Original Research *Feature article*

Development of a novel purification protocol to isolate and identify brain microglia

Deanna Doughty[1,2*], Surendra K Rajpurohit[3*], Amy Trang[1], Ahmet Alptekin[3], Ahmet K Korkaya[1], Bhagelu R Achyut[4], Ali S Arbab[3] and Jennifer W Bradford[1]

[1]Department of Biological Sciences, Augusta University, Augusta, GA 30912, USA; [2]D. D. is Medical College of Georgia, Augusta University, Augusta, GA 30912, USA; [3]Georgia Cancer Center, Augusta University, Augusta, GA 30912, USA; [4]Winship Cancer Institute, Emory University, Atlanta, GA 30322, USA
Corresponding author: Jennifer W Bradford. Email: jebradford@augusta.edu
*These authors contributed equally to this work.

Impact Statement

Here, we describe a novel method for isolating a highly pure population of primary microglia from mouse brain tissue and provide highly specific flow cytometry markers for accurate microglial identity. Microglia are being recognized as having crucial roles in normal brain development and in neurological disorders, and thus, having a method to isolate a highly pure population is important for a wide variety of studies. Historically, microglia have been very hard to work with and identify because they share many common markers with other macrophages. Here, we also provide specific markers to identify these cells from other macrophages. Our novel isolation technique and specific microglial markers should greatly aid in basic CNS biology studies and in CNS disease research.

Abstract

Microglia, the tissue-resident macrophage of the central nervous system (CNS), play a paramount role in brain health and disease status. Here, we describe a novel method for enriching and isolating primary microglia from mouse brain tissue. This isolation method yields a high number of cells from either young or adult mice, and importantly, maintains the health and function of the cells for subsequent cell culture. We also describe flow cytometry methods using novel cell surface markers, including CX3CR1 and Siglec-H, to specifically label microglia while avoiding other bone marrow and/or non-CNS derived macrophages and monocytes, which has been historically difficult to achieve. As microglia are crucial in multiple aspects of biology, such as in normal brain development/function, immune response, neurodegeneration, and cancer, this isolation technique could greatly benefit a wide range of studies in human CNS biology, health, and disease mechanisms. Being able to isolate a largely pure population of microglia could also allow for a more comprehensive understanding of their functional dynamics and role in disease mechanisms, advancement of potential biomarkers, and development of novel therapeutic targets to improve prognosis and quality of life in multiple diseases.

Keywords: Microglia, primary cell isolation, flow cytometry, primary cell culture

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Introduction

Microglia are the resident macrophage of the central nervous system (CNS) and have crucial roles in normal brain homeostasis, function, and immune response.[1] Microglial progenitor cells originate from the mesoderm and migrate from the yolk sac into all regions of the CNS.[2] During a resting state, microglia acquire a specific ramified morphological phenotype[3] and are the most sensitive cell sensors of brain function during both normal and disease states.[4] Microglia play key functional roles in the healthy brain, ranging from phagocytosis of dying neurons and CNS debris to modulating adaptability of animal behavior.[5] The role of microglia as phagocytes in normal and disease states has been studied since their discovery in the 1920s, but their specific contributions to neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, and in the development and progression of cancers, such as glioblastoma,[6] have recently garnered more acute attention. To better understand microglial function in normal and disease conditions, their isolation from brain tissue is essential. However, microglial identification and isolation are not trivial, as they are morphologically and functionally similar to other macrophages, such as those derived from bone marrow cells.[7] The specificity of microglia isolation becomes even more complicated during states of disease or stress, when peripheral blood macrophages (derived from bone marrow) can migrate to the brain in large numbers.[6]

Here, we describe a method that utilizes both mechanical and enzymatic methods of neural tissue dissociation using a

commercially available kit and a Percoll gradient. There are currently several published methods describing microglial isolation; however, these protocols are often cumbersome and/or fail to confirm microglial purity using microglial-specific markers.[8–11] For example, Magnetic-Activated Cell Sorting (MACS) is widely used to isolate immune cells, including microglia.[12] However, in our hands, Miltenyi's popular MACS kits often took an entire day, often up to 12 h, and resulted in very few cells per brain, even after multiple rounds of optimization. It has also been recently demonstrated that T cells isolated using MACS positive selection techniques can retain magnetic properties for extended periods of time, which could significantly alter downstream experiments.[13] Other immune cell types, including microglia, are also likely to retain magnetic properties following MACS. Fluorescence-Activated Cell Sorting (FACS) is another isolation method that can increase cell purity through the use of select markers and can significantly decrease procedure time. However, FACS can activate the isolated microglia, which could render them unusable for downstream experiments involving gene expression analysis or other *in vitro* studies.[14] We found that CNS cells isolated by FACS displayed activated morphology when placed in cell culture, and most cells did not survive long after plating. The lack of microglia viability from multiple published methods, including MACS and FACS, is supported by the literature.[15] Due to the difficulty in isolating a pure population of viable microglia, we have developed a robust microglia isolation protocol capable of efficiently isolating the microglial population with high specificity and viability in cell culture.

