Generation of Neuron-enriched Cultures (Method 1)

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[Abstract] This protocol will guide you through the process for generating midbrain neuronal cultures from late embryo mouse brain. These cultures serve as a useful tool to study molecular pathways during neuronal death in various neurological disorders. This method uses cytosine β-D-arabinofuranoside in the cultures to suppress the proliferation of glial cells. Seven days after the seeding, the neuron-enriched cultures prepared following this protocol will contain less than 10% glia cultures. The concentrations of β-D-arabinofuranoside should be adjusted according to your culture condition. This protocol has been developed and improved over the years by various researchers in Dr. Hong's lab, especially Dr. Bin Liu.

Materials and Reagents

1. Poly-D-lysine (Sigma-Aldrich, catalog number: P7280)
2. MEM (Life Technologies, Gibco®, catalog number: 11090-08)
3. D-Glucose
4. Sterile PBS
5. Trypan blue dye
6. Heat-inactivated fetal bovine serum (FBS) (Life Technologies, Gibco®, catalog number: 16000-044)
7. Heat-inactivated horse serum (HS) (Life Technologies, Gibco®, catalog number: 26050-088)
8. Non-essential amino acids (100 ml) (Life Technologies, Gibco®, catalog number: 11140-050)
9. Sodium pyruvate (100 ml) (Sigma-Aldrich, catalog number: S8636)
10. 200 mM L-glutamine (100 ml) (Life Technologies, Gibco®, catalog number: 25030-081)
11. Penicillin/streptomycin (100 ml) (Sigma-Aldrich, catalog number: P0781)
12. Neurobasal medium (Life Technologies, Invitrogen™, catalog number: 12348-017)
13. 50x B27 serum-free supplement (10 ml) (Life Technologies, Invitrogen™/ Gibco®, catalog number: 21103-049)
14. Poly-D-lysine solution (see Recipes)
15. Maintenance culture medium (see Recipes)
16. Treatment medium (see Recipes)

Equipment

1. Cell culture incubator
2. Centrifuges
3. Dissection microscope
4. Scissors and forceps
5. Laminar hood
6. 24-well plates
7. 50 ml tube
8. 10-ml pipet

Procedure

1. Coating and washing culture plates
   a. In a laminar hood, dilute poly-D-lysine stock solution (5x) with sterile water to 20 µg/ml.
   b. Add 0.25 ml to each well of 24-well plates.
   c. Leave the plates in the hood for 2-3 h or in the in incubator for at least 1 h.
   d. Before use, remove the coating solution.
   e. Wash the wells twice with 1 ml/well of sterile water.
   f. Add 1 ml sterile PBS to each well. Completely remove the PBS right before use.
2. In the animal procedure room, remove embryos from time-pregnant rats or mice at embryonic day 13/14 and place embryos in cold MEM.
3. Under a microscope, dissect out the midbrain portion of the embryonic rat or mouse brains. Remove meninges and blood vessels. Pool tissues and keep in ice cold MEM.
4. In a laminar hood, transfer tissues to a 50 ml tube. Gently triturate the tissues (5-10 times each) first with a 10 ml pipet, then a 1 ml pipet tip fitted to the 10-ml pipet followed with a fitted 200-µl pipet tip.
5. Centrifuge the triturated tissues for 10 min at 6.5x speed setting (~1,500 rpm).
6. Carefully remove the supernatant and resuspend the pelleted cells in 10 ml of maintenance culture medium.
7. Take 30 µl of the cell suspension and mix with 270 µl of Trypan blue dye. Load 10 µl onto a hemocytometer to count cell density.
8. Adjust the cell density to1 x 10^6 cells/ml with maintenance culture medium.
9. Add 0.5 ml of cells to each well of the poly-D-lysine-coated 24-well plate.
10. Place the plates in a humidified 37 °C incubator with 5% CO_2.
11. Two days after initial seeding, add 0.5 ml culture medium containing 10–20 µM cytosine β-D-arabinofuranoside to the cultures to suppress the proliferation of glial cells.

12. At 2–3 d later, change the cultures back to fresh culture medium.
13. Treat seven-day-old cultures that contain less than 0.1% OX-42-immunoreactive microglia and about 8% glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes with desirable reagents or vehicle.

Recipes

1. Poly-D-lysine solution
   Dissolve in 50 ml of ddH_2O to make 5x stock solution.
   Keep as 5.0 ml aliquots at -20 °C.
2. Maintenance culture medium

Reagents	volume	final con.
MEM	380 ml	-
D-Glucose	0.5 g	1 g/L
Heat-inactivated fetal bovine serum	50 ml	10%
Heat-inactivated horse serum	50 ml	10%
None essential nonessential amino acids	5 ml	0.1 mM
Sodium pyruvate	5 ml	1 mM
L-glutamine	5 ml	2 mM
Penicillin/streptomycin	5 ml	50 U/ml/50 µg/ml

   Con.: concentration
   Sterile filter (0.2 µm) and store wrapped in foil at 4 °C.
   *Heat-inactivated at 56 °C for 30 min and stored in 50-ml aliquots at -70 °C.
   ** Stored in 50-ml aliquots at -20 °C.
3. Treatment medium

Reagents	**volume**	**final con.**
MEM	465 ml	-
Heat-inactivated FBS	10 ml	2%
Heat-inactivated HS	10 ml	2%
Sodium pyruvate	5 ml	1 mM
L-glutamine	5 ml	2 mM
Penicillin/streptomycin	5 ml	50 U/ml/50 µg/ml

   Sterile filter (0.2 µm) and store wrapped in foil at 4 °C.

References

1. Gao, H. M., Hong, J. S., Zhang, W. and Liu, B. (2002). Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. *J Neurosci* 22(3): 782-790.