Video Article

Rapid and Refined CD11b Magnetic Isolation of Primary Microglia with Enhanced Purity and Versatility

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Abstract

Microglia are the primary responders to central nervous system insults; however, much remains unknown about their role in regulating neuroinflammation. Microglia are mesodermal cells that function similarly to macrophages in surveying inflammatory stress. The classical (M1-type) and alternative (M2-type) activations of macrophages have also been extended to microglia in an effort to better understand the underlying interplay these phenotypes have in neuroinflammatory conditions such as Parkinson's, Alzheimer's, and Huntington's Diseases. *In vitro* experimentation utilizing primary microglia offers rapid and reliable results that may be extended to the *in vivo* environment. Although this is a clear advantage over *in vivo* experimentation, isolating microglia while achieving adequate yields of optimal purity has been a challenge. Common methods currently in use either suffer from low recovery, low purity, or both. Herein, we demonstrate a refinement of the column-free CD11b magnetic separation method that achieves a high cell recovery and enhanced purity in half the amount of time. We propose this optimized method as a highly useful model of primary microglial isolation for the purposes of studying neuroinflammation and neurodegeneration.

Video Link

The video component of this article can be found at https://www.jove.com/video/55364/

Introduction

Microglia are Myb-independent resident macrophages of mesodermal origin, which differentiate from c-kit+/CD45- erythromyeloid progenitors in the blood islands of the yolk sac[1,2]. Once embryological microglia have colonized the central nervous system (CNS), they transition from an amoeboid to a ramified form[3]. These adult microglia are classified as surveillant since their dynamic ramifications probe the healthy brain parenchyma for potential insults[4]. Although microglia only contribute to approximately 10% of the CNS cell population, their ability to tile amongst each other ensures maximal scanning of the parenchyma[4,5]. Danger-associated molecular patterns (DAMPs), such as α-synuclein[6,7] and amyloid-β[8], or pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS)[9], classically activate microglia to promote an inflammatory response characterized by reversion to the amoeboid active state and the production of nitric oxide, tumor necrosis factor-α (TNFα), interleukin 1β (IL-1β), IL-6, IL-12, and the chemokine C-C motif ligand 2[9,10,11]. In neuroinflammatory conditions such as Parkinson's disease, in which pathogenic α-synuclein has accumulated, a neurodegenerative cycle is created from the death of dopaminergic neurons, which release more aggregated α-synuclein, further promoting classical activation of microglia[7]. Similar to peripheral macrophages, microglia may also have the ability to alternatively activate in the presence of the anti-inflammatory cytokines IL-4 and IL-10, giving them the potential of promoting neural repair and attenuating inflammation[2,11]. Aside from their immunological roles in the CNS, microglia have been described as vital regulators of neuronal circuitry by pruning synapses during development. For example, *Cx3cr1*-KO mice have less dense microglia and reduced synaptic pruning, which leads to an overabundance of dendritic spines, immature synapses, and the electrophysiological patterns of an underdeveloped CNS[12]. Understanding these physiological complexities and the diverse functional roles of microglia in the homeostasis of the CNS is critical to the search for therapeutics targeting neurodegenerative disorders.

In the area of neuroimmunology, *in vitro* experiments are highly desirable because of the greater feasibility for mechanistic studies, the lower maintenance costs, and for being less time- and labor-intensive. Furthermore, the ability to isolate cell populations is critical to delineate the functionality of those target cells under prescribed conditions. Numerous microglial isolation methods exist, but they are limited by their ability to obtain relatively high numbers and purity for broad experimentation[13,14,15]. For example, a cluster of differentiation 11b (CD11b) is a common surface marker of monocytes, macrophages, and microglia[16]. By exploiting CD11b, a method of magnetic separation was first described as a column-based approach that yielded ~99.5% purity and ~1.6 x 10^6 microglia per neonatal brain[17]. Our laboratory recently developed a column-free CD11b magnetic separation method[15], which we performed in a polystyrene tube by tagging CD11b with a monoclonal antibody conjugated to phycoerythrin (PE). A bispecific secondary antibody to PE and dextran complexes with the PE. Once bound, dextran-coated magnetic

particles are introduced, which bind to the dextran end of the antibody complex. Lastly, the polystyrene tube is placed in a magnet for microglial isolation. This approach doubled the yield to ~3.2 x 10^6 microglia per neonatal brain but at the cost of reducing purity to ~97%.