Another issue with many available protocols is the widespread use of pan-macrophage markers to determine the purity of the isolated population. Recent data have shown that commonly used microglial surface markers, such as CD11b and CD45, can vary in expression based on activation and disease state,[16] casting doubt on the accuracy of cell purity numbers previously reported in the literature for commonly used isolation techniques. To overcome this, we utilized the more recently identified markers CX3CR1 and Siglec-H to determine our protocol's efficiency in specifically isolating the microglial population. Here, we provide a detailed, comprehensive protocol to isolate primary microglia from mouse neural tissue, which involves the following steps: Mouse Brain Harvest and Enzymatic/Mechanical Dissociation, Microglial Isolation, and Cell Culture of Primary Isolated Microglia. We also provide flow cytometry analysis using microglial-specific antibodies and fluorescent staining for microglia identification. As this protocol has been designed for isolating microglia that can be used immediately or that can live in cell culture for downstream experiments, we are confident that this newly developed protocol will be very useful for a variety of applications in studying microglia function during both normal and disease conditions.

Materials and methods

Animals

C57BL/6 wild-type animals were used for brain tissue harvest. Juvenile (~4 weeks) and adult mice (~8 weeks or older) were used for protocol design and development purposes. All experiments were performed according to the National Institutes of Health guidelines and regulations. The Augusta University Institutional Animal Care and Use Committee approved all animal experimental protocols (#2014-0691). Animals were housed in a barrier facility with a 12-h light–dark cycle. Food and water were offered *ad libitum*. Euthanasia was conducted prior to tissue harvest as specified by approved Institutional Animal Care and Use Committee (IACUC) protocols.

Table 1. Enzyme and buffer amounts for two juvenile mouse brains (< 1 month old) or one adult brain, lacking cerebellum.

Enzyme mix 1	Enzyme mix 2
50 µL enzyme P (keep –20)	20 µL buffer Y
1900 µL buffer X	10 µL enzyme A (keep –20)

For more than one adult brain or more than two juvenile brains per C tube, scale up the enzyme mixes. Do not exceed the recommended tissue amount for each C tube.

Isolation of primary mouse brain microglia

Mouse brain harvest and enzymatic/mechanical dissociation. Prior to harvesting the brain, perform euthanasia according to IACUC-approved protocol. Dissect the mouse through the head region and remove the brain. For this protocol, the cerebellum and brain stem are typically removed, as these regions generally have a smaller microglial population as compared to the rest of the brain.[17] Move the brain to a labeled petri dish (Corning 430167) with cold Dulbecco's phosphate-buffered saline (DPBS; Gibco #14-287-072) and keep on ice. Prepare Miltenyi Enzyme Mix 1 and 2 (from Neural Tissue Dissociation Kit (P) Miltenyi #130-092-628) in separate tubes according to Table 1. Add Enzyme Mix 1 to a labeled Miltenyi C tube (Miltenyi #130-093-401). Drain DPBS from the petri dish, blot with a Kimwipe, and chop the brain into small pieces with a new razor blade. Add the brain tissue to the C tube along with Enzyme Mix 2. Secure the C tube cap well and invert the tube so that all of the tissue is in the lid of the tube; swirl contents if needed. Insert the C tube with the cap down into the machine and add the heat sleeve. Select and run gentleMACS Octo Dissociator with Heaters program 37C_ABDK_01 (Miltenyi #130-096-427).

Microglial isolation. If cells are to be subsequently cultured, perform the remainder of the procedure under sterile conditions in a biological safety cabinet. After the gentleMACS Octo Dissociator with Heaters program 37C_ABDK_01 finishes, divide the homogenate from one C tube into two 10 mL Potter-Elvehjem tissue grinders (Capital Scientific #T7475-5) and add enough cold Roswell Park Memorial Institute (RPMI) 1640 (Corning #10040CV) to each grinder to make a total volume (homogenized brain tissue + RPMI) of 5 mL. For example, if you have 4 mL of tissue homogenate in the C tube, 2 mL will be added to each of two tissue grinders along with 3 mL of RPMI for a total volume of 5 mL in each grinder. The C tubes can be rinsed with the cold RPMI to capture any remaining tissue. Note, each C tube should be

Table 2. SIP and 70% Percoll solutions.

Stock Isotonic Percoll (SIP)	70% Percoll
9 mL Percoll	3.5 mL SIP
1 mL 10x HBSS	1.5 mL 1x HBSS

SIP: stock isotonic percoll; HBSS: Hank's balanced salt solution. The amount in the chart will give you enough volume for two grinders, plus a little extra. Percoll solutions should be room temperature before using. If making ahead of the procedure, keep at 4°C until the day of the experiment. If culturing cells following isolation, all solutions must be kept sterile.

processed individually – do not combine cell homogenate until instructions specify. Use ~6 strokes of the pestle to obtain a homogenized cell suspension. This additional homogenization step will greatly increase cell yield. Transfer the homogenate from one grinder to a 70 µM cell strainer (Fisher #22-363-548) placed on a new 50 mL tube (Fisher 12-565-271). Wash the 70 µM cell strainer with 2 mL cold RPMI. Total volume should now be 7 mL in each 50 mL tube. Keep tubes on ice.

Prepare the two Percoll (Sigma #P4937) solutions according to Table 2 with 1x HBSS (Gibco #14175095) or 10x Hank's balanced salt solution (HBSS) (Sigma #H4641) and adjust volumes based on how many tissue grinders you are using. Add 3 mL Percoll Stock Isotonic Percoll (SIP) into the 7-mL-filtered cell suspension, and gently invert five times to mix. Add 2 mL of 70% Percoll into a new, labeled 15 mL tube (Fisher 12-565-269). Carefully overlay the 10 mL SIP Percoll/cell suspension mixture onto the 2 mL of 70% Percoll. Critical Step: add the 10 mL cell suspension to the 70% Percoll dropwise to the side of the tube so the two layers do not mix. You can do this slowly with an electric pipetman. Centrifuge samples at 600 × g for 35 min at 10°C. Set centrifuge for very low acceleration (i.e.: Level 1), completely remove the brake, and set the timer to start once speed is reached.

