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Preparation of Rodent Primary Cultures for Neuron–Glia, Mixed Glia, Enriched Microglia, and Reconstituted Cultures with Microglia

Shih-Heng Chen, Esteban A. Oyarzabal, and Jau-Shyong Hong

Abstract

Microglia, neurons, and macroglia (astrocytes and oligodendrocytes) are the major cell types in the central nervous system. In the past decades, primary microglia-enriched cultures have been widely used to study the biological functions of microglia in vitro. In order to study the interactions between microglia and other brain cells, neuron–glia, neuron–microglia, and mixed glia cultures were developed. The aim of this chapter is to provide basic and adaptable protocols for the preparation of these microglia-containing primary cultures from rodent. Meanwhile, we also want to provide a collection of tips from our collective experiences doing primary brain cell cultures.

Keywords

Microglia; Neuron–glia culture; Mixed glia culture; Microglia-enriched culture; Reconstituted neuron–microglia culture

1 Introduction

Although the in vitro study of microglia goes as far back as 1930 [1, 2], it was not until 1986, with the development of a new protocol to selectively isolate and culture microglia from mammalian brain [3], that the use of microglia in cell culture systems of the brain became popular. Since then, and with the advent of several different microglia-containing cell culture protocols and microglia-specific cell markers, our understanding of the physiology of microglia continues to vastly improve.

Several cell culture systems exist to study microglia derived from rodent brains, including mesencephalic neuron–glia, mixed glia, enriched microglia, and reconstituted neuron–microglia cultures. Each culture system can serve different purposes to study the molecular mechanisms and the interactions between microglia and neurons, microglia and astrocytes, or microglia themselves under normal physiological or pathological conditions. Mesencephalic neuron–glia cultures serve as an excellent in vitro model of the midbrain—both in the diversity and ratio of different cell types when compared to the normal physiology of the brain. This culture is predominantly composed of astrocytes (~50 % GFAP immunopositive cells), neurons (~40 % NeuN immunopositive cells), and microglia (~10 % OX-42 immunopositive cells) [4] and is commonly used as a platform to study

neuron–glia interaction, especially in culture models that focus on degeneration or neuroprotection of dopaminergic neurons (which account for 1–2 % of the neurons in this culture)—such as in toxin-induced models of Parkinson’s disease [4–7].

Primary mixed glia cultures are composed of astrocytes (~80 % GFAP immunopositive cells) and microglia (~20 % OX-42 immunopositive cells) [8] and are commonly used to study the interaction between microglia and astrocytes. In mixed glia cultures, microglia grow loosely attached to the confluent monolayer of astrocytes; therefore, microglia can be shaken off to produce a microglia-enriched culture that is composed of more than 95 % microglia, as characterized by immunocytochemical staining with OX-42 [9]. Primary rodent microglia-enriched cultures are widely used to study microglial biology in vitro. Lastly, enriched microglia can be seeded on top of neuron-enriched cultures to generate reconstituted neuron–microglia cocultures which are a useful tool to investigate direct neuron and microglia interaction in vitro.

Over the past decade these protocols have successfully allowed our laboratory to investigate the physiology of microglia. Our hope is to share our experience to help other researchers establish their own microglia-containing primary cultures.

2 Materials

2.1 Equipment Component

1. A guillotine or an acrylic box connected to CO₂ tank for euthanizing animals (*see* Note 1).
2. One pair operating scissors (5½").
3. One pair dissecting scissors (4¼")
4. One pair microdissecting scissors (4½", 17 mm blades).
5. One pair curved dissecting forceps (5").
6. One pair microdissecting tweezers (no. 5).
7. One pair curved microdissecting tweezers (no. 5).
8. Dissection microscope.
9. Sterile petri dishes.
10. Sterile 50 mL conical vials.
11. Sterile 10 and 25 mL serological pipettes.
12. Sterile 200 µL and 1 mL pipette tips.
13. Sterile 70 µm cell strainer (BD Bioscience, San Jose, CA, USA).

¹Rat and mouse dams are euthanized without anesthesia by decapitation and cervical dislocation, respectively, because anesthetic agents have been shown to affect brain chemistry [11]. Rapid asphyxiation with CO₂ gas is a suitable alternative method that appears to have a minimal impact on embryonic brain cells.

14. Sterile tissue culture-grade plates (Corning Inc., Corning, NY, USA) or flasks (BD Bioscience, San Jose, CA, USA).
15. Hemocytometer, 0.4 % Trypan Blue Stain solution (Invitrogen, Carlsbad, CA, USA) and bright-field microscope.
16. Humidified cell culture incubator (37 °C, 5 % CO₂).