Herein, we demonstrate a rapid and refined column-free CD11b magnetic separation protocol (**Figure 1**). This improved method remains as feasible as our original column-free method since the price of the CD11b magnetic separation kit is the same. The completion time is reduced in half, which can be crucial to maximizing cell survival and yield. Notably, the purity achieved from this optimized method is ~>99%, a marked improvement over the purity achieved from the original column-free method developed by our laboratory[15]. Most importantly, CD11b-PE is not utilized, eliminating the need to incubate away from light and allowing the use of the red channel for fluorescence microscopy. Lastly, as in the original CD11b method, an astrocytic fraction of high yield and purity is obtained with this improved method. Astrocytes are the most numerous glial cells in the CNS, leading to the idea that their homeostatic functions are indispensable in relation to pathophysiology[18]. These glial cells play a role in diverse physiological functions such as forming the blood-brain barrier, providing nutrient support, maintaining neurotransmitter homeostasis, forming glial scars in response to injury, neuroprotection, learning and memory, and neuroinflammation, exemplifying their investigatory potential in glial biology[19]. Morphology and functionality of microglia and astrocytes have been ascertained via confocal microscopy, Western blotting, quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), Griess nitrite assay, and the Luminex multiplex cytokine assay. The refinement provided by this protocol offers increased confidence pertaining to microglial or astrocytic purity, broader application of fluorescence microscopy with the availability of the red channel, and saves time, all of which are important for *in vitro* experimentation.

Protocol

Use of the animals and protocol procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA, USA)

1. Growing of Mixed Glial Cultures

1. Decapitate 1- 2 day-old pups quickly with 5.5 inch operating scissors, and place the heads immediately in a 50 mL tube on ice. Note that this decapitation is the mode of euthanasia.
2. **In a laminar airflow hood, make a small incision in the skull and meninges using 4.5 inch straight micro-dissecting scissors. Begin cutting from the caudal end to the rostral end (nose). Get underneath the skin by using the opening formed by the decapitation.**
    1. After the incision, peel one of the hemispheres to the side. Then use a pair of curved or hooked tweezers to remove the entire brain.
3. Immerse the brain(s) in a new 50 mL tube containing 0.25% trypsin-ethylenediaminetetraacetate acid (EDTA) for 15 min in a 37 °C water bath. Use 2 mL of trypsin-EDTA per brain.
4. Wash the brain(s) with fresh Growth Media (10% FBS, DMEM/F12, 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, and 1% non-essential amino acids) by adding and removing the media. Repeat this 4x.
5. For each brain, plate two T-75 flasks containing growth media. Therefore, add 2 mL of growth media per brain to the tube. Thus, it will be an equivalent of 1 mL of homogenized brain with 8-9 mL of growth media per T-75 flask.
6. **Homogenize by triturating the brain(s) with pipettes of differing aperture sizes, in order from largest to smallest. When it is visible that the brain tissue is not getting smaller, transition to the next pipette. At the end of the trituration, the suspension should be clear, with no visible chunks.**
    1. Use a 25 mL pipette, 5 mL pipette, and then a 10 mL pipette sequentially.
7. Pass each homogenous brain suspension through a 70 µm cell strainer to make it into a single cell culture.
8. For each homogenized brain, plate two T-75 flasks containing growth media, as described in step 1.5 (1 mL of homogenized brain with 8-9 mL of growth media per T-75 flask).
9. Change growth media after 6 d and grow until isolation on the 16th day.