Following centrifugation, remove the top "fatty" raft layer by vacuum aspiration, then slowly remove the layer above the cell "halo" until about 1 mL of liquid above this halo remains. See Figure 4. Aspirate carefully so as not to remove the microglial fraction in the halo. Use a 1 mL pipette to remove the cell halo (about 2–2.5 mL) into a new, labeled 15 mL tube. Combine the two divided parts of each sample into the same 15 mL tube at this step. Centrifuge the cells 7 min at 900 × g at 4°C to pellet cells, while ensuring the brake and acceleration are reset to full break and acceleration. Aspirate the supernatant, resuspend the cells in desired media, count cells, and proceed with cell processing for desired endpoints (see below for cell culture and flow cytometry procedures). If red blood cells are still present, add 1 mL prepared 1x Red Blood Cell Lysis Buffer (Miltenyi #130-094-183) to the cell pellet and pipet to mix. Incubate cells in the refrigerator with the RBC lysis buffer for no longer than 5 min. Fill the tube to 14 mL with media, inverting to mix. Centrifuge for 10 min at 900 × g at 4°C to pellet cells and proceed to cell counting and desired endpoint experiments.

Cell culture of primary isolated microglia

All procedures must be performed under sterile conditions in a biological safety cabinet. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. Cell culture dishes were pre-coated with poly-D-lysine. To coat the plates, 10 mg poly-D-Lysine (Sigma P7886) was resuspended in 100 mL tissue culture grade water according to the "Cell Culture" directions from the manufacturer. 75 µL of the resuspended poly-D-lysine was transferred to the wells of a 24-well cell culture dish and the plate was gently rocked by hand for 5 min to evenly coat the wells. Any remaining poly-D-lysine solution was aspirated, and the wells were washed with tissue culture grade water. The wash water was then aspirated, and the plates were allowed to dry completely, at least 30 min, inside the sterile biological safety cabinet.

Following the final spin and cell counting of the Microglial Isolation Procedure above, 2.5×10^5 cells in a volume of 150 µL warm media per well were plated in the coated wells. Media recipe: Neurobasal Media (Gibco/Fisher 21-103-049)/ 2% B27 (Gibco/Fisher 17-504-044)/ 1% GlutaMAX (Gibco/Fisher 35-050-061)/ 1% Penicillin-Streptomycin (ATCC 30-2300). For best results, gently pipette cells into the center of the well and allow them to settle in the hood at room temperature for 30 min. After this 30-min period, fill wells to 1 mL (for a 24-well plate) with warm media and place in culture incubator. After 24 h, gently pipette out 500 µL of media from each well without disturbing the settled cells and replace with 500 µL fresh, warmed full neurobasal media.

Comparison of various microglial isolation methods

Microglia were isolated using a variety of common methods. For MACS isolation, the Neural Tissue Dissociation Kit (P) (Miltenyi 130-092-628) was used and was followed by the MojoSort Mouse CX3CR1 Selection Kit (480056 BioLegend); all manufacturer directions were followed. For FACS, tissue was homogenized and filtered through a 70 µm cell strainer into a single cell suspension and blocked for 10 min at room temperature with FcR blocker (STEMCELL Technologies #18730). The samples were then centrifuged at 1200 rpm for 5 min, and the supernatant was aspirated. The cell pellet was resuspended in 100 µL of DPBS, and 1.5 µL of each antibody (CX3CR1 APC [Biolegend 149008], CD11b Brilliant Violet 421 [Biolegend 101235]) was added. The samples were vortexed and incubated at room temperature for 30 min in the dark. Then 200 µL PBS was added to each sample, and cells were sorted by a BD Biosciences FACSAria II SORP. Two different Percoll gradients were also used in the comparison experiment. Prior to Percoll gradient centrifugation, brain tissue was homogenized using a 10-mL Potter-Elvehjem tissue grinder. The homogenate was filtered with a 70 µm cell strainer, and the cell suspension was subjected to Percoll gradient centrifugation. The methods for the 70%/37%/30% gradient[18] and the 70/30% gradient[19] have been previously published.

Post isolation, cells were grown for 24 h in cell culture dishes that were pre-coated with poly-D-lysine, in full neurobasal media, according to the *Cell Culture of Primary Isolated Microglia* protocol, as described above. Isolated cells were counted using a hemocytometer and Trypan Blue cell viability dye (Corning 25900CI), except following FACS, in which

Table 3. Microglial labeling components for flow cytometry.

