Isolation and Purification of Primary Rodent Astrocytes

Astrocytes are a major cell type in the mammalian central nervous system (CNS). The ability to obtain virtually pure populations of these cells makes it possible to study their function as isolated cells or in mixed populations where they support the growth and survival of surrounding neurons. Unlike other mature CNS cells, mature astrocytes maintain the lifelong ability to reenter the cell cycle. The first isolation procedure described (see Basic Protocol) takes advantage of the proliferative ability of these cells, as does the second (see Alternate Protocol), except that no antibody or complement treatment is required. A procedure for detecting glial fibrillary acidic protein (GFAP), which is present in most astrocytes in vivo and virtually all astrocytes in vitro and is a useful marker for assessing the purity of cultures, is also presented (see Support Protocol).

BASIC PROTOCOL

ISOLATION AND CULTURE OF ASTROCYTES FROM RODENT CEREBELLM

Neonatal animals from postnatal day 0 (P-0) to P-7 are decapitated, and the scalp as well as the entire dorsal portion of the cranium are removed. The cerebellum is isolated and freed from the remainder of the brain, the meninges and pia mater are stripped free and discarded, and the remaining tissue is enzymatically and mechanically dissociated. The cells are pelleted and resuspended for density gradient centrifugation. The astrocyte-enriched population is plated and then freed of contaminating neurons and fibroblasts by antibody-mediated cytolysis. This protocol can be used for one animal or an entire litter; animals from different litters should be processed separately.

Materials

- Mice aged P-0 to P-7
- PBS/glucose (see recipe)
- 60% and 30% Percoll (see recipes)
- Trypsin/DNase solution (see recipe)
- DNase solution (see recipe)
- PBS/glucose (see recipe) containing 5 mg/liter MgSO₄
- DMEM/10% FBS (APPENDIX 2A) containing 2% glucose
- 0.4% trypan blue solution (e.g., Life Technologies)
- Tissue culture plates coated with poly-L- (or poly-D-) lysine (APPENDIX 2A)
- PBS (APPENDIX 2A)
- Horse serum (optional)
- 0.05% trypsin/0.53 mM EDTA (Life Technologies)
- Anti-Thy 1.1 or 1.2 antibody; depending on the species and strain of animals used as the source of astrocytes (if monoclonal, IgM antibodies are preferable)
- Dulbecco’s minimum essential medium (DMEM)
- Rabbit or guinea pig complement (Life Technologies)
- Fetal bovine serum (FBS, APPENDIX 2A; optional)
- Dimethyl sulfoxide (DMSO; optional)

Dissecting instruments:
- 1 pair microdissecting scissors (~3.5-in.)
- 1 pair dissecting scissors (~6.5-in)
- 2 pairs no. 5 Dumont forceps (~4.75-in.)
- 1 pair Semkin dissecting forceps (~6-in)
- 1 pair curved no. 5 Dumont forceps (optional)
100-mm petri dishes (Falcon)
5-ml and 15-ml conical tubes (Falcon)
9-in. Pasteur pipets (one flame polished) and bulb
Swinnex 13-mm filter units (Millipore) with 20-µm nylon mesh filters (Fisher)
100-mm tissue culture plates (Falcon)
1-ml syringes
18-, 20-, and 23-G needles
Humidified 37°C, 7% CO₂ incubator
Bright-field microscope equipped for epifluorescence
Additional reagents and equipment for determining cell number and viability with a hemacytometer and trypan blue, and trypsinizing cells (See *CPMB APPENDIX 3F* and *APPENDIX 1A* in this manual)

**NOTE:** The following procedures must be carried out under sterile conditions on a clean bench or in a laminar flow hood.

**NOTE:** All protocols using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

### Prepare instruments and work area
1. Sterilize the instruments, preferably in an autoclave, or by dipping in ethanol and flaming. Autoclave the Swinnex filters after they have been assembled with nylon mesh in place.

2. In the lid of a petri dish, make several small puddles (approximately five times more than the number of pups to be harvested) of ∼100 µl PBS/glucose, and a single puddle of ∼400 µl.


4. For discontinuous Percoll gradients (one gradient for each two to three cerebella to be dissected), add 3 ml of 60% Percoll to the bottom of a 15-ml conical tube. Carefully and slowly overlay 2 ml of 30% Percoll such that there is a sharp separation between the two concentrations.