2.2 Reagents and Solutions Component

1. Neuron–Glia Dissection Buffer: Minimum Essential Medium (MEM) (cat. No. 11090, Invitrogen, Carlsbad, CA, USA).
2. Mixed Glia Dissection Buffer: Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (cat. No. 11330, Invitrogen, Carlsbad, CA, USA).
3. Poly-D-lysine working solution: dilute 100 µg/mL poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) in ddH₂O to a final concentration of 20 µg/mL. Store at 4 °C.
4. Cytosine β-D-arabinofuranoside (ara-C) (Sigma-Aldrich, St. Louis, MO, USA) solution: dilute 10 mM of stock solution in Neuron–Glia Maintenance Medium to the desired final concentration.
5. Neuron–Glia Maintenance Medium (for 500 mL): combine 380 mL of MEM, 50 mL of heat-inactivated fetal bovine serum (FBS), 50 mL of heat-inactivated horse serum (HS), 5 mL of 100× nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 5 mL of 100 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 5 mL of 200 mM L-glutamine, 5 mL of 5,000 U/mL penicillin and 5,000 µg/mL streptomycin, and 0.5 g of D-glucose. Stir gently to dissolve, filter sterilize, and store in the dark at 4 °C.
6. Mixed Glia Maintenance Medium (for 500 mL): combine 430 mL of DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 5 mL of 100× nonessential amino acids, 5 mL of 100 mM sodium pyruvate, 5 mL of 200 mM L-glutamine, and 5 mL of 5,000 U/mL penicillin and 5,000 µg/mL streptomycin. Stir gently to dissolve, adjust pH to 7.2, add 50 mL of heat-inactivated FBS, filter sterilize, and store in the dark at 4 °C.

3 Methods

3.1 Preparing for Primary Culture

1. All cell culture plates and flasks are pre-coated with poly-D-lysine before seeding with single cell suspension. Coat 24- and 6-well plates with 0.5 and 1 mL of the poly-D-lysine working solution, respectively, and 25 mL to 175 cm² culture flasks. Place in 37 °C incubator for at least 1 h.
2. After coating, rinse the culture plates or flasks three times: twice with sterile ddH₂O and once with sterile PBS. The volume of ddH₂O and PBS in different culture plates and flask is 1 mL in 24-well plate, 2 mL in 6-well plate, and 25 mL in 175 cm² flask. Store culture plates and flasks in PBS at 37 °C until immediately before cell seeding.

3. Preheat aliquoted culture medium in water bath at 37 °C, and sterilize dissection instruments and work surfaces with 70 % ethanol beforehand.

3.2 Mesencephalic Neuron–Glia Culture Protocol

1. Euthanize two to four rat dams at gestation day 14–15 and mouse dams at gestation day 13–14 (*see* Notes 1 and ²).
2. Lay the rodents on their back and clean the surface of abdominal area with 70 % ethanol. Lift the abdominal skin with the curved dissecting forceps, and using operating scissors make a vertical incision to expose the abdominal muscle layer. Using the dissection scissors make a vertical incision through the muscle layer to expose the abdominal cavity. Carefully remove the uterine horns and place them in a petri dish filled with ice-cold Neuron–Glia Dissection Buffer.
3. Carefully remove the embryos from the amniotic membrane and transfer them into a petri dish with ice-cold Neuron–Glia Dissection Buffer and place on ice. Rinse the embryos twice with ice-cold Neuron–Glia Dissection Buffer and transfer them into a new petri dish with ice-cold Neuron–Glia Dissection Buffer. Place this dish on top of a petri dish filled with ice and set on the floor of a dissection microscope.
4. Under the microscope, hold down the body of the embryo with a pair of microdissecting tweezers, and make two perpendicular incisions with the microdissecting scissor to cut out the mesencephalic region of the brain (*see* Fig. 1a) [10]. Insert one blade of the scissors into the tubular structure, and butterfly the tissue by making an incision down the dorsal midline (*see* Fig. 1b, c). Carefully separate the tissue from the meninges, and transfer the ventral midbrain tissue into a 50 mL conical tube filled with 10 mL of ice-cold dissection Neuron–Glia Dissection Buffer.
5. In a biological safety cabinet, triturate the midbrain tissues into a single cell suspension by slowly passing the tissue through a 10 mL serological pipette until the tissues are smaller than the opening of the 1 mL pipette tip. Fit a sterile 1 mL pipette tip to the end of the 10 mL serological pipette, and continue to triturate the tissue until it is smaller than the opening of a 200 μ L pipette tip. Remove the 1 mL pipette tip and fit a 200 μ L pipette tip to the end of the 10 mL serological pipette and triturate the tissues three to five times and strain the cell suspension through a 70 μ m cell strainer into a fresh 50 mL conical tube (*see* Note 3).
6. Centrifuge the cell suspension at $430 \times g$ for 6 min at 4 °C.

