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TITLE:

Dissection and Isolation of Murine Glia from Multiple Central Nervous System Regions

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glia, regional heterogeneity, oligodendrocyte precursor cell, microglia, astrocyte, in vitro culture, immunocytochemistry

SUMMARY:

Here we present a protocol for in vitro isolation of multiple glial cell populations from a mouse CNS. This method allows for the segregation of regional microglia, oligodendrocyte precursor cells, and astrocytes to study the phenotypes of each in a variety of culture systems.

ABSTRACT:

The methods presented here demonstrate laboratory procedures for the dissection of four different regions of the central nervous system (CNS) from murine neonates for the isolation of glial subpopulations. The purpose of the procedure is to dissociate microglia, oligodendrocyte progenitor cells (OPCs), and astrocytes from cortical, cerebellar, brainstem, and spinal cord tissue to facilitate further in vitro analysis. The CNS region isolation procedures allow for the determination of regional heterogeneity among glia in multiple cell culture systems. Rapid CNS region isolation is performed, followed by the mechanical removal of meninges to prevent meningeal cell contamination of glia. This protocol combines gentle tissue dissociation and plating on a specified matrix designed to preserve cell integrity and adherence. Isolating mixed glia from multiple CNS regions provides a comprehensive analysis of potentially heterogenous glia while maximizing the use of individual experimental animals. Additionally, following dissociation of regional tissue, mixed glia are further divided into multiple cell types including microglia, OPCs, and astrocytes for use in either single cell type, cell culture plate inserts, or co-culture systems. Overall, the demonstrated techniques provide a comprehensive protocol of broad applicability for careful dissection of four individual CNS regions from murine neonates and includes methods for the isolation of three individual glia cell types to examine regional heterogeneity in any number of in vitro cell culture systems or assays.

INTRODUCTION:

Glia are necessary for proper neuronal function in the CNS. They are composed of three major subpopulations, astrocytes, oligodendrocytes, and microglia, each with a different, yet indispensable role[1]. Without the proper glial cell diversity and activity, neuronal function would be severely impacted, leading to CNS impairment. Glia are capable of influencing neurotransmission, and each cell type does so in a unique manner. Glial cells in the brain have the capacity to communicate amongst themselves, as well as with neuronal cells, in order to facilitate proper CNS function[2]. Oligodendrocytes increase the speed of electrical transmission through the formation of a myelin sheath, which facilitates the clustering of ion channels at the nodes of Ranvier, the sites of neuronal action potential generation[3]. Microglia are critical for the pruning of synapses by monitoring synaptic transmission and "rewiring" neuronal connections following injury[4]. In addition, microglia are the most abundant resident immune cell of the CNS, acting as the primary form of host defense against pathogens[5]. Astrocytes can regulate synaptic transmission between neurons by modifying the concentration of extracellular potassium[6]. They also have roles in controlling local blood flow[7], releasing and taking up neuromodulatory elements[8], and have a key role in blood-brain barrier maintenance[9]. Thus, each glial subtype is critical for CNS function, as defects in any type have long been associated with a wide variety of pathological states, including psychiatric diseases, epilepsy, and neurodegenerative conditions[10].

The greatest obstacle in the study of CNS pathobiology is the inability to investigate human cells in the context of their microenvironmental niche. Human biopsy tissue is most collected post-mortem and cells can easily be damaged or lost during the extraction and processing. Furthermore, it is a challenge to keep human cells alive and viable in vitro for any length of time without deriving immortalized cell lines from tumors, at which point they no longer accurately reflect their normal physiological properties[11,12]. Additionally, there is a significant amount of regional heterogeneity among individual glia cell types[13-15], and obtaining regional CNS samples from individual patients is nearly impossible. As such, it is necessary to develop alternative models to study the contribution of regional glia in specific CNS disorders.

Here, we describe an in vitro system using mouse CNS region-specific isolation of multiple glial subpopulations, allowing for the manipulation and quantification of microglia, oligodendrocyte precursor cells (OPCs), which give rise to mature oligodendrocytes, and astrocytes. Each population can be independently isolated and subjected to a wide variety of experimental techniques including drug or molecule treatment, immunocytochemistry, protein/RNA extraction and analysis, and other co-culture systems depending on experimental necessity. Additionally, this isolation technique yields high cell number, allowing for the characterization and investigation of each glial population in a high-throughput manner. It also enables the study of CNS cell differentiation, growth, and proliferation in response to a wide variety of microenvironmental stimuli in a controlled manner in order to avoid confounding factors which are typically present in an in vivo setting. Lastly, this cell isolation technique facilitates the manipulation of glial cell populations within different CNS regions to investigate how regional glia interact with each other and respond to varying stimuli, allowing for precision and reproducibility.

PROTOCOL:

NOTE: All animal studies were authorized and approved by the Cleveland Clinic Lerner Research Institute Institutional Animal Care and Use Committee.

1. Prepare media and supplies for dissection

NOTE: All buffer and media recipes are provided in **Table 1**. This procedure is done under sterile conditions in a tissue culture designated biosafety cabinet.

1.1. Prepare and sterile filter mixed glia media (MGM). This can be done the day before and stored at 4 °C.

1.2. Dilute fibronectin at a 1:100 concentration with sterile H_2O. The volume of diluted fibronectin will depend of the number of pups and CNS region used for the experiment. Use one T25 flask for 1 cortex, one T25 flask for 2-4 cerebella, one T25 flask for 2-4 brain stems and one T25 flask for 2-4 spinal cords.

NOTE: Combining tissue from the same region using the criteria listed above will allow adequate dissociation without altering the protocol described below. Combining more tissue than described in Step 1.2 will require further optimization of dissociation reagent concentrations.

1.3. Pipette 3 mL of diluted fibronectin into T25 flasks at room temperature and allow to sit for 2 min.

1.4. Aspirate the fibronectin and allow flasks to dry overnight with flask lid loosened.

2. Cortex, cerebellum, brainstem, and spinal cord dissection

NOTE: This procedure can be done on the benchtop and requires a dissection scope. Use strict aseptic technique for all steps of the procedure and minimize tissue exposure to the room air. Keep all media chilled on ice during dissection to ensure maximal tissue preservation. Alternatively, this procedure could be done in a hood that allows the use of an internal dissection scope.