	Negative control	Single-color control samples							Test samples (Exp 1)	Test samples (Exp 2)
	Negative control	CX3CR1 control	CD11b control	CD45 control	CD49d control	Siglec-H control	F4/80 control	7AAD control	Test samples	Test samples
	No color	APC	Brilliant Violet 421	FITC	PE	PE	FITC	7AAD	APC, BV, FITC, PE, 7AAD	APC, PE, FITC, 7AAD
Isolated cell sample (µL)	200	200	200	200	200	200	200	200	200	200
CX3CR1 APC (µL)	0	**2**	0	0	0	0	0	0	**2**	**2**
CD11b BV 421 (µL)	0	0	**2**	0	0	0	0	0	**2**	0
CD45 FITC (µL)	0	0	0	**2**	0	0	0	0	**2**	0
CD49d PE (µL)	0	0	0	0	**2**	0	0	0	**2**	0
Siglec-H PE (µL)	0	0	0	0	0	**2**	0	0	0	**2**
F4/80 FITC (µL)	0	0	0	0	**0**	0	**2**	0	0	**2**
7AAD	0	0	0	0	0	0	0	**2 µL** at time of analysis	**2 µL** at time of analysis	**2 µL** at time of analysis

7AAD: 7-aminoactinomycin D. Tube contents for microglial staining for analysis by flow cytometry.

the cells were counted by the BD Biosciences FACSAria II SORP. Brains were harvested from mice aged 4 months and younger. Each method was performed in triplicate with the exception of the 70%/30% Percoll gradient, which was performed in duplicate.

Flow cytometry characterization of isolated brain cells

Mouse microglia were isolated according to the directions of our novel procedure above. 1.5 mL microcentrifuge tubes were labeled and components added according to Table 3. Components were mixed briefly by vortexing, and tubes were covered with foil and incubated for 45 min at 4°C. Samples were centrifuged for 5 min at 500 × g at 4°C; the cell pellet should be visible in the bottom of the tube after spinning. The majority of the supernatant was discarded with the exception of ~90 µL, so as not to disturb the cell pellet. The cell pellet was then gently resuspended by pipetting with the remaining ~90 µL of supernatant. The tubes were covered with foil and incubated for at least 1 h at 4°C (the samples can be stored overnight in the refrigerator at this point, if needed). The samples were then incubated for 45 min at room temperature and covered with foil. 400 µL 1x PBS/2 mM EDTA (Corning Cellgro# 21-031-CV) was then added into all samples and mixed well by pipetting; 200 µL 0.5M EDTA was added to 45 mL of 1x PBS to make the working solution. Then 2 µL of 7-aminoactinomycin D (7AAD) was added to the appropriate samples and incubated for 10 min (see Table 3). Samples were mixed well prior to the start of analysis. A BD Biosciences FACSAria II SORP Cell Sorter (4-laser, 18-color system) and a BD Biosciences LSRII were operated by the Georgia Cancer Center Flow Cytometry Core Facility at Augusta University. BD Biosciences FACSDiva v8.0.1 software was used to analyze the data. Antibodies used for cell labeling included CX3CR1 APC (BioLegend 149008), CD11b Brilliant Violet 421 (BioLegend 101235), CD45 FITC (BioLegend 103108), CD49d PE (BioLegend 103607), Siglec-H PE (BioLegend 129605), F4/80 FITC (BioLegend 123107), and 7AAD (BioLegend 420403).

Immunocytochemistry staining of isolated brain cells

Microglia isolated using the procedure described above were seeded at a concentration of 0.5×10^6–1×10^6 cells/well onto poly-D-Lysine (Sigma P7886)-coated (described above) glass coverslips that had been placed in 6-well cell culture plates (Corning/Fisher 353046). 24-h post isolation, once the cells had adhered to the coverslips, the cell culture media (see recipe above in "Cell Culture" section) was aspirated and cells were washed with 1x PBS. The wash buffer was aspirated, and cells were blocked in blocking buffer (1x PBS/1% BSA) for 60 min at room temperature. The blocking buffer was then aspirated and the Alexa Fluor 488 anti-mouse/human CD11b antibody (M1/70 clone) (BioLegend #101217) was diluted to 2 µg per mL in antibody dilution buffer (1x PBS/ 1% BSA). The cells were incubated in primary antibody overnight at 4°C, with the plates covered with foil. Following incubation with the primary antibody, the wells were washed 3x with 1x PBS at RT for 5 min each. Plates were kept covered with foil during washing. Following washing, the cover slips were mounted on glass slides with Prolong Gold Mounting media with DAPI (CST #8961). Slides were kept in the dark and allowed to cure overnight at room temperature. Slides were imaged using a Zeiss Axiovert 200 fluorescent microscope with Axiocam 305 Color Camera and a 488 nm laser. Images were analyzed using Zen 3.0 Blue Edition imaging software.

Phagocytosis assay

Microglia were isolated and seeded onto poly-D-Lysine-coated plates in complete neurobasal media, according to the procedures above. Mouse embryonic fibroblast cells were plated in a 24-well plate with Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS). Following overnight culture and cell adherence, the growth medium was removed and cells were washed with PBS. Cells were then stained with Carboxyfluorescein succinimidyl ester (CFSE) (ThermoFisher, C34554) according to the manufacturer's protocol. After staining, the CFSE was removed