2. Isolation of Microglial Cells

1. **After 16 days, remove the growth media from the flask and place it in a fresh 50 mL tube. Add 3 mL 0.25% trypsin-EDTA to each T-75 flask. Shake the flasks for 5 min at RT on an orbital shaker.**
    1. Centrifuge the removed growth media at 0.4 x g for 5 min and use to stop the trypsin-EDTA reaction in the subsequent step.
2. After shaking for 5 min, add a minimum of 4 mL of growth media (fresh or the used media from 2.1.1) to stop the trypsin-EDTA reaction.
3. Triturate to ensure that all the cells have been detached.
4. After trituration, pass the cells through a 70 µm cell strainer to make it into a single cell culture, perform a cell count, and then spin down the cells at 0.4 x g for 5 min.
5. For every 100 x 10^6 cells (roughly 15 x T-75 flasks), use 1 mL of Recommended Media (2% FBS, DPBS (calcium and magnesium chloride-free), 1 mM EDTA) to resuspend the cell pellet.
   NOTE: All following steps are tailored for a 1 mL separation.
6. Take a 5 mL polystyrene tube, add 1 mL of Recommended Media, and mark the meniscus. Add Recommended Media up to 2.5 mL and mark this as well. Discard the Recommended Media and transfer the re-suspended cells to the 5 mL polystyrene tube.
7. Add 50 µL of rat serum for every 1 mL of suspended cells. Incubate for 5 min at RT.
8. Prepare selection cocktail by mixing 25 µL of component A and 25 µL of component B. These components are proprietary.
9. Add 50 µL of the selection cocktail to the cells. Incubate for 5 min at RT.
   NOTE: For purer cultures, repeat step 2.9 (recommended but not mandatory).
10. Vortex microspheres for 45 s. Add 80 µL of microspheres per 1 mL of sample. Incubate for 3 min at RT.
11. Bring the volume to 2.5 mL in the polystyrene tube by adding the Recommended Media.

12. Put the tube in the magnet for 3 min at room temperature. Adjust the incubation to increase purity. Slowly pour out the Recommended Media into a 15 mL tube with the magnet still in the polystyrene tube.
13. Repeat step 2.12 three more times. Additional magnetic incubations may be performed to increase purity.
14. Add 3 mL of growth media and count the number of cells using a cell counter.
15. Plate cells accordingly in Poly-D-Lysine (PDL)-coated plates for treatments. Treat the cells 48 h after seeding inPDL-coated plates. This allows the cells to recover from the stress of separation.
    NOTE: Check the purity of the culture using immunocytochemistry as described previously[15].
16. Plate the negative fraction (collected in a 15 mL tube), which mostly contains astrocytes, in T-75 flasks in the growth medium.
17. After at least 6 h of incubation in 37 °C incubator, change the medium and let it grow O/N. Split the astrocytes the next day for treatments.

Representative Results

Microglia isolated using CD11b Positive Selection kit II have high purity

Primary mouse microglia were isolated using the above mentioned protocol and plated on poly-D-lysine-coated coverslips to check the purity of isolation. Ten thousand cells were plated per well and immunocytochemical analysis was performed using ionized calcium-binding adaptor molecule 1 (Iba1) as a marker of microglia and glial fibrillary acidic protein (GFAP) as a marker of astrocytes to check for the purity of the isolated microglia. The isolated culture only expressed Iba1 without GFAP expression (**Figure 2A**), suggesting that the culture isolated was pure microglia. Unlike other previously published methods for primary microglial separation, which achieved ~97% purity, using this method we obtained ~>99% purity in half the time. To further validate the purity of this culture, we ran a Western blot[20] for Iba1 and GFAP. Immunoblot analysis further revealed that this isolation from the mixed glial cultures was a nearly pure microglial culture (**Figure 2B**).

The modified isolation procedure does not have any auto-fluorescence from magnetic beads

The red channel cannot be used for immunocytochemistry (ICC) when using the previous CD11b isolation kit for microglial separation as mentioned by Gordon *et al.*[15] because of the use of PE labeling during separation. Using this new PE-free isolation kit, we can use the red channel for immunocytochemical analysis (**Figure 3**). From this ICC, it is evident that this new method enables us to use all the channels for flow cytometry or other fluorescent imaging studies.

