Video Article

Isolation and Culture of Mouse Cortical Astrocytes

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Abstract

Astrocytes are an abundant cell type in the mammalian brain, yet much remains to be learned about their molecular and functional characteristics. *In vitro* astrocyte cell culture systems can be used to study the biological functions of these glial cells in detail. This video protocol shows how to obtain pure astrocytes by isolation and culture of mixed cortical cells of mouse pups. The method is based on the absence of viable neurons and the separation of astrocytes, oligodendrocytes and microglia, the three main glial cell populations of the central nervous system, in culture. Representative images during the first days of culture demonstrate the presence of a mixed cell population and indicate the timepoint, when astrocytes become confluent and should be separated from microglia and oligodendrocytes. Moreover, we demonstrate purity and astrocytic morphology of cultured astrocytes using immunocytochemical stainings for well established and newly described astrocyte markers. This culture system can be easily used to obtain pure mouse astrocytes and astrocyte-conditioned medium for studying various aspects of astrocyte biology.

Video Link

The video component of this article can be found at https://www.jove.com/video/50079/

Introduction

Astrocytes are a very abundant cell type in the central nervous system (CNS). The ratio of astrocytes to neurons is 1:3 in the cortex of mice and rats, whereas there are 1.4 astrocytes per neuron in the human cortex [1]. Interest in astrocyte function has increased dramatically in recent years. A key function of astrocytes is their role in providing structural and metabolic support to neurons [2,3]. Newly discovered roles for astrocytes cover a broad spectrum of functions. These include guiding the migration of developing axons and certain neuroblasts during development [4-6], functions in synaptic transmission, synapse strength and information processing by neural circuits [7-9], roles in blood-brain barrier (BBB) formation [10] and integrity [11-13] and regulation of the cerebrovascular tone [14]. Another major feature of astrocytes is their response to injury. Under pathological conditions astrocytes become reactive and further upregulate the expression of the intermediate filament glial fibrillary acidic protein (GFAP) and inhibitory extracellular matrix (ECM) proteins [15,16]. Reactive astrocytes demarcate the injury site from healthy tissue by forming a glial scar, which consists mainly of astrocyte secreted ECM proteins of the chondroitin sulfate proteoglycan (CSPG) family, the major factors that inhibit axonal regeneration after CNS injury [15-17].

Astrocytes originate from radial glial (RG) cells during late embryogenesis and early postnatal life. After astrocyte specification has occurred, astrocyte precursors migrate to their final positions, where they begin the process of terminal differentiation. *In vivo*, astrocytes appear to be mature three to four weeks after birth as indicated by their typical morphology [18,19]. A subpopulation of RG cells convert into subventricular zone astrocytes (type B cells). Both, RG and type B cells function as astrocyte-like neural stem cells (NSCs) during development and in the adult, respectively. Like astrocytes, RG and type B cells also express the astrocyte-specific glutamate transporter (GLAST), brain lipid-binding protein (BLBP), and GFAP, indicating that these markers cannot be exclusively used to specifically label adult astrocytes. In contrast to adult parenchymal astrocytes, which do not divide in the healthy brain, RG and type B cells exhibit stem cell potential such as the capacity to self-renew. Dysregulation of astrocytes has been implicated in numerous pathologies, including Alzheimer's disease [20,21], Huntington's disease [22], Parkinson's disease [23], Rett syndrome [24] and Alexander's disease [25]. Moreover, astrocytes react to all insults of the CNS, leading to astrocyte activation and astrocytic glial scar formation [16,26]. The astrocytic glial scar that forms following brain trauma or spinal cord injury is thought to be the prime barrier preventing neuronal regeneration [15].

The development of reliable methods to isolate and maintain purified populations of cells has been essential to our understanding of the nervous system. Pioneering work by McCarthy and de Vellis enables investigators to date to prepare nearly pure cultures of astrocytes from neonatal rat tissue [27]. Much has been learned about astrocyte biology using this method, which is presented here in a slightly modified form for isolating mouse cortical astrocytes. Complementing *in vivo* studies, astrocytes as well as conditioned medium obtained using the described *in vitro* culture, are valuable tools to further gain insights into astrocyte functions.

Protocol

1. Isolation and Plating of Mixed Cortical Cells

Mixed cortical cell isolation for astrocyte cultures can be performed using P1 to P4 mouse pups. In order to achieve proper astrocyte density it is necessary to use 4 mouse pup cortices per T75 tissue culture flask. Therefore, volumes in the following protocol are calculated for a cell preparation using 4 mouse pups.

