Epidermal growth factor human (EGF)
   Dissolved at 50 μg /ml in sterile DMEM/F12 Ham containing 1% BSA
   Aliquoted (100 μl) and stored at -20 °C
   Use within 6 months
3. Fibroblast growth factor-basic human (bFGF)
   Dissolved at 50 μg /ml in sterile DMEM/F12 Ham containing 1% BSA
   Aliquoted (50 μl) and stored at -20 °C
   Use within 6 months
4. Bovine serum albumin (BSA)
   Dissolved at 300 mg /ml in D-PBS
   Sterile filtered (0.22 μm)
   Stored at 4 °C
   Use within 6 months
5. Neurosphere basal medium

Reagents	Volume	Final conc.
DMEM/F12 Ham	487.33 ml	
EGF	500 μl	50 ng/ml
bFGF	500 μl	50 ng/ml
BSA	1.67 ml	0.1%
N2 supplement	5 ml	1%
Penicillin-streptomycin	5 ml	100 IU/ml and 100 μg/ml, respectively

6. Trypsin
   Dissolved at 1 mg /ml in D-PBS containing 10 mM EDTA
   Sterile filtered (0.22 μm)
   Aliquoted (4 ml) and stored at -20 °C
   Use within 6 months
   For the dissociation of minced brain tissue, mix the reagents as follows

Reagents	Volume	Final conc.

Trypsin	1 ml	100 µg/ml
DNase I	1 ml	400 µg/ml
D-PBS	8 ml	

Acknowledgments

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References

1. Gage, F. H. (2000). Mammalian neural stem cells. *Science* 287(5457): 1433-1438.
2. Iwamaru, Y., Takenouchi, T., Imamura, M., Shimizu, Y., Miyazawa, K., Mohri, S., Yokoyama, T. and Kitani, H. (2013). Prion replication elicits cytopathic changes in differentiated neurosphere cultures. *J Virol* 87(15): 8745-8755.