2.1. Wipe all areas with 70% ethanol.

2.2. Pipette 10 mL of PBS/antibiotic solution (PAS) into a 10 cm Petri dish. Prepare one Petri dish for each pup to be dissected. Set on ice to keep the solution chilled.

2.3. In a disinfected tissue culture hood, pipette 9 mL of DMEM (with no additives) into 4 separate 15 mL conical tubes, one for each CNS region, replace tube caps, and put on ice. Place ice bucket with tubes within reaching distance from the dissection scope.

2.4. Place 10 cm Petri dish prepared in step 2.2 on disinfected dissection scope stage.

2.5. Anesthetize and euthanize mouse pup according to institutional protocol and remove the head by rapid decapitation with sharp scissors.

NOTE: Postnatal day (P) 3-5 pups are used. Older pups have a more developed CNS and may not be an adequate source of expanding glial cells. Younger pups (P) 0-2 may yield a higher number of glia and can be used; however, spinal cord tissue is very difficult to dissect at this young age due to size.

2.6. Clean the pup's skin using 70% ethanol.

2.7. Using fine scissors, cut the cutaneous layer along the midline of the head of the animal, starting caudally and moving rostral, until reaching the snout. Avoid cutting deeply into the skull to avoid any tissue damage.

2.8. Angling the head down, pull cutaneous layer to each side of the skull and using spring scissors, make an incision along the skull midline, starting at the foramen magnum, again cutting caudal to rostral.

2.9. With fine-tipped forceps, pull the skull halves to the right and left sides, exposing the cortex, cerebellum, and brainstem.

2.10. Once exposed, gently lift the brain out of the skull and into the 10 cm Petri dish prepared in Step 2.2. Ensure the brain remains undamaged to preserve anatomical structure, with the hindbrain attached.

2.11. Using fine, curved-tipped forceps with the point of the forceps facing upward, pinch off the cerebellum. Remove the meninges and place in designated 15 mL conical tube in ice bucket.

2.12. Ensure that the brainstem is directly ventral to the cerebellum and is visible after removal of the cerebellum. Remove it with fine-tipped forceps, remove meninges, and place in designated 15 mL conical tube in ice bucket.

2.13. Separate the midbrain from the cortex, remove cortical meninges, and place in designated 15 mL conical tube in ice bucket.

2.14. To remove the spinal cord, place the decapitated mouse pup in a supine position (lying face upward) with the severed vertebral column elevated towards the investigator.

2.15. Spray again with 70% ethanol.

2.16. Cut along the lateral sides of the vertebral column, in a rostral to caudal direction, through the rib cage until reaching the hind limbs. While cutting, push back the internal organs until the vertebral column is visible.

2.17. Cut along each lateral side of the vertebral column until it is isolated and place in the 10 cm Petri dish prepared in Step 2.2.

2.18. With ventral side up, using fine spring scissors, alternate cutting the right and left sides of each vertebrae until reaching the lumbar region to expose the spinal cord tissue.

2.19. Gently remove the spinal cord and the meninges with fine-tipped forceps under the dissecting microscope. Place in designated 15 mL conical tube in ice bucket.

2.20. Repeat steps 2.5.-2.19 for each pup, combining tissue from the same region to fit the criteria outlined in step 1.2 for each prepared T25 flask.

NOTE: Remove the meninges as completely as possible. If a significant amount of meninges remain, the fibroblast-like phenotype of meningeal cells will outgrow and overwhelm the cell culture. Multiple spinal cords, of equal phenotype, can be combined in order to generate a specific cell culture. Take care to ensure that overcrowding of cells does not occur, which may lead to glial apoptosis and differential phenotypes.

3. Tissue dissociation

NOTE: All the following procedures are carried out in a sterile tissue culture designated biosafety cabinet using aseptic technique and sterile materials.

3.1. Add 1 mL of 0.05% trypsin containing 0.53 mM EDTA to each 15 mL conical tube of 9 mL DMEM and tissue to begin tissue lysis.

NOTE: DMEM contains a high amount of calcium chloride, which can act as an inhibitor of trypsin. If dissociation is not complete following the outlined steps, Hanks' Balanced Salt Solution without calcium or magnesium can be used. After trypsinization, the enzyme can be neutralized by adding a trypsin inhibitor, although calcium should be added back to the solution as it is a cofactor for DNase I, which is used in subsequent steps.

3.2. Triturate with a 10 mL pipette approximately 20x.

3.3. Transfer the cell suspensions to empty 50 mL conical tubes.

3.4. Incubate the solution at 37 °C, 5% CO_2 for 15 min, gently agitating the lysates after 8 min.

3.5. Add 5 mL of MGM and 200 µL (5 mg/mL) DNase I to each tube for a final concentration of 50 µg/mL.

3.6. Triturate each lysate with a 10 mL pipette 10x.

3.7. Let the cell suspensions sit for 3 min at room temperature to allow non-dissociated tissue to settle at the bottom of the tubes.

3.8. Transfer the cell suspensions to new 50 mL conical tubes, leaving behind the non-dissociated tissue.

NOTE: The lysis and trituration steps described above significantly limits the amount of non-dissociated tissue.

3.9. Centrifuge the tubes at 300 x *g* for 3 min at 4 °C without brake.

3.10. Aspirate the supernatant and resuspend the remaining cell pellets in 5 mL of MGM.

3.11. Triturate the pellet with a 5 mL pipette 20x.

3.12. Plate the 5 mL cell suspensions on coated T25 flasks.

3.13. Incubate the cells at 37 °C, 5% CO_2 and change media initially after 24 h to remove cell debris.

NOTE: Some protocols recommend an initial media change after 72 h. Optimization of this step may be required.

3.14. Perform a 100% media change with MGM every 48-72 h until cells are 80% confluent (approximately 5-7 days).

NOTE: All media must be warmed to 37 °C before media changes.

4. Microglia isolation

4.1. Once mixed glia cultures have reached 80% confluency, prepare flasks for shaking by tightening lids and sealing with paraffin film.

4.2. To remove microglia from mixed glial cultures, secure flasks horizontally on an orbital shaker inside of a 37 °C incubator. Shake flasks at 15 x *g* for 1 h.