1. Before starting the dissection procedure, prewarm 30 ml of astrocyte culture media (DMEM, high glucose + 10% heat-inactivated fetal bovine serum + 1% Penicillin/Streptomycin; see table) to 37 °C. Coat one T75 flask with 20 ml of poly-D-lysine (PDL) at a concentration of 50 µg/ml in cell culture grade water for 1 hr at 37 °C in the CO_2 incubator.
2. For the dissection procedure, prepare all necessary reagents and materials. You will need: surgical scissors, smooth fine forceps, flat tip forceps, paper towels, waste bag, 70% ethanol and 2 dissecting dishes (3.5 cm diameter) on ice filled with 2 ml HBSS each.
3. Gently hold and spray the head and neck of the mouse pup with 70% ethanol. The animal is sacrificed by decapitation using the scissors.
4. Perform a midline incision, posterior to anterior, along the scalp to reveal the skull.
5. Cut the cranium carefully from the neck to the nose. Two additional cuts are performed to allow further access to the brain: The first cut is made anterior of the olfactory bulbs, the other one inferior of the cerebellum to disconnect the cranium from the skull base.
6. Using the flat tip forceps, the cranial flaps are gently flipped to the side and the brain is taken out and placed into the first dissecting dish filled with HBSS. Place the dish back on ice and continue harvesting all 4 brains.
7. The remainder of the dissection procedure is performed under a stereomicroscope. First, the olfactory bulbs and the cerebellum are removed using the fine dissecting forceps (**Figure 1B**).
8. In order to retrieve the cortices, grab the posterior end of the brain with the fine forceps, perform a midline incision between the hemispheres, insert a second set of forceps to the created grove and peel away the plate-like structure of the cortex from the brain.
9. Carefully dissect the meninges from the cortex hemispheres by pulling with the fine forceps (**Figure 1D'**). This step avoids contamination of the final astrocyte culture by meningeal cells and fibroblasts. Transfer the prepared cortex hemispheres into the second dish filled with HBSS and return it onto ice. Continue accordingly with all 4 cortices.
10. Finally, cut each cortex hemisphere into small pieces using sharp blades (approximately 4 to 8 times).
11. Under sterile conditions, transfer cortex pieces into one 50 ml Falcon tube and add HBSS to a total volume of 22.5 ml.
12. Add 2.5 ml of 2.5 % trypsin, mix and incubate the tissue in the water bath at 37 °C for 30 min. Mix by occasional shaking every 10 min.
13. Centrifuge for 5 min at 300 x g to pellet cortex tissue pieces.
14. Carefully remove supernatant by decantation. In order to avoid losing the tissue pellet you may mechanically retain it using a pipet. Dissociate the tissue into a single cell suspension by adding 10 ml astrocyte plating medium and vigorous pipetting using a 10 ml plastic pipette until tissue pieces are dissociated into single cells (20 to 30 times). Adjust volume to 20 ml using astrocyte plating medium. You can proof the dissociation of the cortex tissue into single cells by counting using a hematocytometer. One preparation of 4 mouse pup cortices should yield 10-15 x10^6 dissociated single cells.
15. Aspirate PDL from the T75 culture flask, plate the dissociated single cell suspension and incubate at 37 °C in the CO_2 incubator.

2. Obtaining an Enriched Astrocyte Culture

1. Change the medium 2 days after plating of the mixed cortical cells and all 3 days thereafter.
2. After 7 to 8 days, when astrocytes are confluent and overlaying microglia sit exposed on the astrocyte layer or are already detached from the astrocyte layer (**Figure 2**), shake the T75 flask at 180 rpm for 30 min on an orbital shaker to remove microglia. Discard the supernatant containing microglia or if you wish to culture and examine microglia, spin it down and plate for culture [28,29].
3. Add 20 ml fresh astrocyte culture medium and continue shaking the flask at 240 rpm for 6 hr to remove oligodendrocyte precursor cells (OPC). Since some OPCs will not completely detach from the astrocyte layer, continue to shake vigorously by hand for 1 min in order to prevent OPC contamination. Discard the supernatant or spin it down and plate, if you wish to culture OPCs [29].
4. Rinse the remaining confluent astrocyte layer twice with PBS, aspirate the PBS, add 5 ml trypsin-EDTA and incubate in the CO_2 incubator at 37 °C. Check detachment of astrocytes every 5 min and enforce detachment of astrocytes by hitting the flask against the palm of your hand (2-3 times).
5. After astrocytes are detached from the culture flask, add 5 ml of astrocyte culture medium, spin cells at 180 x g for 5 min, aspirate supernatant and add 40 ml fresh astrocyte plating medium. One T75 tissue culture flask should yield around 1x10^6 cells after the first cell split. Plate cells in two T75 culture flasks and incubate at 37 °C in the CO_2 incubator. Change the medium every 2 to 3 days.
6. 12-14 days after the first split astrocytes are plated in the appropriate cell concentration 24-48 hr before performing the experiment. One T75 tissue culture flask should yield around 1.5-2 x10^6 cells after the second cell split.