Isolated microglial cultures functionally respond to LPS stimulus

To verify that the isolated microglia are functionally active, we treated the cells with LPS, a widely used stimulant[9] to activate microglia, for 24 h before probing for various pro-inflammatory factors. Classical microglial activation is accompanied by the release of nitrite and various pro-inflammatory cytokines into the media. Hence, we used multiple complementary assays to confirm the functional activity of our isolated microglia. First, we used the Griess assay to show that LPS dramatically induced nitrite secretion from the isolated microglia (**Figure 4A**). To further validate the activity of isolated microglia, we used qRT-PCR to show (messenger ribonucleic acid) mRNA levels of *NOS2*, another hallmark of microglial inflammation, significantly enhanced with LPS treatment (**Figure 4B**). Next, we used a bead-based multiplex assay, Luminex[21], to verify that LPS significantly stimulated the secretion of pro-inflammatory cytokines from the microglial culture (**Figure 5A**). Furthermore, we verified via qRT-PCR analysis that LPS treatment enhanced the gene expression levels of several pro-inflammatory cytokines including *IL-1β* and *TNFα* (**Figure 5B**). All these data together suggest that primary microglia isolated using this newly refined method are functionally active and show a similar activation profile as primary microglia isolated using previously published methods.

Isolated microglia can be used for signaling studies

Using the previous methods of microglial isolation, running Western blot for signaling studies was difficult and infeasible due to low yield and purity. Previously, we have shown that Fyn, a Src family kinase, is involved in a pro-inflammatory signaling cascade in microglial cells[9]. Here we plated one million microglial cells in a 12-well plate and after 48 h, we collected the cells to run Western blots for native Fyn and Src kinases phosphorylated at tyrosine residue 416 (p-Src-Y416). We were able to detect both Fyn and p-Src-Y416 levels in our isolated microglial cells (**Figure 6**), showing that this method is applicable to Western blotting techniques for signaling studies.

The negative fraction from the microglial separation contains astrocytes that can be used for signaling studies

Astrocytes are key players in neuroinflammation, and understanding the signaling mechanisms behind astrocytic inflammation and neuron-astrocyte cross-talk is important[19]. Here, we show that the negative fraction from the microglial separation contains GFAP-positive astrocytic cells (**Figure 7A**). Also, our Western blot analysis for Fyn and p-Src-Y416 (**Figure 7B**) showed that both of these proteins can be detected in the negative fraction as well. These results together show that the negative fraction is an ideal preparation for astrocytic studies aimed at identifying proteins important for inflammatory signaling cascades.

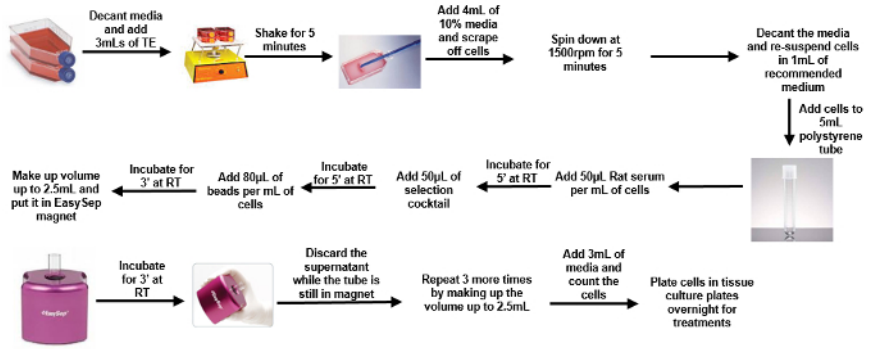

Figure 1: Schematic of Isolation of Microglial Cells from 1-2 Days-post-natal Pups. Please click here to view a larger version of this figure.