4.3. Remove media and pipette into a 15 mL conical tube. Rinse flasks twice with 3 mL warm MGM, adding wash to the 15 mL conical tubes. These are the microglia.

4.4. Add 5 mL of warm, fresh MGM to the culture flasks.

4.5. Reseal flasks with paraffin film, secure flasks horizontally on shaker, and shake at 15 x *g* at 37 °C for 15 h for separation of OPCs from astrocytes.

NOTE: This step can be done overnight, but the 15 h shake time is critical as excess time may result in cell death.

4.6. Centrifuge the supernatant from step 4.3. at 300 x *g* for 3 min and culture microglia according to standard protocols[16] or use for a biological assay.

5. Oligodendrocyte precursor cell isolation

NOTE: When plating OPCs following initial isolation, they must be plated on a poly-D-lysine-coated surface (sterile plate or cover slip). Prepare these materials prior to the completion of this section.

5.1. Following 15 h shake, remove the supernatant from flasks and plate on sterile 100 mm Petri dish.

5.2. Incubate the supernatant at 37 °C, 5% CO_2 for 30 min, swirling after 15 min to remove remaining microglia, as these will very quickly adhere to the dish. Non-tissue culture-treated Petri dishes may be used for this step.

5.3. Remove non-adherent cell supernatant, count, and plate on a poly-D-lysine-coated surface. Typically, 7,500-10,000 OPCs are plated/cm^2.

5.4. Incubate at 37 °C, 5% CO_2 for at least 1 h (up to 6 h), then gently aspirate 95% of media, and slowly add warm OPC Media, pipetting media against the wall of the well to minimize disruption of OPCs. Change media every 48 h until cells are ready for use.

NOTE: It is critical that only one well is changed at a time. OPCs are sensitive and are especially intolerant to dry conditions. The addition of PDGF-AA in OPC media is to delay OPC maturation into oligodendrocytes. This factor may be excluded from culture media if the experimental focus is mature oligodendrocytes.

6. Astrocyte isolation

6.1. Following 15 h shake, remove supernatant and rinse flasks 2x with warm 1x PBS.

6.2. Add 4 mL of 0.05% trypsin containing 0.53 mM EDTA and incubate at 37 °C, 5% CO_2 for 5 min or until cells have lifted. To ensure astrocytes have lifted, visualize them using a standard wide-field microscope. Astrocytes will appear spherical following

trypsinization.

6.3. Once astrocytes have detached, stand flask vertically and add 4 mL MGM. Triturate to mix.

6.4. Pipette astrocytes into a 15 mL conical tube, centrifuge at 300 x *g* for 5 min.

6.5. Resuspend astrocytes in Astrocyte Media and plate on fibronectin-coated surface as described in step 1.2. or use for biological assay.

NOTE: 20 ng/mL murine fibroblast growth factor can be added during the first media change to help establish the astrocyte culture. Additionally, standard gelatin coating may be a low-cost alternative to fibronectin.

7. Identification and isolation of microglia, OPCs, astrocytes, and mature oligodendrocytes using immunocytochemistry

7.1. Once plated cells have reached appropriate confluency, gently remove media and fix adherent cells using 4% paraformaldehyde (PFA) for 10 min. Do this step in a biosafety cabinet.

NOTE: When aspirating or adding solutions, pipette with gentle pressure to prevent cell detachment.

7.2. Slowly aspirate PFA then wash cells 3x with 1x PBS for 5 min.

7.3. Prepare appropriate blocking solution using 10% serum and 0.1% Triton X-100 in 1x PBS.

NOTE: Serum source reflects the host animal in which the secondary antibody was raised. For example, if the secondary antibody is goat anti-rabbit, the appropriate blocking serum is normal goat serum. Likewise, if the secondary antibody is donkey anti-rabbit, the blocking serum should be normal donkey serum.

7.4. Add blocking solution until cells are completely covered. Block for 1 h at room temperature.

7.5. Prepare an antibody diluent solution (9 mL of 1x PBS, 0.01 g bovine serum albumin, 30 µL Triton X-100). Alternatively, antibodies can be diluted in the blocking solution described in step 6.3.

7.6. Dilute primary antibodies specific for the following in antibody diluent or blocking solution:

7.6.1. Microglia: ionized calcium binding adaptor molecule 1 (Iba1) at a 1:250 dilution (2.4 µg/mL).

7.6.2. OPCs: neural/glial antigen 2 (NG2) at a 1:200 dilution (5 μg/mL).

7.6.3. Mature oligodendrocytes: myelin basic protein (MBP) at a 1:400 dilution (concentration is lot-dependent and optimization may be necessary).

7.6.4. Astrocytes: glial fibrillary acidic protein (GFAP) at a 1:400 dilution(0.25 μg/mL)[17].

NOTE: Do not mix primary antibodies raised in the same species.

NOTE: GFAP will reliably label white matter astrocytes. For grey matter astrocytes, an alternative marker may need to be used.

7.7. Incubate primary antibody overnight at 4 °C (with gentle agitation is preferable).

7.8. Wash 3x with 1x PBS for 5 min to remove the primary antibody.

7.9. Incubate cells with appropriate secondary antibodies at a 1:400 dilution in antibody diluent or blocking solution protected from light.

NOTE: Secondary antibodies are conjugated to a fluorophore which may be interchanged depending on available microscope parameters. Once fluorescent secondary antibodies have been applied, protect from light as much as possible.

7.10. Incubate secondary antibody for 1 h at room temperature, limiting exposure to light.

7.11. Wash 3 times with 1x PBS for 5 min to remove excess secondary antibody.

7.12. To label nuclei, use a 1:1,000 DAPI/PBS solution and incubate cells for 5 min at room temperature in the dark.

7.13. Wash 3x with 1x PBS for 5 min in the dark.

7.14. To mount, apply mounting media and allow to dry in the dark before imaging.

NOTE: Mounted slides can be stored at room temperature for 2-3 days. For long term storage, move slides to 4 °C. Image within one week for maximal signal. All representative images have been imaged on a confocal microscope; however, inverted fluorescent microscopes are also recommended.