Representative Results

Upon isolation of the complete mouse brain (**Figure 1A**), the cerebellum and the olfactory bulbs have to be removed (**Figure 1B**). The cortices are peeled of the mouse brain stem (**Figure 1C**) and meninges of the individual cortex (**Figure 1D'**) are carefully removed (**Figure 1E**). Meninges are obvious by the meningeal artery system and incomplete removal results in contamination of the final astrocyte culture by meningeal cells and fibroblasts.

After plating of the mixed cortical cell suspension, some astrocytes already attach to the culture flask within 24 hr (**Figure 2A**). However, the cell culture supernatant will contain cell debris and dying neurons for the first 3 to 5 days (**Figure 2A-C**), as the culture medium favors survival and growth of glial cells. Once astrocytes are confluent and microglia sit exposed on the astrocyte layer, usually at day 7 after isolation of mixed cortical cells, astrocytes should be separated from microglia and OPCs by shaking (**Figure 2D**). 2 days after the first split cells show astrocyte

morphology. At this point astrocyte density is low and astrocytes are voluminous (**Figure 2E**, black arrows mark one cell). The expected yield at this point is low with about 4-6 x10^5 cells per T75 tissue culture flask. However, the cells still proliferate and mature in culture at this timepoint. Astrocytes are usually used for experiments at day 21 to 28 (**Figure 2F**) to ensure a mature phenotype of isolated astrocytes. At this point the expected yield is about 1.5-2 x10^6 cells per T75 tissue culture flask.

The astrocyte culture purity has been characterized by electron microscopy morphological studies, cell marker expression and pharmacological responsiveness [27]. Contamination of the astrocyte culture can be examined by using marker for microglia (IBA-1) and OPCs (O4). The obtained astrocyte culture purity and maturity should be examined by immunocytochemical examination using an antibody against the GFAP protein. It is expected that the majority of the cells show an intensive, filamentous signal (**Figure 3**). Our isolation and culture method of cortical astrocytes resulted in an astrocyte purity of greater than 98%, revealed by using an antibody against the GFAP protein (**Figure 3**). If the procedure was successful, contaminating cells are rare (<2%) and contain microglia and OPCs, which can be stained by using IBA-1 and O4, respectively. If desired, further analysis of other astrocyte markers, such as Aquaporin-4, BLBP, S100B and GLAST may be used. In addition to GFAP, these markers were all expressed by the 28 days old astrocyte cultures. Recent gene expression profiling of purified astrocyte populations revealed new potential astrocyte markers, such as the folate metabolic enzyme ALDH1L1 [30,31], which is also expressed by the majority of the isolated cortical astrocytes (**Figure 3**).
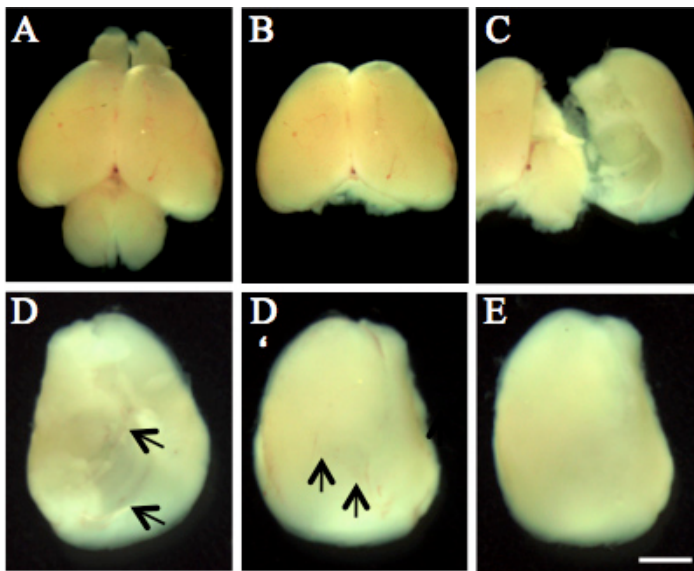