   *It is essential that the gradients be handled with extreme care so the interface is maintained. The gradients are stable for ~1 hr after pouring.*

5. Place one 1-ml aliquot of trypsin/DNase and two to six 1-ml aliquots of DNase (depending on the number of animals to be taken; about one aliquot per five animals) in a 37°C water bath for use in steps 12 and 14.

### Harvest the cerebellum
6. Anesthetize the pups by cooling them on ice one at a time. Once the animal has stopped moving, decapitate with the large scissors, drop the head into a 100-ml petri dish, and grasp the snout with large forceps using the left hand (for right-handed individuals).

7. Slide the closed points of the small scissors between the scalp and the cranium and separate the scalp from the underlying tissue by sliding the scissors back and forth. Cut away the skin thus-loosened and discard.

8. Open the small scissors, insert one point through the foramen magnum (at the base of the skull), angling the blade to the extreme side of the head, and cut along the side of the skull until the coronal suture is reached. Repeat the process on the other side,
then cut across the cranium and lift it away from the underlying tissue with a fine
forceps.

At this stage, you will be looking at a dorsal view of the brain and the cerebellum will be
exposed.

9. Place the blades of either a straight or curved forceps under the cerebellum, and gently
lift it away from the remainder of the brain (see Fig. 3.5.1). Place each cerebellum
harvested into a separate small puddle in the lid of the petri dish.

Strip the membranes
10. Move the petri dish lid with the cerebella to the stage of a dissecting microscope.
Using the Dumont forceps, hold the tissue with one forceps, and peel away the
meninges and pia mater from both sides of the cerebellum.

If blood vessels are observed on the surface of the tissue, they are most likely associated
with the membranes, and should be removed along with the associated membranous
material. This step is critical to reduce the number of contaminating fibroblasts.

11. Move each stripped cerebellum without the dissociated membranes to the larger
puddle in the dish lid.

Enrich astrocyte population
12. Transfer the cleaned cerebella to a 5-ml tube, rinse with PBS/glucose, and remove
the fluid. Immediately add 1 ml trypsin/DNase solution, making sure that the fluid
is in contact with all of the tissue in the tube. Cap the tube and transfer it for 3 min
to a 37°C water bath.

13. Remove the trypsin/DNase solution and carefully wash the tissue three times by
adding ~2 ml PBS/glucose, allowing the tissue to settle to the bottom of the tube, and
then removing PBS/glucose with a Pasteur pipet and bulb, making sure that the pellet
is undisturbed.

14. After removing the last wash solution, add 1 ml DNase solution. Beginning with the
18-G needle, draw up the tissue and DNase solution and expel it back into the tube.
Repeat this procedure for a total of fifteen times, then repeat (fifteen times each) with
the 20- and 23-G needles.
15. Draw up the cell suspension and discard the needle. Using sterile technique, attach the Swinnex filter unit onto the syringe and pass the cell suspension through the filter into a fresh 5-ml tube.

16. Centrifuge the tube 1 min at 150 × g, 4°C, in a tissue culture or clinical centrifuge.

17. For each gradient (two to three cerebella) used, resuspend the pellet in 1 ml of a 1:1 mixture of DNase solution and PBS/glucose/MgSO₄ (e.g., 5 ml for five gradients). Without disturbing the gradient interfaces, overlay the gradients with 1 ml of the cell suspension, and cap tube.

18. Transfer the gradients to a centrifuge and spin 10 min at 150 × g, 4°C.

   The astrocyte-enriched fraction migrates to the buffer/30% Percoll interface, and the neuron-enriched fraction migrates to the 30%/60% interface (see Fig. 3.5.2).

19. Transfer the gradient back to a laminar flow hood. Place a rubber bulb on a flame-polished Pasteur pipet and expel all of the air from the bulb (do not introduce bubbles into the gradient). Insert the pipet into the gradient just above the astrocyte-enriched layer and carefully aspirate the cells at the interface; the cells will adhere to one another, so lift them as a carpet of material. Transfer the cell suspension to a fresh 15-ml conical tube, top off the tube to 15 ml with PBS/glucose, and centrifuge 10 min at 150 × g, 4°C.