REPRESENTATIVE RESULTS:

Representative data shown below illustrates that IFNγ signaling influences OPC differentiation and maturation. Without the presence of IFNγ receptor (IFNγR), cortical OPCs do not differentiate into mature myelinating oligodendrocytes as readily, which is evidenced by the

absence of MBP staining (**Figure 1**). Since oligodendrocytes and astrocytes are derived from a common progenitor, we analyzed GFAP expression, which labels astrocytes. We found that IFNγR-deficient cells strongly express GFAP suggesting that they may be adopting an astrocytic phenotype, corroborating earlier reports[19].

Additional evidence for regional heterogeneity in CNS cells is evidenced by varying astrocyte morphology as seen in astrocytes from the cortex, cerebellum, brainstem, and spinal cord (**Figure 2**). Of note, astrocytes from the same region may also exhibit morphological heterogeneity, supporting the notion that this glial subtype is highly dynamic. The differences in cellular architecture is suggestive of functional diversity and thus the ability to isolate glial populations is necessary to study phenotypic responses in the absence and presence of microenvironmental stimuli.

Oligodendrocytes are critical for the myelination of neuronal axons and are necessary for proper CNS repair and function. OPCs give rise to their mature counterparts, making it critical to understand the biology behind their ability to differentiate. Cytokine signaling significantly influences stem and immune cell behavior. Thus, it is important to understand how regional responses of OPCs may vary to differential cytokine stimulation (**Figure 3**), which may impact their ability to differentiate into mature myelinating oligodendrocytes.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative data showing OPC differentiation in WT and IFNγR$^{-/-}$ mice in the presence of exogenous IFNγ. Cortical OPCs were isolated from **(A)** WT and **(B)** IFNγR$^{-/-}$ P4 mouse pups following the procedure outlined above. Cells were treated with 1 ng/mL IFNγ for 48 h, then fixed and stained to delineate cell differentiation. OPCs were labeled for NG2 and GFAP to identify those which were adopting an astrocytic phenotype. Likewise, OPCs were also labeled for NG2 and MBP to identify those that were differentiating into mature oligodendrocytes. Scale bar = 20 μM.

Figure 2: Representative data demonstrating regional heterogeneity in astrocyte morphology. Astrocytes from cortex, cerebellum, brainstem, and spinal cord were isolated from P4 mouse pups using the protocol described above and labeled for GFAP (green) by immunocytochemistry following 48 h in culture. Scale bar = 20 μM.

Figure 3: Representative data demonstrating differential responses of regional OPCs to cytokines. OPCs were isolated from the brainstem and spinal cord of P4 mouse pups using the protocol described above. Cells were treated with increasing concentrations (1-10 ng/mL) of **(A)** IFNγ or **(B)** interleukin (IL)-17 in order to investigate the differential influence of cytokine on the ability of regional OPCs to differentiate into myelinating oligodendrocytes. Following a 48 h incubation with specified cytokines, OPCs were fixed and labeled for NG2 (green) and MBP (red). Scale bar = 20 μM. Data represent means ± SEM. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by 2-way ANOVA.

Table 1. Buffer and Media Recipes.

DISCUSSION:

In this protocol, we describe the isolation of the three major glial cell subpopulations from mouse CNS: microglia, OPCs, and astrocytes. A major setback for the investigation of neurodegenerative and neuroinflammatory CNS diseases is the lack of primary human cells and tissues, particularly those that are regional and from the same patient. In most instances, human CNS cell lines are derived from transformed, immortalized cancer cells which may not be accurate representations of their normal physiological behavior[20-22]. Thus, alternative methods are necessary to study CNS cell phenotypes in a controlled manner. Furthermore, the diversity of neurological glial cell populations makes it necessary to investigate each subtype both independently of one another, as well as in co-culture conditions in order to recapitulate both their cell autonomous and non-autonomous functions. Glial cells have a wide variety of critical functions in the CNS ranging from neuronal support[23], learning/cognition[24,25], and CNS immunological responses[26]. As such, it is necessary to understand the molecular and cellular functions of each glial subpopulation in a physiological and pathological context. In order to do so, we provide here a reliable method for the extraction and isolation of viable glia subtypes. Due to practical and ethical constraints in human subject's research, animal models are currently the most relevant surrogates for human glial cell biology. In particular, mice are ideal model animals as their genome can be manipulated and analyzed to further dissect particular molecular mechanisms underlying health and disease. Therefore, the successful removal and separation of murine microglia, OPCs, and astrocytes is a key tool to investigate the functions of glia during physiological, neurodegenerative, or neuroinflammatory conditions.

This protocol can be optimized to explore CNS cell regional heterogeneity. It is becoming increasingly clear that glia exhibit regional heterogeneity in form and function. Astrocytes are regionally diverse and display distinct morphology depending on their location within the CNS[27]. Furthermore, the density of astrocytes and their mitotic index can define anatomical regions, supporting the hypothesis that regional astrocyte heterogeneity may reflect molecular and functional differences based on their location within the CNS[28]. Microglial regional heterogeneity is also under active investigation, although the underlying mechanisms and functional consequences of microglia diversity in CNS development or behavior are currently unclear. However, it is known that adult microglia display diversity in cell number, cell and subcellular structures, and molecular signatures[29]. Moreover, recent advances in multiplexed mass cytometry have further defined the regional heterogeneity of microglia, analyzing cellular phenotype from five different CNS regions of nine human donors, allowing for large-scale immunophenotyping of human microglia[30]. Currently, such approaches are in their nascent stages, making animal studies a viable solution for the study of regional glia in CNS disease development. Finally, regional heterogeneity has also recently been described in oligodendrocytes. Single-cell RNA sequencing on 5072 individual cells from 10 regions of juvenile and adult CNS identified 13 distinct subpopulations across different stages of differentiation[31]. Importantly, it was also found that as oligodendrocytes matured from OPCs, their transcriptional profiles diverged and their functional phenotypes changed, highlighting oligodendrocyte heterogeneity within the CNS[31].