Figure 1. Dissection of postnatal (P3) mouse cortex. A) Whole brain. B) Brain after removal of olfactory bulbs and cerebellum. C) Isolation of cortices by peeling off the plate-like structure of the cortex from the brain. D, D') Cortex from ventral and dorsal site with meninges (black arrows indicate meningeal arteries). E) Cortex without meninges. Scale bar, 1.5 mm.
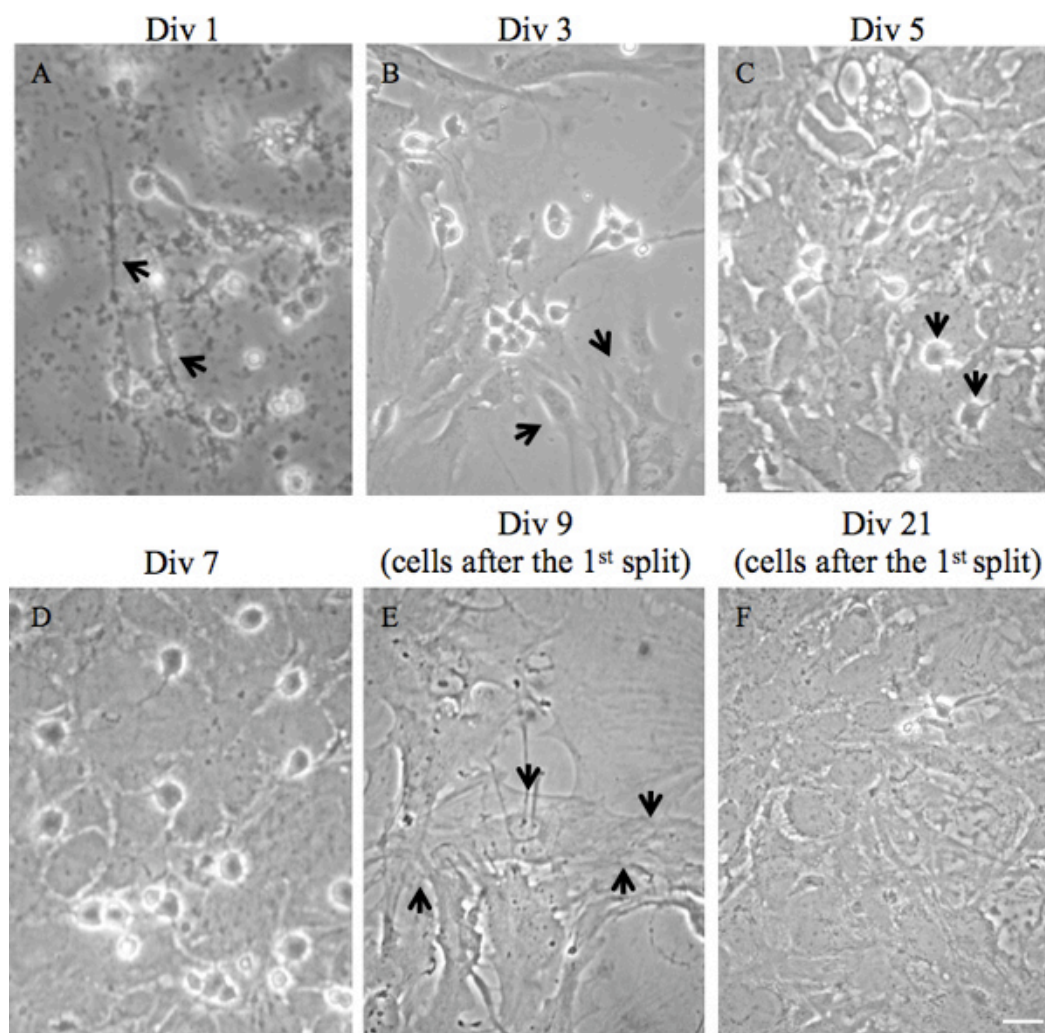
Figure 2. Morphological overview of isolated mixed cortical cells and pure astrocyte culture at different timepoints after isolation. A) 1 day after plating of mixed cortical cells. First astrocytes are attached to the bottom of the flask (black arrows) and dying neurons are in the supernatant. B) 3 days after plating of mixed cortical cells. Astrocyte layer is forming (black arrows). Neurons are almost