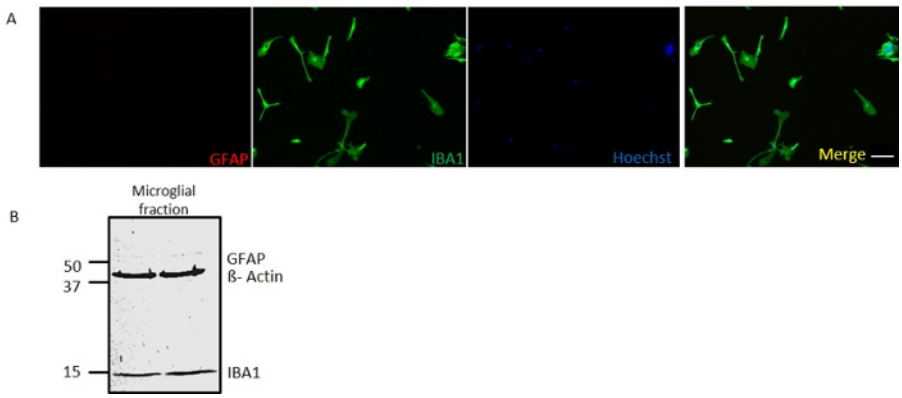

Figure 2: Microglia Isolated using CD11b-positive Selection Kit II Have High Purity. (**A**) Immunocytochemistry of isolated microglial culture probing for GFAP in red channel and IBA1 in green channel. (**B**) Immunoblot of isolated microglial culture probing for GFAP (~51 kDa), IBA1 (~15 kDa) and β-Actin (~42 kDa). Scale bar = 20 µm. Please click here to view a larger version of this figure.

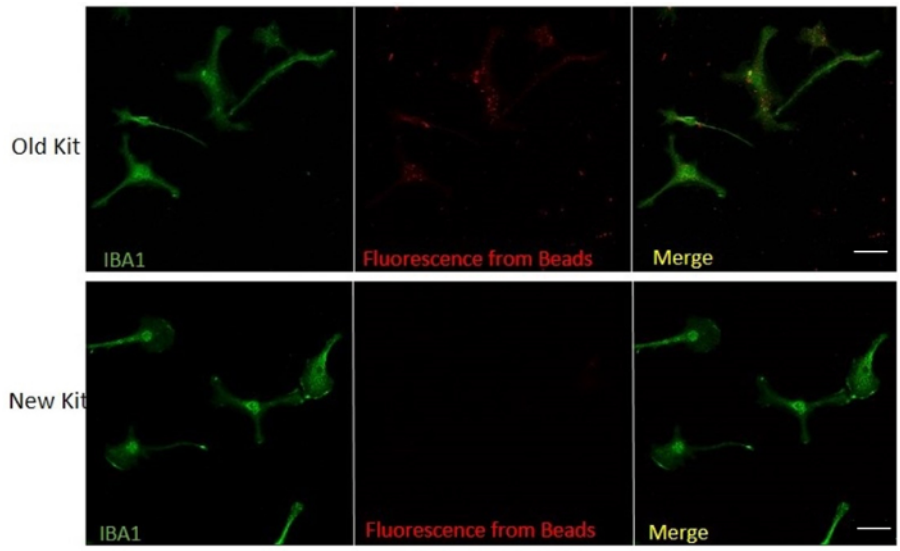

Figure 3: The Modified Isolation Procedure does not Have any Auto-fluorescence from Magnetic Beads. Immunocytochemistry of isolated microglial culture using the new isolation method and old (original) method probing for IBA1 in green channel and PE fluorescence in red channel. Scale bar = 30 µm. Please click here to view a larger version of this figure.

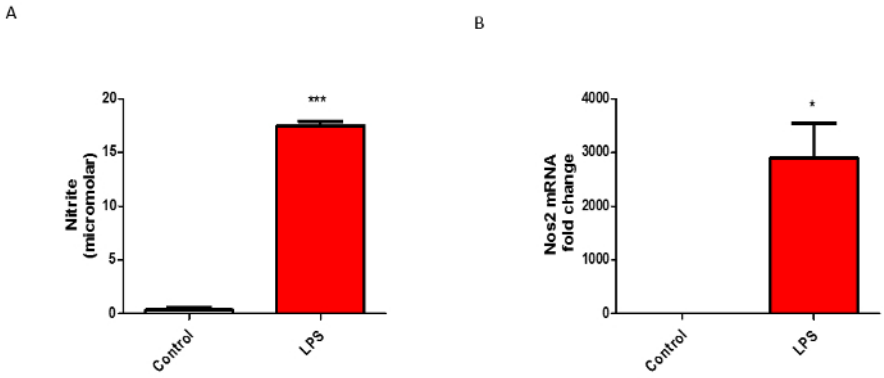

Figure 4: LPS Stimulation Increased Nitrite Production in Microglial Culture. (**A**) Isolated microglial cells treated with 1 µg/mL LPS for 24 h and the treatment medium were collected to determine nitrite release by Griess assay. (**B**) q-RT-PCR for Nos2 from isolated microglia treated with LPS for 24 h. The figures represent the mean ±SE from 2 or more independent experiments. Data were analyzed using Student's t-test (*p <0.05, **p <0.01, ***p <0.001). Please click here to view a larger version of this figure.