Thus, understanding regional heterogeneity of the various resident CNS cells in the context of their diverse neighboring neurons and other glia may provide important rationale for the future development of novel therapies to treat neuroinflammatory and neurodegenerative disorders. While this protocol focuses on the extraction, isolation, and identification of glial subpopulations, it provides a convenient starting point for the examination of their function. Furthermore, it can be adapted and combined with transgenic mouse models in order to study genetic mechanisms associated with glial cell biology. It can also be used to examine the responses of glial cells to each other in co-culture assays. The outlined steps represent a cost-efficient and high-throughput method of extracting and isolating different CNS glial populations which can then be adapted to a wide variety of experimental parameters. It should be noted; however, that the method described here utilizes neonates due to the lower levels of myelination and high density of proliferating glia. For these reasons, it is technically more feasible to isolate viable glia from neonates compared to adult animals. The phenotypic differences in neonatal glia compared to adult glia should thus be considered during experimental design and data interpretation.

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DISCLOSURES:

The authors have conflicts of interest to disclose.

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29 Tan, Y.-L., Yuan, Y., Tian, L. Microglial regional heterogeneity and its role in the brain. *Molecular Psychiatry.* **25** (2), 351-367 (2020).
30 Bottcher, C. et al. Human microglia regional heterogeneity and phenotypes determined by multiplexed single-cell mass cytometry. *Nature Neurosciences.* **22** (1), 78-90 (2019).
31 Marques, S. et al. Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science.* **352** (6291), 1326-1329 (2016).