absent. C) 5 days after plating of mixed cortical cells. First microglia and OPCs on top of a astrocyte layer (black arrows). D) 7 days after plating of mixed cortical cells. Astrocyte layer is completely confluent. E) After removing microglia and OPCs by vigorous shaking and 2 days after splitting, attached cells show astrocyte morphology with low density (arrows indicate one cell). F) Astrocyte layer shows high density 2 weeks after the first split. Scale bar, 10 μm.
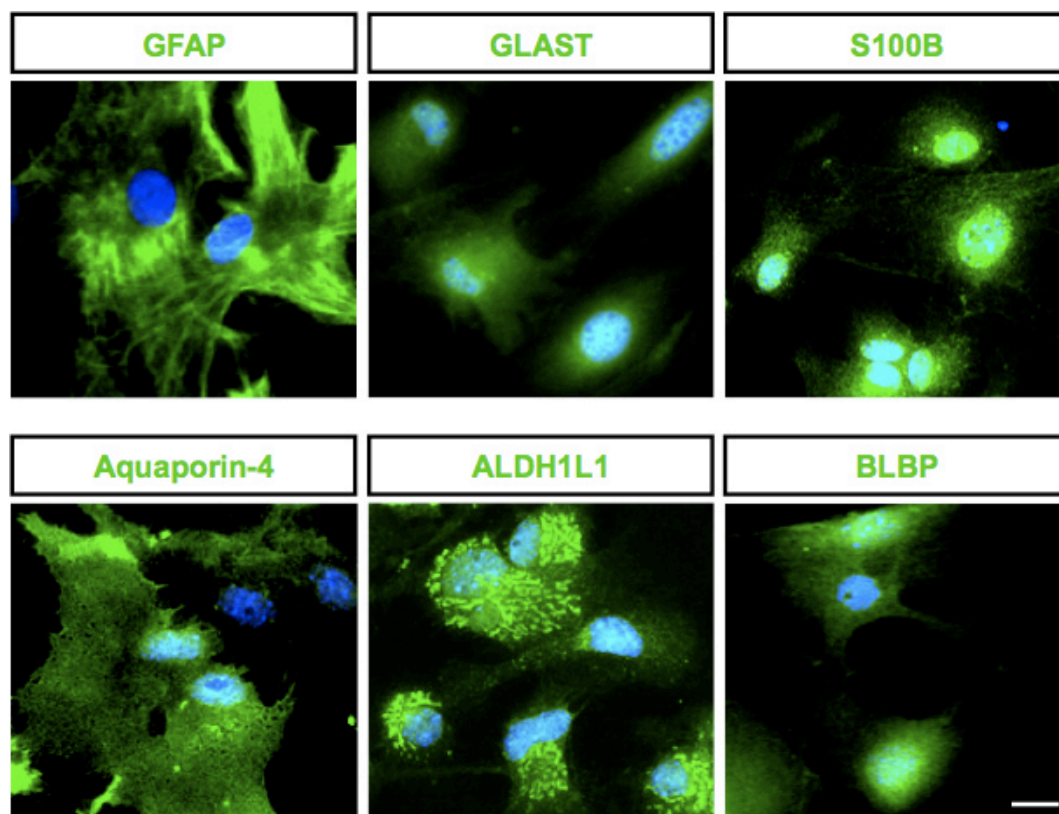
Figure 3. Purity of primary astrocyte culture. Immunolabeling of primary mouse astrocyte cultures with the markers GFAP, GLAST, S100B, Aquaporin-4, ALDH1L1 and BLBP (all green) revealed pure primary astrocyte culture. Nuclei are stained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 10 µm.