   OPTIONAL: The neurons may be harvested if desired. Pellet cells, wash with PBS/glucose, and plate the cells on polylysine-coated plates with 5% horse serum.

20. Resuspend the pellet in DMEM/10% FBS/2% glucose, determine the cell number and viability using trypan blue and a hemacytometer (CPMB APPENDIX 3F), and plate at 2.5–5 × 10⁴ cells/ml on polylysine-coated plates, using 1 ml DMEM/10% FBS/2% glucose for every 10 mm² tissue culture area.

   IMPORTANT NOTE: Incubate all cultures at 37°C in a humidified incubator buffered with 7% CO₂, and use sterile technique appropriate for tissue culture in carrying out procedures.

   At this point, there will be numerous contaminating neurons, and a small number of fibroblasts.
**Purify astrocytes by antibody-mediated cytolysis**

21. Two days after the establishment of astrocyte-enriched cultures, trypsinize the cells with trypsin/EDTA solution. Wash the cells three times with PBS/glucose, remove the last wash, and add 1 ml trypsin/EDTA for every 30 mm² tissue culture surface area. Check frequently under the microscope.

  The procedure outlined in steps 21 to 27 will deplete the culture of all Thy 1–expressing cells, including neurons and fibroblasts, which are the contaminating cell types in this preparation.

22. As soon as the cells begin to loosen from the plate, tap the plate firmly against a hard surface to facilitate lifting of the cells. Once the majority of cells are in suspension, add 3 vol DMEM/10% FBS/2% glucose, transfer to 15-ml conical tube, and centrifuge 5 min at −150 × g, 4°C.

23. Resuspend the pellet in 5 ml DMEM/10% FBS/2% glucose and centrifuge 5 min at −150 × g, 4°C. Resuspend the pellet in 1 ml monoclonal antibody supernatant, or antibody diluted in DMEM/10% FBS/2% glucose as recommended by the manufacturer for cytolysis. Incubate the cells 1 to 2 hr on ice.

24. Spin cells in a refrigerated centrifuge 5 min at −150 × g, 4°C. Resuspend in 3 ml DMEM and add 1 ml complement. Incubate 1 hr at 37°C.

25. Add DMEM to full volume of the tube (15 ml) and centrifuge the cells 5 min at −150 × g, 4°C. Resuspend the pellet in DMEM and centrifuge 5 min at −150 × g, 4°C. Repeat.

26. Plate the cells at 5 × 10⁴ cells/ml in DMEM/10% FBS/2% glucose on polylysine-coated plates and incubate.

  These cells are essentially pure astrocytes, and can now be grown, used, or passaged.

27. Passage the cells when they reach confluence, splitting them 1:4.

  Cells can be frozen at the time of passage for future use. Harvest the cells as in steps 21 and 22, resuspend the pellet in 1 ml of 95% heat-inactivated FBS/5% DMSO, and freeze overnight at −80°C in a cryotube. The following day, move the cryotube to liquid nitrogen for long-term storage.


**ENRICHMENT OF ASTROCYTES BY DIFFERENTIAL ADHESION**

This procedure can be used in place of steps 21 to 27 of the Basic Protocol. The advantage of this method is that antibody and complement treatment is not required. The disadvantage is that it will remove many, but not all, contaminating neurons. In addition, fibroblasts will be spared, and eventually they will overtake the culture.

**Additional Materials** (also see Basic Protocol)

- PBS (Appendix 2A), 37°C

1. Complete Basic Protocol steps 1 to 16. Then resuspend the cells in 1 to 2 ml DMEM/10% FBS/2% glucose and determine the concentration and viability using trypan blue and a hemacytometer (CPMB APPENDIX 3F). Plate the cells at 5 × 10⁴ cells/ml in DMEM/10% FBS/2% glucose on poly-L- (or poly-D-) lysine-coated plates and incubate in a humidified 37°C, 7% CO₂ incubator, as in Basic Protocol, step 20.

2. Two to three hours after plating, dislodge the loosely adherent neurons by aggressively agitating the plate, taking care not to spill the medium over the sides of the
plate or otherwise contaminate the culture. Firmly tap the plate against a benchtop, then gently wash the surface of the culture with PBS warmed to 37°C. Monitor the culture after each wash to be sure that astrocytes are not inadvertently removed.