²An accurate estimate of embryonic age is extremely important when culturing dopaminergic neurons—this is because cells must be transplanted during a critical 2-day window during which dopaminergic neurons are undergoing differentiation. Transplants outside this critical window result in poor survival. The embryonic age can be confirmed by comparing the crown-to-rump length (CRL) (*see* Fig. 2) of your embryos to the average lengths (*see* Table 2) [12, 13]. Differences in CRLs may also be attributed to differences among strains or animals with partial or complete genetic ablations. Thus it is important to conduct pilot studies to determine the most suitable critical time window for your cultures.

³The frequency of tissue trituration varies among cell culture users. The number of passages may depend on the pipette-aid and the size and quantity of brain tissue. A gradual decrease in the size of the tip in respect to the decrease in the size of the tissue reduces the sheering pressure exerted on tissue resulting in less cellular rupturing (optimally less than 10 % of the total cells).

7. Decant the supernatant and resuspend the pellet in 10 mL of warm Neuron–Glia Maintenance Medium.
8. Using Trypan Blue Stain solution and a hemocytometer, determine the cell count and viability of the cell suspension. Adjust the density of the viable cells with warm Neuron–Glia Maintenance Medium to 1×10^6 cells/mL. Cap and invert the conical tube to ensure a thorough mixture of the cell suspension. Add 0.5 mL per well of the cell suspension to a poly-D-lysine-coated 24-well plates (*see* Note 4). Gently agitate the plate in several directions to ensure even seeding, and place it in a humidified 37 °C, 5 % CO₂ incubator. Avoid stacking plates because it may affect the cellular air exchange of the plates.
9. Gently supplement each well with 0.5 mL of warm Neuron–Glia Maintenance Medium on the third day after seeding. Be sure to add medium along the side of the wells to avoid disturbing the cellular monolayer.
10. Cells are ready for treatment 7 days after their initial seeding. The cellular composition of the neuron–glia culture can be determined by immunocytochemistry staining (*see* Note 5).

3.3 Mixed Glia Culture Protocol

1. Wipe the head of 0–1-day-old rodent pups with 70 % ethanol and euthanize them by decapitation.
2. Remove the whole brain by securing the rostral end of the head (dorsal side upward) with a pair of tweezers and making an incision through the foramen magnum towards the ear and curving up (near the eye sockets) towards the coronal suture. Peel back the skin and skull to expose the brain with a pair of tweezers and gently scoop up the entire brain, and immerse it in a petri dish filled with Mixed Glia Dissection Buffer. Place this dish on top of a petri dish filled with ice and set on the floor of a dissection microscope.
3. Remove the olfactory bulbs, cerebellum, and brain stem using microdissecting tweezers and curved microdissecting tweezers. Dissociate the remaining brain tissue into cerebral hemispheres and midbrain. Carefully remove all the meninges and blood vessels from each tissue. Pool the brain tissue into a 50 mL conical tube with 15 mL of ice-cold Mixed Glia Dissection Buffer.
4. In a biological safety cabinet, triturate the pooled cerebral hemisphere and midbrain tissues into a single cell suspension by slowly passing the tissue through a 10 mL serological pipette until the tissues are smaller than the opening of the 1 mL pipette tip. Fit a sterile 1 mL pipette tip to the end of the 10 mL serological pipette, and continue to triturate the tissue until it is smaller than the opening of a 200 µL

⁴Based on our laboratory's experience, optimal growth of neuron–glia cultures has been observed in 24-well plate from Corning but not BD Falcon.

⁵Immunocytochemical staining using cell type-specific markers (*see* Table 3) is routinely used to determine the cellular heterogeneity of the neuron–glia culture—which should be composed of ~50 % astrocytes, ~40 % neurons, and ~10 % microglia. A variation in the ratio of microglia, either above or below 10 % of the total cells, may result in highly variable experimental results. Thus, it is important to monitor the cellular heterogeneity of the neuron–glia culture to ensure cell culture stability from batch to batch.

pipette tip. Remove the 1 mL pipette tip and fit a 200 μ L pipette tip to the end of the 10 mL serological pipette and triturate the tissues three to five times and strain the cell suspension through a 70 μ m cell strainer into a fresh 50 mL conical tube (*see* Note 3).

5. Centrifuge the cell suspension at $430 \times g$ for 6 min at 4 °C.
6. Decant the supernatant and resuspend the pellet in 10 mL of warm mixed glia culture maintenance medium.
7. Using Trypan Blue Stain and a hemocytometer, determine the cell count and viability of the cell suspension. Adjust the density of the viable cells with warm Mixed Glia Maintenance Medium to acquire the proper seeding concentration (*see* Table 1).
8. Refresh the medium every three days by completely removing and replacing the spent medium. Add 25 mL for flasks, 1 mL/well for 24-well plates, and 3 mL/well for 6-well plates (*see* Note 6).
9. Cells are ready for treatment 7–14 days after their initial seeding, depending on size of the culture container. Cultures seeded on 6- or 24-well plates are ready for treatment 7 days after seeding (*see* Note 7).