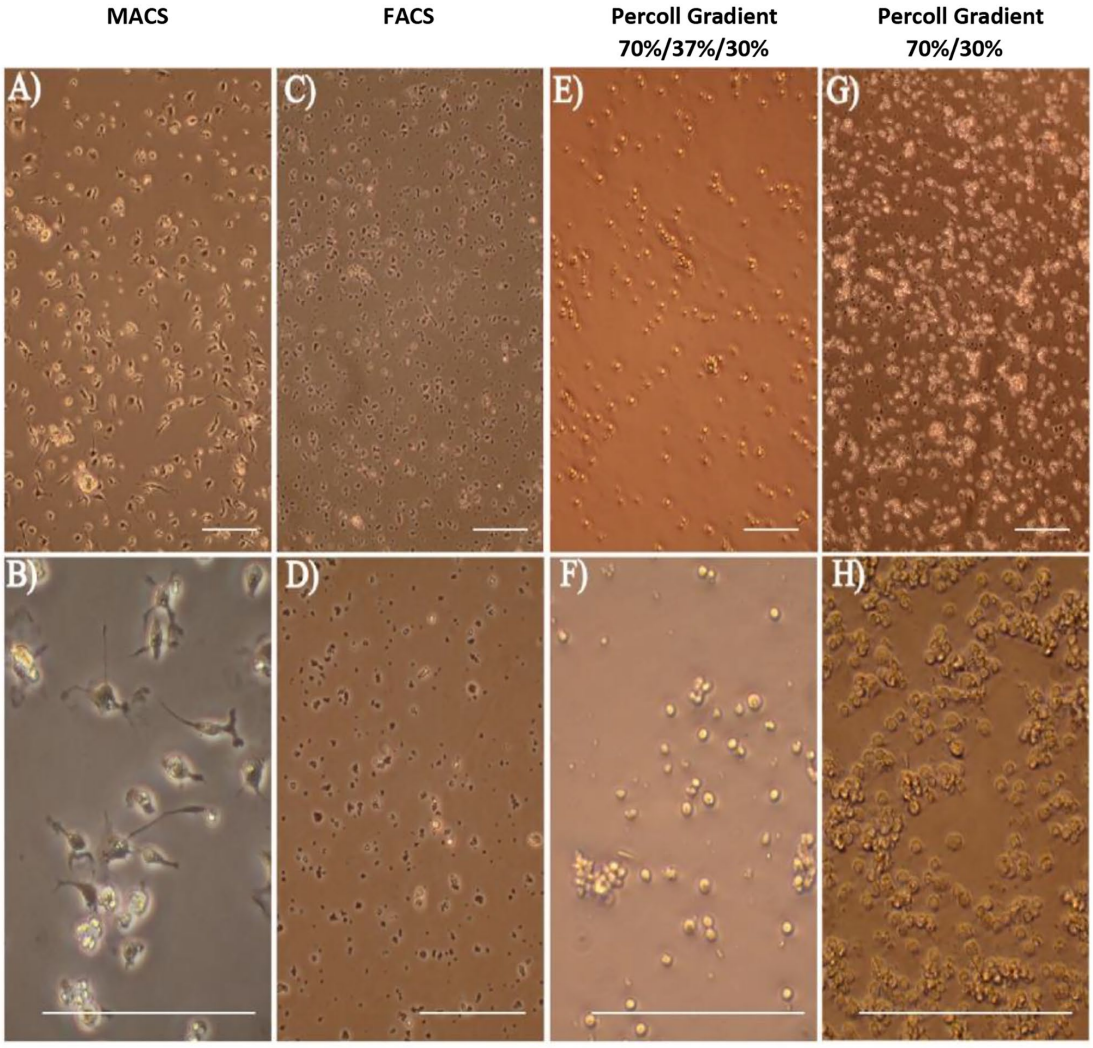

Figure 1. Cells isolated using common microglial isolation methods failed to robustly survive in culture. Isolated cells grown in cell culture for 24 h. Microglia isolated using several different methods were grown on poly-D-lysine-coated plates for 24 h, according to the described protocols in the "Methods" section. (A–B) Cells isolated using MACS with CX3CR1 beads. Images taken with (A) 10x objective lens and (B) 40x lens. (C–D) Cells isolated using FACS with anti-CD11b/CX3CR1 antibodies. Images taken at (C) 10x and (D) 20x. (E–F) Cells isolated using a 70%/37%/30% Percoll gradient. Images taken at (E) 10x and (F) 40x. (G–H) Cells isolated using 70%/30% Percoll gradient. Images taken at (G) 10x and (H) 40x. Images were taken with a Motic AE2000 inverted light microscope outfitted with a Canon EOS DSLR camera. Scale bars = 50 µm. (A color version of this figure is available in the online journal.)

and the culture medium was added. To detect phagocytosis, 2 µL of FluoSpheres™ Carboxylate-Modified Microspheres red fluorescent beads with a 2.0 µm size (ThermoFisher #F8826) were added. Microglial phagocytosis was tested in the presence of lipopolysaccharide (10 ng/mL). After 3 h of incubation with beads, microglial cells and mouse embryonic fibroblasts were fixed with 3% formalin and washed with PBS. Images were acquired using Keyence fluorescence microscopy. Images were obtained using Z-stack mode (10–12 images from above to below the cells) and reconstructed using two to three images through the central part of the cells.

Results

Issues with established microglial isolation techniques

To answer several research questions, it became imperative for our group to be able to work with a highly pure population of microglia isolated from mouse brain. Thus, we initially tried published protocols and other proprietary kits to obtain a pure population of primary microglia. Microglia were isolated by MACS, FACS, and two different Percoll gradient methods. Microglia were isolated using MACS with the microglial-specific antibody, CX3CR1. Although the cells isolated using MACS showed a ramified morphology (Figure 1[A] and [B]), which is an indication of them being in a "resting" or inactivated state,[20,21] this method yielded the fewest cells of the methods we tried, which averaged around 3.3×10^5 cells per brain (Figure 2). It is also important to note that even though the cells showed a ramified morphology, cells isolated by positive selection MACS techniques can retain magnetic properties which may alter downstream experimental results.[13]

FACS, a commonly used method to isolate CNS cells, including microglia, was also attempted. FACS is popular due to shorter tissue processing time and the potential to