Washes may be repeated until most of the contaminating neurons are removed. The washes should be carried out in a laminar flow tissue culture hood.

3. Following the final wash, add DMEM/10% FBS/2% glucose to the cultures and return them to the incubator (see Basic Protocol, step 26).

Further trypsinization and passage will remove contaminating neurons. If desired, this culture can be treated with antibody and complement to remove undesired cells.

**Support Protocol**

**IMMUNOHISTOCHEMICAL DETECTION OF GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)**

GFAP is expressed by most astrocytes in vivo and virtually all astrocytes in vitro, and it is a useful marker for assessing the purity of these cultures. Cells that are GFAP-negative are contaminants, most likely fibroblasts.

**Materials**

- Astrocyte culture (see Basic Protocol or Alternate Protocol)
- Glass coverslips or 8-well slides (Lab-Tek, Nalge Nunc) coated with poly-L- (or poly-D-) lysine (APPENDIX 2A)
- PBS (APPENDIX 2A)
- 100% methanol, ice-cold
- Blocking reagent: 10% goat serum/0.1% Triton X-100 in PBS
- Anti-GFAP monoclonal antibody (clone GA-5; Boehringer Mannheim)
- Anti-mouse antiserum conjugated with chromofluor or enzyme

1. Before passaging cells starting in step 21 of the Basic Protocol, coat either glass coverslips or a Lab-Tek 8-well slide with polylysine. Plate $3 \times 10^3$ to $1 \times 10^4$ cells per slide well or $1 \times 10^4$ cells per coverslip in DMEM/10% FBS/2% glucose and culture overnight under standard conditions.

2. Rinse the cells twice with PBS. Cover the cells with ice-cold 100% methanol and let sit 8 min at $-20^\circ$ C.

3. Rinse the cells three times with PBS or CMF-PBS and add 300 µl blocking reagent. Incubate 1 hr at room temperature.

4. Dilute anti-GFAP monoclonal 1/20 in blocking reagent. Add to slides in blocking reagent and let sit 1 hr at room temperature.

5. Wash slides three times for 5 min each in PBS.

6. Dilute anti-mouse antiserum conjugated to a chromofluor or enzyme per the manufacturer’s instructions in blocking reagent, add to slides, and incubate in the dark 1 hr at room temperature.

7. Wash three times for 5 min each in PBS.

8. Coverslip and view under fluorescence optics (UNIT 2.1).
REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps, and filter sterilize all solutions unless otherwise indicated. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

**DNase solution**
7.5 mg DNase (e.g., DP DNase, Worthington)  
15 ml DMEM  
Do not adjust pH  
Store up to 12 months at −20°C

**Isoperc**
90 ml Percoll (Pharmacia Biotech)  
10 ml 10× PBS (APPENDIX 2A)  
Store up to 6 months at 4°C

**PBS/glucose**
900 ml 1× PBS (APPENDIX 2A); calcium- and magnesium-free  
2 g glucose  
2 ml 0.5% (w/v) phenol red  
Adjust pH to 7.4 with 1 M HCl or 1 N NaOH, as needed  
Adjust volume to 1 liter with H2O  
Store up to 2 years at 4°C

**Percoll, 30%**
30 ml Isoperc (see recipe)  
60 ml PBS/glucose (see recipe)  
Store up to 6 months at 4°C

**Percoll, 60%**
60 ml Isoperc (see recipe)  
30 ml PBS/glucose (see recipe)  
0.1 ml 0.4% trypan blue (e.g., Life Technologies), filter sterilized  
Store up to 6 months at 4°C

**Trypsin/DNase solution**
150 mg trypsin (TRL trypsin, Worthington)  
15 mg DNase (DP DNase, Worthington)  
15 ml PBS/glucose (see recipe) containing 5 mg/ml MgSO4  
90 µl 1 N NaOH  
Store up to 1 year at −20°C

**COMMENTARY**

**Background Information**
During development of the mammalian CNS, neurogenesis is largely completed by the onset of gliogenesis. Despite the temporal difference in the appearance of neurons and glial cells, they share a common progenitor, as demonstrated by thymidine labeling (Fujita, 1967) and retroviral labeling (Price et al., 1987; Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Wetts and Fraser, 1988). In the absence of trauma or transforming events, neurons and astrocytes reach a numerical and functional equilibrium that is maintained for the life of the organism (Sturrock, 1975). However, astrocytes are capable of reentering the cell cycle throughout life, either in response to injury (Latov et al., 1979) or after transforming events. Neurons are able to inhibit astrocyte proliferation in a contact-dependent manner (Hatten, 1985, 1987; Weinstein et al., 1990, 1991), and it is likely that, despite the proliferative potential of astrocytes in vivo, they are held in a quiescent state by the presence of neuronal elements.