3.4 Enriched Microglia Culture Protocol

1. Grow mixed glial cultures (*see* Subheading 3.3) in 175 cm² culture flasks for about 14–16 days after seeding, tightly cap the flasks, and seal them with parafilm (*see* Note 8).
2. Stack the flasks on a flat platform shaker and shake the flasks at 180 rpm for 30 min to 1 h at 37 °C (*see* Note 9). After shaking, collect the medium into 50 mL conical tubes, and centrifuge the cell suspension for 6 min at $430 \times g$ at 4 °C.
3. Resuspend the cell pellet in an appropriate volume of Mixed Glia Maintenance Medium.
4. Using Trypan Blue Stain and a hemocytometer, determine the cell count and viability of the cell suspension. Adjust the density of the viable cells with warm Mixed Glia Maintenance Medium to 1×10^6 cells/mL. Seed the cell suspension into either a 96-, 24-, or 6-well plate with 0.1, 0.5, and 2 mL of the cell suspension per well, respectively. Store cells overnight in a humidified incubator (37 °C, 5 % CO₂).
5. Cells are ready for treatment the next day.

⁶The ability of the cells to adhere to the culture plates may be damaged if cultures are perturbed during the first three days. Gently agitate the plates or flasks before refreshing the medium to dislodge any cellular debris—reducing potential endogenous ligands that could activate microglia via damage-associated molecular pattern receptors.

⁷The growth of microglia may be delayed in different strains or transgenic mice—pilot studies may be required to optimize the microglia composition in the culture.

⁸Covering the filter cap of flasks with parafilm prevents the fluctuation of pH in medium and reduces the risk for contamination.

⁹The speed and duration of shaking is relatively slower and shorter than other protocols, resulting in fewer but more pure cell suspensions of microglia. The time of shaking should be optimized by individual to reduce Type II astrocyte and oligodendrocyte contamination.

3.5 Reconstituted Neuron–Microglia Coculture Protocol

1. Grow neuron–glia cultures (*see* Subheading 3.2) in 24-well plates for 48 h, and gently supplement each well with 0.5 mL of warm Neuron–Glial Maintenance Medium containing ara-C with a final concentration of 5–15 μ M (*see* Notes 10 and 11).
2. Refresh the medium at day 6 after seeding by completely removing and replacing the spent ara-C-containing medium with warm neuron–glia medium containing a cell suspension of enriched microglia, seeding at 5×10^4 cells/well. Store cells overnight in a humidified incubator (37 °C, 5 % CO₂).
3. Cells are ready for treatment the next day.

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¹⁰The consistency of the neuron–glia culture is important during the proper preparation of neuron-enriched cultures. Once the culture is consistent, optimize the treatment concentration of ara-C to ensure the highest glial toxicity and lowest neurotoxicity.

¹¹Ara-C and leucine methyl ester (LME) are routinely used to ablate glial cell and microglia, respectively, from culture systems. It is important to note that as little as 1% microglial contamination can result in a detectable proinflammatory response.

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Fig. 1.

Illustration of the steps to isolate the mesencephalic region. **(a)** Separate the rostral forebrain (use the traverse sinus as hallmark) and the caudal hindbrain from the mesencephalic region as indicated the *dotted lines*. **(b)** Butterfly the tissue by inserting the microdissection scissors through the inside of the mesencephalic region of the neural tubule and slicing open the tissue along the dorsal midline. **(c)** Remove the meninges from the butterflyed mesencephalic tissue

Fig. 2.
The crown-to-rump length (CRL) of the embryo is the greatest distance from the top of the skull to the buttock

Table 1

Seeding concentration for primary murine mixed glia cultures

	Mouse	Rat
6-well plate	1.5×10^6 cells/well	1×10^6 cells/well
24-well plate	1.5×10^5 cells/well	1×10^5 cells/well
175 cm ² flask	5 brains/flask	2.5 brains/flask

Table 2

Average CRL measurements from E13 to E15 rodents as derived from data compiled by Butler and Juurlink [12]

Gestation days	Mouse	Rat
E13	7–8 mm	7–9 mm
E14	9–10 mm	10–11 mm
E15	10.5–11.5 mm	12.5–13 mm

Table 3

Markers used to identify brain cells

Cell type	Marker
Astrocytes	GFAP
Microglia	OX-42 Iba-1
Neurons	NeuN MAP-2
Dopaminergic neurons	TH
Oligodendrocytes	MBP