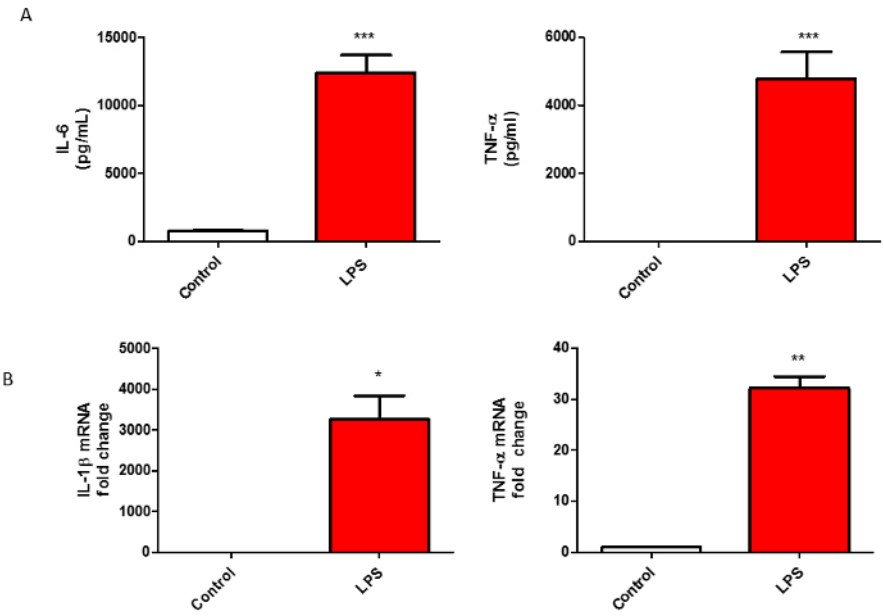

Figure 5: LPS-induced Pro-inflammatory Cytokine Release from Microglial Cells. (**A**) Luminex multiplex assay was performed on treatment medium collected from isolated microglial cells treated with 1 µg/mL LPS for 24 h. (**B**) q-RT-PCR for IL-1βand TNFα from isolated microglia treated with LPS for 24 h. The figures represent the mean ±SE from 2 or more independent experiments. Data were analyzed using Student's t-test (*p <0.05, **p <0.01, ***p <0.001). Please click here to view a larger version of this figure.

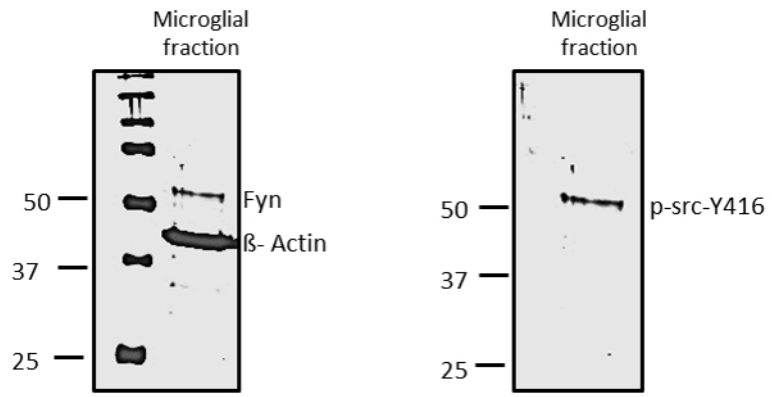

Figure 6: Isolated Microglia can be used for Signaling Studies. Western blot analysis shows that Fyn and p-Src-Y416 can be detected from microglia isolated with our newly refined method.