Figure 1 Figure 1

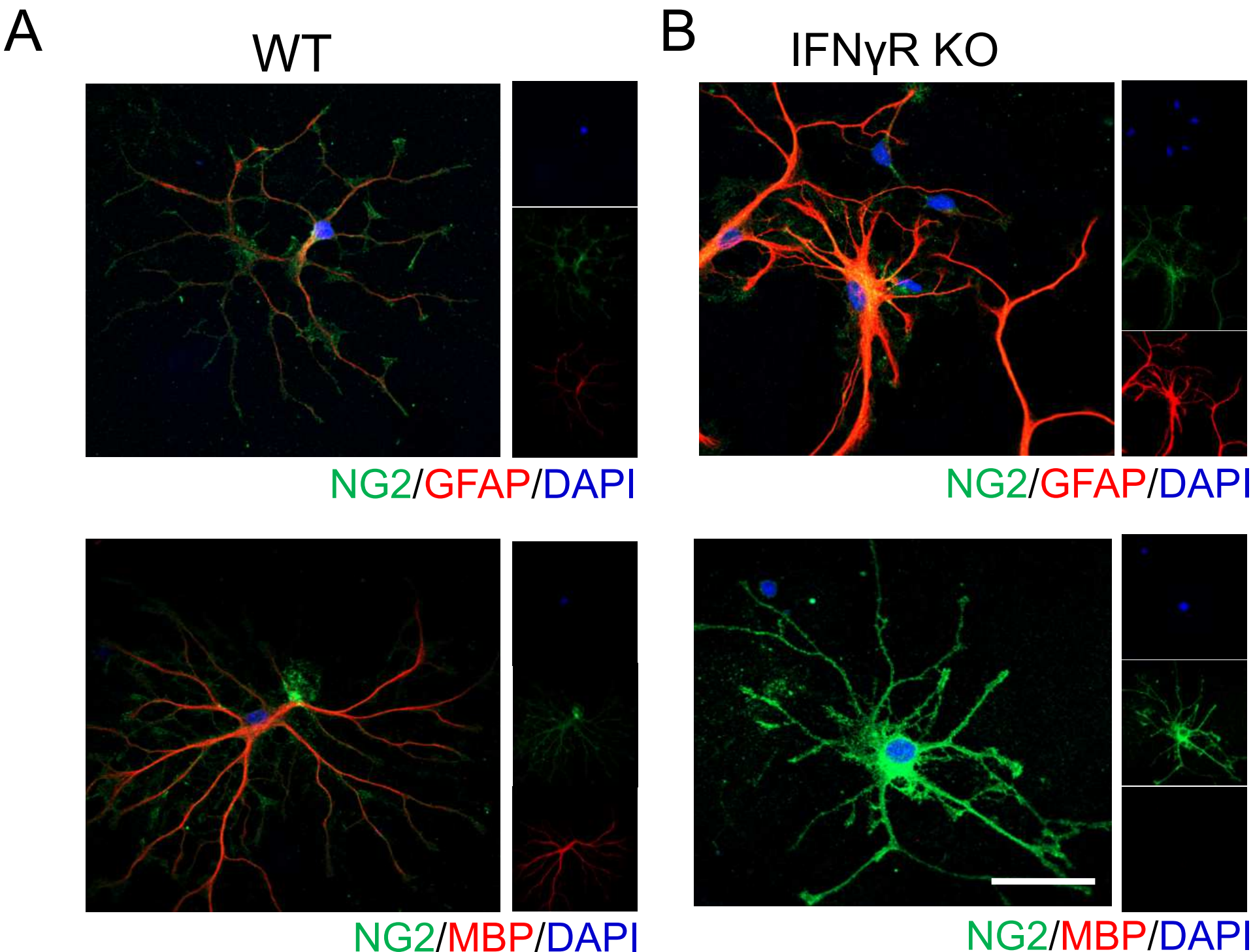

Figure 2

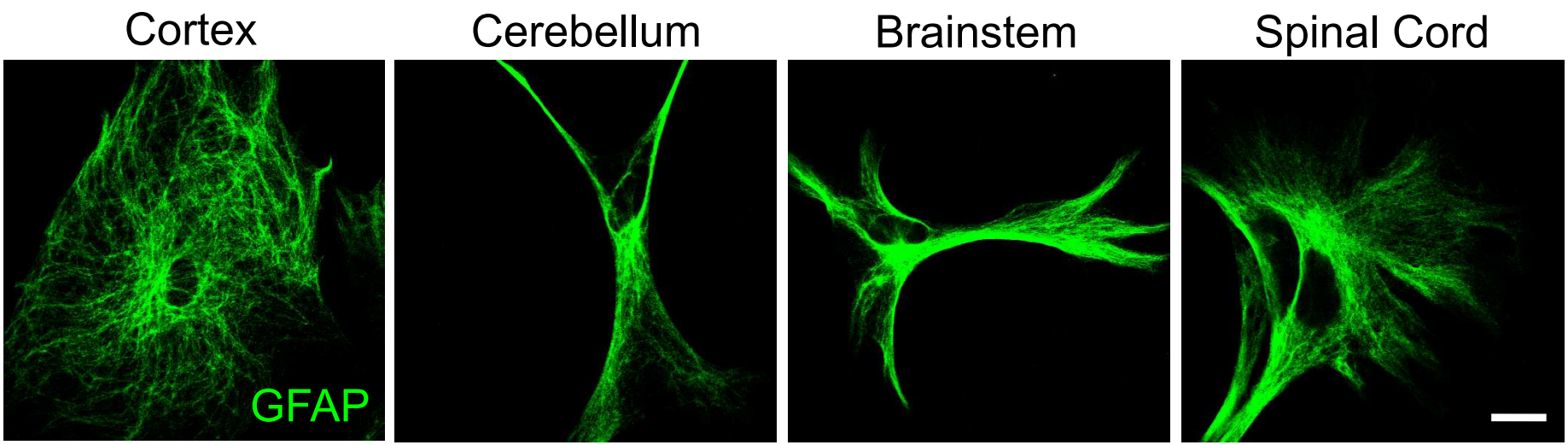

Figure 3

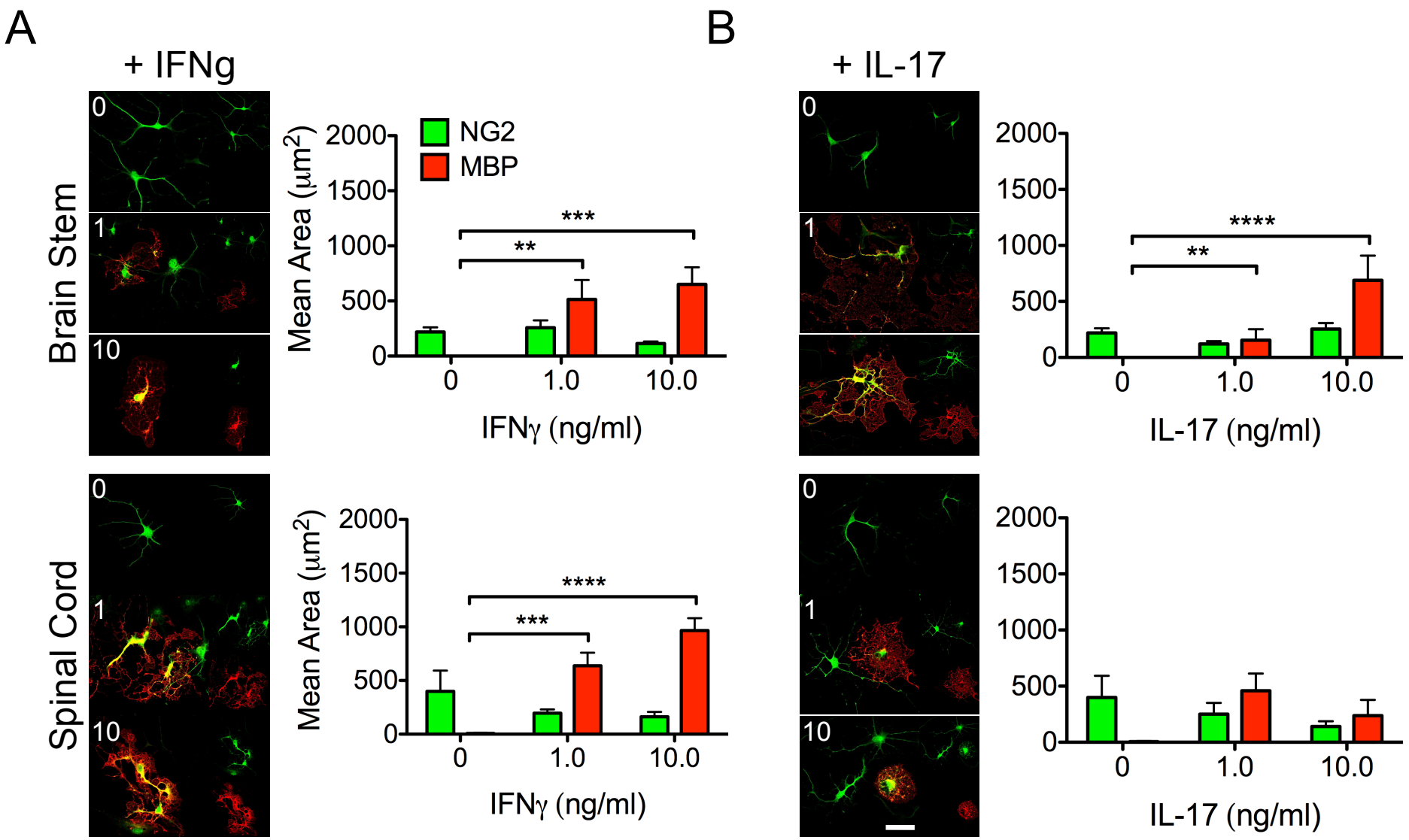

Table 1. Buffer and Media Recipies

PBS/Antibiotic Solution (PAS):

1.0 mL	100X Antibiotic/Antimycotic containing 10,000 units/mL penicillin and 10,000 μg/r
25 μg/mL	Amphotericin B
99 mL	1X PBS

Mixed Glia Media (MGM)

88 mL	1X DMEM (high glucose, w/L-glutamine, w/Na pyruvate)
10 mL	Heat-inactivated FBS
1.0 mL	L-Glutamine (100X)
1.0 mL	Antibiotic/Antimycotic

OPC Media (50mL)

49 mL	Neurobasal media
1.0 mL	B27 supplement (50X)
10 ng/ml	PDGF-AA

NOTE: PDGF-AA is added fresh prior to each media change.

Astrocyte Media (1 L)

764 mL	MEM with Earle's salts containing glutamine
36 mL	Glucose (use 100 mg/mL stock for final concentation of 20 mM)
100 mL	Heat-inactivated FBS
100 mL	Heat-inactivated horse serum
10 mL	Glutamine (use 200 mM stock if not included in stock medium)

OPTIONAL: 10 ng/mL recombinant mouse epidermal growth factor

NOTE: Sterile filter all media and store at 4°C until used.