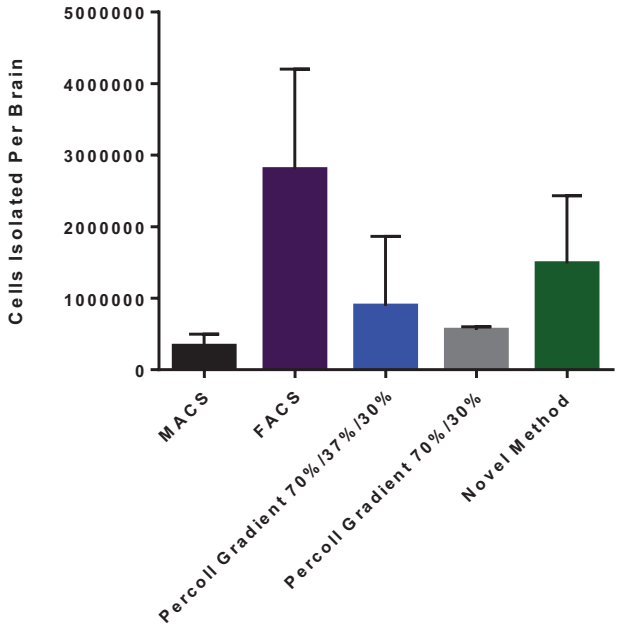

Figure 2. Numbers of cells isolated using common microglial isolation methods. Microglia were isolated by MACS with CX3CR1 beads (black bar), FACS with anti-CD11b/CX3CR1 antibodies (purple bar), a 70%/37%/30% Percoll gradient only (blue bar), a 70%/30% Percoll gradient only (gray bar), or the novel isolation method described in this article (green bar). (A color version of this figure is available in the online journal.)

obtain a more highly pure cell population than other techniques, such as a Percoll gradient only, but the actual cell sort can take a very long time.[22] Microglia were isolated by FACS using CX3CR1 and CD11b antibodies. A large number of CD11b + /CX3CR1 + cells were isolated, which averaged 2.8×10^6 cells per brain (Figure 2). However, very few cells survived after plating, and mostly dead cells and debris remained 24 h after isolation (Figure 1[C] and [D]).

As Percoll gradients are also commonly used to isolate microglia, we tried two different methods in our attempt to isolate a pure microglial population: a 70%/37%/30% gradient and a 70%/30% gradient. The 70%/37%/30% gradient yielded a good number of microglia, which averaged 8.9×10^5 cells per brain (Figure 2). However, 24 h after isolation, the cells that lived exhibited a very rounded morphology in culture, which is indicative of them being in a reactive or activated state[20,21] (Figure 1[E] and [F]). The 70%/30% Percoll gradient method yielded an average of 5.6×10^5 cells per brain (Figure 2). Fewer cells were isolated compared to the 70%/37%/30% gradient method; however, 24 h after isolation, more cells were still alive, albeit in a rounded morphology (Figure 1[G] and [H]).

Production of a novel microglial isolation protocol

As we were unable to find a microglial isolation protocol that worked for our needs, we decided to develop our own. The protocol we established uses the Miltenyi Neural Tissue Dissociation Kit (P) followed by manual tissue dissociation and a Percoll density gradient separation step. Figure 3 gives an overview of the main isolation steps, which are described in great detail in the "Methods" section. For protocol design and development purposes, C57BL/6 wild-type animals were used for brain tissue harvest, and cells were successfully isolated from juvenile and adult mice. We consistently found that between 6.5×10^5 and 2×10^6, microglia can be expected from juvenile mouse brains (< 1 month old), and between 4×10^5 and 8×10^5, microglia can be expected from one older mouse brain (> 1 month old). Younger animals typically yielded more cells than older adults and are recommended for this process.

The Percoll gradient is an important step in the microglial isolation process, as this technique can effectively remove myelin and other debris and separate cells based on size.[19] To help identify the microglial layer following centrifugation, we have provided a picture of the tube at this step (Figure 4). The microglial layer is a subtle, gray-white layer located at the interface of the lighter pink 30% Percoll layer and the darker pink 70% Percoll layer. This can be seen at the arrow in Figure 4. Like some other methods, our novel method also uses a 70%/30% Percoll gradient, but the tissue homogenization step prior to the gradient is drastically different, which might greatly aid in cell survival. Our novel method yielded an average of 1.49×10^6 cells per brain (Figure 2). Importantly, 24 h post isolation, the cells had a more ramified morphology and cell survival was better than the other methods tested (Figure 5). When compared to several different methods of microglial isolation, our novel method yielded a comparable, if not higher, number of microglia, which had good survival and morphology in cell culture following isolation.

Cell culture of isolated microglia

We found that the isolated microglia grew best on poly-D-lysine-coated cell culture plates in neurobasal media containing 2% B27 supplement, 1% GlutaMAX, and 1% Penicillin-Streptomycin. Gently resuspending the cells by pipetting in a small amount of media, pipetting them into the center of the coated plate/well, and allowing them to settle in the hood at room temperature for 30 min increased cell survival. The remaining media appropriate for the size of the well/plate can be added after the 30-min period. We also found that replacing half of the media from each well 24 h after plating instead of replacing all of the media resulted in better cell survival. Figure 5 shows images of cells taken 24 h after isolation using our novel method and culture. Immunofluorescent staining of isolated microglial cells was performed to gain a general sense of cell purity. Isolated microglia were grown in cell culture on poly-D-lysine-coated coverslips and stained with Alexa Fluor 488 anti-mouse/human CD11b antibody. The coverslips were mounted onto slides with DAPI Prolong Gold. CD11b is a pan macrophage/monocyte marker and is frequently used for microglial staining purposes. As can be seen in Figure 6, the isolated microglia have positive CD11b staining. As the microglia were isolated from non-disease mice, it is not expected that significant numbers of peripheral blood-derived macrophages would be in the brain.