The protocols outlined in this unit take advantage of the proliferative potential of these
cells. Astrocytes can be plated and then expanded in culture for a desired purpose. Interestingly, when astrocytes are co-cultured with neurons, they revert to a quiescent phenotype and display many of their in vivo properties, such as process extension (Weinstein et al., 1991), trophic support of neurons (Hatten et al., 1988), and upregulation of transporters (Swanson et al., 1997). The dissections described in this unit are carried out on neonatal rodent cerebellum because this tissue develops postnatally, and as a result, there is less debris and a greater yield of viable cells in comparison to other postnatal brain regions. The use of a step gradient takes advantage of the density differences between large, membranous astrocytes, and small, granule cell neurons, which at this developmental state have little cytoplasm and therefore are denser than astrocytes. These procedures, which were first described by Hatten (1985), have been modified and have the advantage of yielding pure astrocyte cultures. The addition of an antibody and complement cytolysis step removes contaminating fibroblasts, which, if left in the culture, would overtake the astrocytes during cell expansion. Alternatively, astrocytes can be harvested from postnatal cortex (Dobrenis et al., 1995), but this procedure takes considerably more time and has a lower yield of astrocytes based on amount of starting material. It is also very difficult to get rid of contaminating cell types in cultures generated using this method.

Critical Parameters and Troubleshooting

Tissue isolation prior to dissociation. Once deprived of its blood supply, CNS tissue rapidly degrades. Anecdotal evidence suggests that the degradation is more rapid if the cerebellum is left in situ after the animal is decapitated. For this reason, the protocol suggests that the animals be sacrificed one at a time, and the cerebellum removed from each before moving on to the next animal. In addition, to prevent tissue drying and subsequent death, it is critical that the cerebellum be completely covered with PBS/glucose when it is placed in a small volume of fluid during tissue preparation.

Remove clumps of tissue prior to gradient centrifugation. Filtering the single cell suspension through nylon mesh prior to loading the gradient is necessary to optimize the yield of cells and to minimize the number of neurons in the astrocyte-enriched fraction. If Swinnex filters are unavailable, the cell suspension can be centrifuged for 2 min at 1 × g at room temperature to allow the large clumps to settle out prior to loading the gradients. The large clumps can thereby be omitted when loading onto Percoll.

Washing adherent cells. Care must be taken not to over-wash the cells, as the astrocytes can be blown off the plate with exuberant washing. Gently passing a fine stream of PBS over the surface of the cells repeatedly should remove the loosely adherent neurons.

Anticipated Results

The Basic Protocol is designed to yield cultures that are close to 100% pure astrocytes. The Alternate Protocol yields a more mixed cell population containing fibroblasts and some contaminating neurons as well as astrocytes.

Time Considerations

In order to achieve the desired goal of obtaining nearly pure astrocytes, a minimum of 4 days are required. The dissections, gradient centrifugation, and initial plating must all be done on Day 1. The author recommends antibody and complement treatment be done on Day 3, which will give the cells an opportunity to recover and begin dividing. On Day 4, the purity of the cells can be assayed by assaying for GFAP (see Support Protocol). At this point, if no more cells are required, they can be used as needed. If more astrocytes are needed, the cultures can be expanded until the required number has been reached. The cells have a cell-cycle time of 24 to 28 hr. If they are split 1:4 they will need to be split every 4 to 5 days. Cells have been continuously grown for up to 3 months, after which they stained for GFAP and had the morphological appearance of astrocytes.

Literature Cited


**Key References**

Hatten, 1985. See above.

First description of the use of a discontinuous gradient for separation of astrocytes and neurons.

Weinstein, et al., 1990. See above.

Describes in detail the analysis of astrocyte response to a neuronal cell line.

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