ɳL streptomycin

Name of Material/ Equipment	Company
0.05% Trypsin and 0.53 mM EDTA	Gibco
12-Well Plates	Greiner Bio-One
1X PBS pH 7.4	Gibco
32% Paraformaldehyde	Electron Microscopy Sciences
50 mL, 25 cm^2 cell culture flask	Greiner Bio-One
Antibiotic-Antimycotic 100X	Gibco
B-27 Supplement 50X	Gibco
Bovine serum albumin	Sigma
Confocal Microscope	Zeiss
DAPI	ThermoFisher
DMEM (1X), high glucose with Na pyruvate	Gibco
Dnase I	Sigma
Fetal bovine serum heat inactivated	Gibco
Fibronectin from bovine plasma	Sigma
Fine stitch Scissors	Sklar
Goat anti-rabbit IgG Alexa Fluor 488	Invitrogen
Goat anti-rat IgG Alexa Fluor 555	Invitrogen
Hanks' Balanced Salt Solution (w/o Ca or Mg)	ThermoFisher
L-glutamine, 200mM	Gibco
Murine epidermal growth factor	ThermoFisher
Murine IFN-γ	Peprotech
Murine PDGF-AA	Peprotech
Neurobasal	Gibco
Normal goat serum	Sigma
Operating Scissors	Surgi-OR
Poly-D-Lysine 12 mm #1 German Glass Coverslip	Corning Biocoatt
Prolong Gold Antifade Reagent	Cell Signaling Technology
Rabbit anti-Iba1	Wako
Rabbit anti-NG2 Chondroitin Proteoglycan	Millipore
Rat anti-GFAP	ThermoFisher
Rat anti-myelin basic protein	Abcam
Sharp Tip Scissors	Surgi-OR
Stereo Microscope	Leica
Tissue Forceps	Sklar
Triton X-100	Fisher Bioreagents
Trypsin Inhibitor (from chicken egg white)	Sigma

Catalog Number	Comments/Description
25300054	Tissue dissociation
665 180	Cell culture plate
10010031	Standard reagent
15714-S	Fixative
690 175	Cell culture (T25) flask
15240-096	Media component
17504-044	Media component
A9647-50G	Antibody diluent
LSM 800	Confocal for imaging
D1306	Nuclear stain
11995040	Media component
10104159001	Tissue dissociation
A3840001	Media component
F1141-1MG	Cell adherent
64-3260	Dissection tools
A11008	Secondary staining antibody
A21434	Secondary staining antibody
14170120	Tissue dissociation
20530081	Media component
PMG8044	Media component
315-05-20UG	Media component
315-17	Media component
21103-049	Media component
G9023	Blocking solution component
95-272	Dissection tools
354086	Cell adherent
9071S	Mounting Media
019-19741	Primary antibody
ab5320	Primary antibody
13-0300	Primary antibody
ab7349	Primary antibody
95-104	Dissection tools
S4 E Stereo Zoom Microscope	Microscope for dissection
66-7644	Dissection tools
BP151-100	Cell permabilization
10109878001	Tissue dissociation

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April 14, 2020

Dr. Vineeta Bajaj
Review Editor
Journal of Visualized Experiments
1 Alewife Center #200
Cambridge, MA 02140

Dear Dr. Bajaj:

Attached you will find the revised manuscript entitled "Dissection and isolation of murine glia from multiple central nervous system regions" by Sinyuk and Williams. The manuscript was judged to require revisions before publication in *JoVE*. We wish to thank the editor and reviewers for insightful recommendations and have addressed their concerns, marked as red text in the manuscript, as follows:

Editorial Comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.* The manuscript has been thoroughly proofread and we are confident it is now suitable for publication.
2. *Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. Please include one inch margin on all sides.* The JoVE-provided template was used for formatting and we believe all criteria have been met.
3. *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.* There are no commercial trademark symbols listed in the manuscript.
4. *Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.* The correct numbering format is now used.
5. *Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."* Passive voice has been removed to provide more direct instruction throughout the protocol.
6. *The Protocol should contain only action items that direct the reader to do something.* The protocol now only contains action items.
7. *Each step can have 2-3 actions per step.* Each step contains no more than 2-3 actions.

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8. *Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).* The proper unit for centrifuge speed is now used.
9. *Please ensure you answer the "how" question, i.e., how is the step performed?* We have ensured the steps are outlined in a clear fashion to answer how the step is performed.
10. *2.5 Please include the age of the pups used for the study. Don't you perform CO2 asphyxiation for euthanasia?* The age of the pups is included in the Note prior to the Section 2 protocol and we have indicated that the institutional policies and procedures should be followed with regard to euthanasia.
11. *7.5: Please include all the antibody used to perform the study with dilutions and citations.* Antibodies and dilutions are now listed in Step 7.5.
12. *Please make a separate table for all the buffers and media recipe and upload it separately as .xlsx file to your editorial manager account. Please do not embed in the manuscript text. Please ensure that the legend is included in the figure/table legend section.* We have included a separate table for all media and solution recipes and have included a table legend in the figure/table legend section.
13. *There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.* We have reduced the amount of highlighted text to fit within the 2.75 page limit for filmable content.
14. *There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.* Please see above comment.
15. *As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:*
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We feel as though we have covered the necessary topics for understanding the importance and applicability of the described technique in the discussion and have made notes throughout the protocol identifying critical steps and alternatives.
16. *Figures: For all the images obtained with a microscope, please include a scale bar and define it in the figure legend.* Scale bars are now included in the figures and noted in the figure legends.
17. *Please sort the materials table in alphabetical order.* The materials table is now in alphabetical order.

Reviewer 1 Comments:
Major Concerns:
-Steps 2.14-2.17 - I am having a hard time envisioning these procedures. Can authors confirm that they mean "supine" vs "prone" and "ventral" vs "dorsal". This may be aided by video. We thank the reviewer for pointing this out. We have edited the text to make the procedure clearer.
-Representative results - It may be helpful to discuss somewhere that astrocytes and OPCs derive from a similar progenitor cell. Without this, it is confusing that you would have astrocytes in your OPC culture. This is

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a great suggestion. We have pointed out the common progenitor in the representative results section.