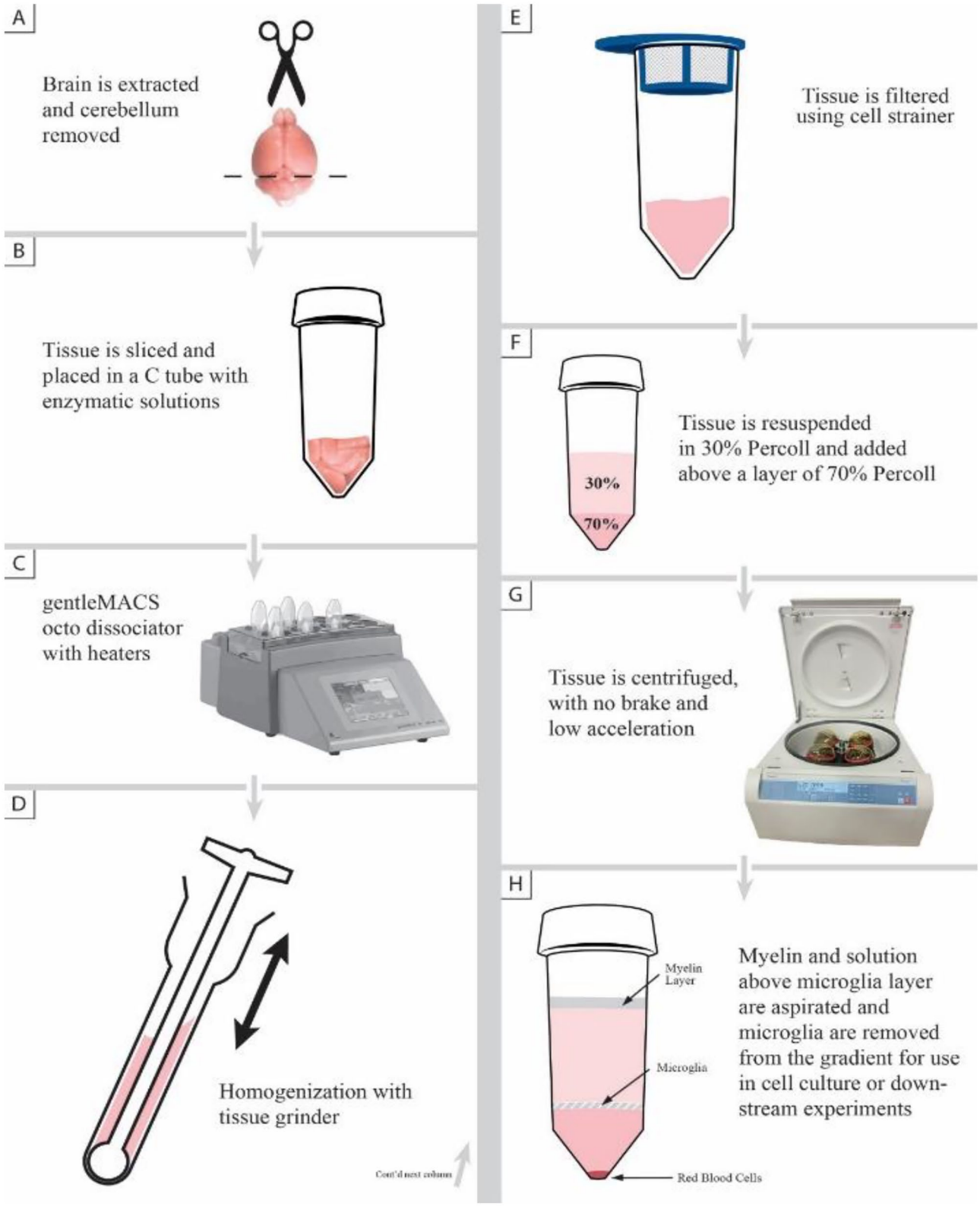

Figure 3. Schematic representation of isolation.
Overview of the major steps of the microglial isolation protocol, including: brain tissue harvest and homogenization, Percoll gradient centrifugation, and microglial enrichment. Images in panels C and G were modified from Miltenyi Biotec Inc. and Marshal Scientific, respectively. (A color version of this figure is available in the online journal.)