Discussion

The method outlined here is based on the astrocyte culture preparation from rodent neonatal brains, originally described by McCarthy and de Vellis in 1980 [27]. The modified method of the isolation and culture of cortical astrocytes from postnatal P1 to P4 mouse brain presented here is fast, yields pure primary astrocytes and is highly reproducible. This technique can easily be transferred to isolate astrocytes from other species, such as from rat or pig and from other brain regions, such as the spinal cord. Whereas astrocyte progenitor cell isolation from neonatal brain by the McCarthy and deVellis method generates highly proliferative cells, the cell proliferation and propagation of isolated astrocytes of postnatal P1-P4 mouse pups is limited. After splitting astrocytes once at 7 days *in vitro* (DIV), they will grow to confluency and mature. *In vivo*, most astrocyte proliferation is largely complete by P14 [32]. Here, we suggest to use astrocytes for experiments at day 21 to 28 DIV (Figure 2F) to ensure the mature phenotype of isolated astrocytes. Due to the intrinsic restraint to proliferate astrocyte cultures should not be splitted more than 3 times.

Critical steps in the described isolation method are the digestion of cortices and the following trituration of the digested tissue for obtaining the single cell suspension. Therefore, it is necessary to optimize trypsin concentration and digestion time in order to obtain a single cell suspension after trituration of the brain cortex tissue for 20-30 times. In order to minimize variation trypsin should be aliquoted and freeze-thaw cycles should be avoided. The described procedure relies on plating the mixed cortical cells on PDL-coated culture flasks, which assures the binding of astrocytes and promotes a confluent astrocyte layer several days after plating. While PDL-coating is not necessary for astrocyte maintenance after their separation from microglia and oligodendrocytes, it may be performed for certain downstream applications such as immunofluorescene staining. Good timing of the first cell split is important for cell integrity and yield. If astrocytes are cultured beyond they reached confluency, you may lose the majority of cells due to insufficient detachment during the first cell split. This cannot be overcome by increasing the time for detachment, since extensive incubation time with trypsin negatively influences cell integrity. In contrast, plating the cortical cell suspension too scarcely will result in insufficient formation of a confluent astrocyte cell layer. The best time for the first cell split is at 7 to 8 days after plating of mixed cortical cells, when astrocytes are confluent and microglia cells sit on the topmost position of the astrocyte layer.

In the described cortical cell culture, astrocytes show cellular heterogeneity (**Figure 3**), as it has been described for astrocytes *in vivo* [33,34]. However, defining diverse astrocyte morphology and functionality has been hampered by the limited number of markers to identify and distinguish potentially heterogeneous astrocyte subtypes. A well characterized marker of mature fibrous and reactive astrocytes is GFAP. However, GFAP is barely expressed by mature protoplasmic astrocytes, limiting its use as a marker for all astrocytes and it is also expressed by RG cells during development and by B cells in the adult, restricting its use as a stage-specific marker. Other markers of astrocytes, including GLAST, ALDH1L1 or BLBP, are also expressed by immature astrocytes and therefore do not exclusively mark mature astrocytes. Finally, mature astrocyte markers such as GFAP, Aquaporin-4, and S100B (**Figure 3**) are increasingly up-regulated during postnatal maturation.

In our hands, astrocyte cultures at an age of 4 weeks have characteristics of mature astrocytes *in vivo*. Using primary astrocyte cultures we could identify the molecular mechanism how the blood born protein fibrinogen induces astrocyte activation [17]. Our studies revealed that fibrinogen is a carrier of latent TGF-β. Treatment of primary astrocytes with fibrinogen led to active TGF-β formation and the activation of the TGF-β/ Smad signaling pathway in astrocytes [17,26]. These results have been confirmed using fibrinogen injections *in vivo*. Furthermore, astrocytes control other cell types by the secretion of substances, which can be analyzed by harvesting astrocyte-conditioned medium and applying this conditioned medium to other cell types. We previously used astrocyte-conditioned medium to analyze in a functional assay how conditioned medium of fibrinogen-treated astrocytes affects neurite outgrowth. Reactive astrocytes express and secrete proteins of the CSPG family, which inhibit neurite outgrowth [16]. Indeed, conditioned medium from fibrinogen-treated astrocytes significantly decreased both neurite length and the percentage of cells showing neurite outgrowth [17].

The isolation and culture of cortical astrocytes described in this protocol provides a powerful tool for investigating astrocyte biology, since their manageability in diverse applications can greatly complete their investigation *in vivo*. However, it should be kept in mind that the obtained astrocytes have been cultured *in vitro* and while they reflect many astrocyte characteristics, they also differ from in vivo astrocytes. Therefore, other methods of direct selection and isolation of astrocytes by immunopanning [35] or antibody-based FACS isolation [36] represent new avenues to further investigate the fundamental properties of astrocytes.

Disclosures

No conflicts of interest declared.

Acknowledgements

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