Minor Concerns:
-*Step 2.7 - Specify that this is the head, and not the body.* This is now specified.
-*Step 2.20-2.21 - If dissecting more than 1 pup, are all tissues from similar region combined together into a single 15 ml conical tube, or are they kept separate? Ie, if dissecting 3 spinal cords for a T25 flask, are they combined? What about 6 spinal cords? Does this affect the volume of DMEM/Trypsin?* We have clarified the optimum number of tissues to be combined in Step 1.2 and 2.20, commenting on the dissociation reagent requirements in an associated Note.
-*Steps 3.9, 4.6, 6.5 - Authors say to centrifuge at 1200 RPM, what is this in g-force?* All centrifuge speeds are now listed as g-force.
-*Step 5 - Authors note "If replating OPCs, they must be plated on poly-L-lysine coated surface." Is this referring to the first replating after the mixed glial culture or only subsequent replatings? Similar to this, will glial cells proliferate, or should cells be plated in their final experimental plates after mixed glial cell culture?* We appreciate this comment. The note was confusing and it is now simplified for clarity.
-Step 5.3 - *Authors say to count the cells. Is there a certain density that cells should be plated at, or should the total volume just be kept constant?* The recommended plating density has been added to Step 5.3.
-*Step 7.5 - What is antibody diluent solution? Is this something specific?* The contents of the antibody diluent solution have been added to this step with an alternate solution in the note below.
-*Figures - Authors should include scale bars.* All figures now include scale bars.

Reviewer 2 Comments:
The representative results on page 6 line 327 need more quantitative data. Even though this is listed as representative results it is to strong wording to say that INFγR is required for differentiation of OPCs into oligodendrocytes without presenting any quantitative data on this. This is especially true as the culture conditions used for OPCs are likely to delay differentiation compared to differentiation protocols using SATO media and that high serum or B27 concentrations may increase the number of precursors that turn into astrocytes. Similar in line 333 and onwards when commenting on the different morphology observed in representative pictures of astrocytes from different brain regions presented in figure 2 more experimental evidence is required. Astrocytes in culture can have quite diverse morphologies even when isolated form the same brain region so a more quantitative analyses of morphology is required to support the stated differences between brain regions. We agree and thank the reviewer for these comments and recommendations. Regarding Figures 1 and 2, we have edited the representative results section so as to not make such strong conclusions since we do not show quantitative data.

Minor Concerns:
Page 2_Line 113 The authors use mouse pups at the age P3 to P5- it may be word mentioning that using P0 to P2 pups may give a higher yield of glia cells. We have added a Note at the beginning of Step 2 to address this point.
Page 4 _line 189 (3.13) In our experience not changing the media after 24 hours, but waiting to 72 hours will increase the numbers. We thank the reviewer for this insight. We have added a Note mentioning a media change after 72 h as an alternate.
Page 5 _line 216 (5.2) May be worth mentioning that this step could be done using non-tissue culture treated. This is now mentioned in this Step.

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Page 5 Line 220 (5.4) Is worth mentioning that the OPC media used is likely to delay the differentiation of OPC into oligodendrocytes, due to the addition of PDGF. (this explain why the authors apparently do not see any MBP positive cells in non-treated cultures. We have added a note to address this important point.

Reviewer 3 Comments:
Minor Concerns:
Line169
The purpose of this step is to further dissociate the cells using Trypsin. The authors add Trypsin/EDTA into DMEM which contain a high amount of Calcium (Calcium chloride dihydrate 265mg/L) which is an inhibitor of trypsin. This dissociation is better achieved in media such as Hank's without calcium or magnesium (HBSS Ca/Mg free, Thermofisher Cat14170120). After trypsinization, the enzyme should be neutralized by adding trypsin inhibitor (e.g. from Chicken egg white, Sigma 10109878001). Calcium should also be added since it's an important coactivator for DNase treatment in a subsequent step. We thank the reviewer for pointing this out. We have added a Note following Step 3.1 detailing the suggestion as an alternative approach and have added the suggested HBSS and trypsin inhibitor to the materials list.

Line 184 and wherever centrifugation is used
For centrifugation, the authors should provide the Relative Centrifugal Force (RCF) instead of the RPM. Since the latter is dependent on the centrifuge models or the rotor used. We agree and RCF is now used throughout the manuscript.

Line 237
Astrocytes do well on variety of substrates. Standard Gelatine coating is a cheap alternative to fibronectin. This is a great suggestion. It is now mentioned in a Note following Step 6.5.

Line 239
Astrocytes thrive in the presence of the mitogen FGF2 (20ng/ml). We thank the reviewer for this insight; however, we do not have experience with FGF2 and thus feel uncomfortable recommending its use without a specific manufacturer and catalogue number.

Line 264
In this line and anywhere the use of antibodies are mentioned, the concentration of the antibody should be provided as μg/ml since these antibodies are available from different manufacturers and often have different specifications. The concentrations of antibody used (where available) are now listed in Step 7.6 with the exception of anti-MBP. The manufacturer (Abcam) was contacted to get the specific concentration for the MBP antibody lot used and we were told the concentration for this antibody was not measured, to follow specific dilution instructions. We commented on this in the manuscript.

Line 245
(7.1) In this line the authors use plates, whereas line 288 (7.14) the authors refer to slides. We thank the reviewer for pointing this out. We have corrected this discrepancy by using verbiage applicable to either.

Line 353 and wherever magnification is used.
Scale bars are more accurate and should be used in lieu of magnifications. Scale bars are now used in all

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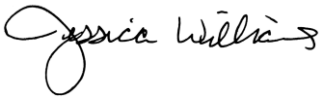
figures.

Line 353
The authors should indicate that the use of a confocal is optional. An inverted fluorescence microscope is the tool of choice. A Note highlighting this point has been added to the end of the protocol.

Figure 3.
The images (10ng IL17 or TNFg) show colocalization of NG2 (green) and MBP(red). These markers do not normally overlap. The author should provide larger images with their respective color splits similar to those of Figure 1. This is an excellent point; however, using the Barres RNA-Seq database, we found that *Cspg4,* which encodes NG2 in mice, is expressed by newly formed oligodendrocytes, which would also express MBP. Additionally, we have now provided larger images with color separation. We thank the reviewer for this suggestion.

Thank you for your consideration of our revised manuscript and we look forward to hearing from you.

Best wishes,



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