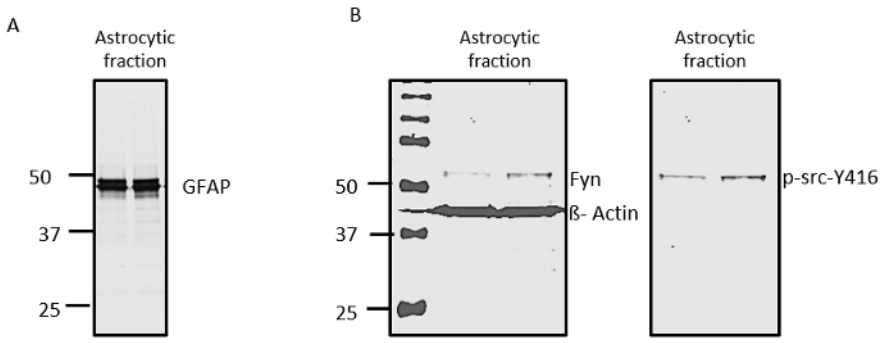

Figure 7: The Negative Fraction from the Microglial Separation Contains Astrocytes that can be used for Signaling Studies. (**A**) Western blotting shows that the negative fraction contains GFAP-positive cells**.** (**B**) Western blot analysis shows that Fyn and p-Src-Y416 can be detected from the GFAP-positive cells. Please click here to view a larger version of this figure.

Parameters	Current method	Modified method
Cost	$620	$620
Time	55 min	25 min
Fluorescence	Yes (red channel)	No
Purity	~97%	~99%

Table 1: Comparative Analysis between the Refined Microglial Isolation Method and the Original Method of Isolation.

Discussion

Older microglial isolation methods have limited recoveries that are not appropriate for various protein analyses by Western blot and RNA analyses by qRT-PCR. The differential adherence and mild trypsinization methods are two common approaches with low microglial yields[13,14,15]. The column-based CD11b approach also has low recovery, but achieves greater purity than differential adherence and mild trypsinization[13,14,15,16,17]. Our original column-free CD11b approach greatly improved the isolated yields, making it suitable for protein and RNA analyses such as Western blotting and qRT-PCR, but at the cost of decreased purity[15].

Our newly modified method retains the yields of the original method and increases the purity slightly from ~95-97% to ~>99%, but in half the completion time, which is critical for cell survival. Crucial steps within the protocol can assure optimal microglial isolation. While decapitating the pups, care should be taken to keep the heads on ice and to dissect in a laminar airflow chamber within 5-10 min. Prolonged brain excision times will make it difficult to obtain the full brain because it begins to lose its solidity at ambient temperatures, compromising the microglial yields. Notably, if step 2.9 is repeated at least once, there is an observable increase in purity and viability. If the yield from the separation is low, the separation can be repeated using fewer flasks per mL of recommended media, or by increasing the volume of separation (>1 mL). Decreasing cell crowding generally will improve purity, viability, and yield. The magnetic incubation time may be extended in step 2.12 to increase purity. This can assure proper magnetic binding of the microspheres that are attached to the microglia. Furthermore, additional magnetic incubations may be performed in step 2.9 for the sake of increasing purity and yield.

The feasibility of the protocol is equivalent to or better than the original CD11b method; however, the new method is refined and shorter. Another significant advantage gained from this refined method is the option of utilizing the red channel for fluorescence microscopy, which is occupied by PE in the original method (**Table 1**)[15]. Though we get high yield and purity of microglia with the method, the astrocytic yield obtained from this separation is less pure since it retains some fibroblasts. As described in step 2.17, after plating the astrocytes and incubating for 6 h, the growth media should be replaced with fresh media. Astrocytes are the first to attach to the flask, while much of the fibroblasts remain in suspension. The replacement of the media generally will remove the majority of the contaminating fibroblasts. Although this technique assures a high purity of astrocytes, a secondary purification method to achieve purer cultures is warranted.

Overall, this modified CD11b isolation method generates highly pure primary microglial cells and astrocytes in a short amount of time. The shorter isolation time generally improves cell survival rates. It provides a useful model system for elucidating the signaling mechanisms underlying both microglial and astroglial biology.

Disclosures

The authors declare that they have no competing financial interests.

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