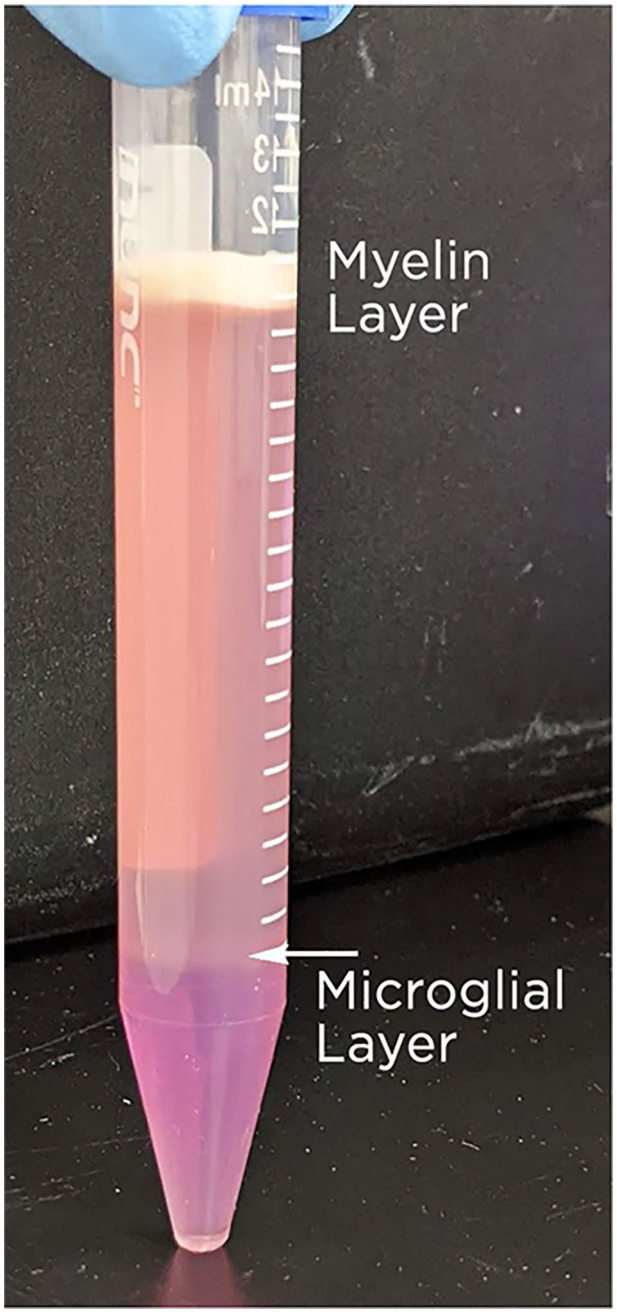

Figure 4. Gradient layers.
Picture shows the various layers produced following centrifugation of the homogenized brain sample mixed with Percoll. The microglial layer is a subtle, gray-white layer located at the interface of the lighter pink 30% Percoll layer and the darker pink 70% Percoll layer. (A color version of this figure is available in the online journal.)

Phagocytic activity of isolated microglia

To test the functionality of microglia isolated using our novel method, we performed a phagocytosis assay. As can be clearly seen in Figure 7, microglia isolated using our novel technique are efficiently able to phagocytose multiple fluorescent particles following 3 h of exposure, whereas mouse embryonic fibroblasts could not. This functional assay demonstrates that microglia isolated using this novel protocol maintain their ability to perform normal immune roles, such as phagocytosis of foreign particles.

Flow cytometry analysis of isolated microglia

Flow cytometry analysis was used to help assess the purity of our isolated microglia. Our microglial marker selection criteria came from multiple sources[6,23–26] and are summarized in Table 4. We first stained our isolated cells with commonly used markers to identify microglia, $CD11b^{+}/CD45^{-/low}$. In our isolated microglial population, we had a very low rate of CD45 positive staining (2–11.2%), which was to be expected, as CD45 highly labels blood leukocytes and not CNS leukocytes[23] (Table 5). The range can be potentially explained by the fact that microglia can upregulate CD45 expression when activated.[6,25]

However, the number of isolated cells that stained positive for CD11b was lower than we were anticipating (19.1–25.3 % in two different pooled samples) (Table 5), considering the robust CD11b immunofluorescent staining of our isolated microglial population (Figure 6). Overall, only 21.5–28% of the isolated cells were $CD11b^{+}CD45^{-/low}$. CD49d is a lymphocyte marker and was used as an internal, negative control (Table 5). While $CD11b^{+}CD45^{-/low}$ markers have been used extensively in the literature to identify microglia using flow cytometry, more recent studies have highlighted a growing number of issues with using just these markers alone, including lack of microglial specificity and ability to alter expression status depending on cell activation. Therefore, we examined our isolated cells for CX3CR1 expression and found 83.9–85.8% positivity (Figure 8 and Table 5). All $CD11b^{+}$ cells were also $CX3CR1^{+}$ (Figure 8, Table 5, Supplementary Figure 1). To confirm the high CX3CR1 staining and low CD11b staining of our isolated cells, we performed another microglial cell isolation and stained the cells with just these two markers. This second round of flow cytometry data, also consisting of three pooled brains, showed an even higher level of CX3CR1 positivity (92.5%), and CD11b positivity (57%) was more than double of what was seen previously (Figure 9, Table 6, and Supplementary Figure 2).

Due to the variability in the CD11b flow results, we decided to examine cell surface markers more recently identified as microglial-specific and less likely to label other types of macrophages as well. These markers included CX3CR1 along with Siglec-H and F4/80. Analysis of our isolated microglial population shows these cells are again, highly positive for CX3CR1, with positivity rates around 92% (Figure 10, Table 7, and Supplementary Figure 3). Even more striking is the positivity rate for the marker Siglec-H, which stained around 98% of the isolated cells (Figure 10, Table 7, and Supplementary Figure 3). Positivity rate for F4/80 was roughly 77% (Table 7). See the Supplementary Materials section for all flow cytometry histograms.

Discussion

Microglia are being increasingly recognized as essential in normal CNS function and as key cells altered in many types of neurological diseases. Microglia are easily distinguished

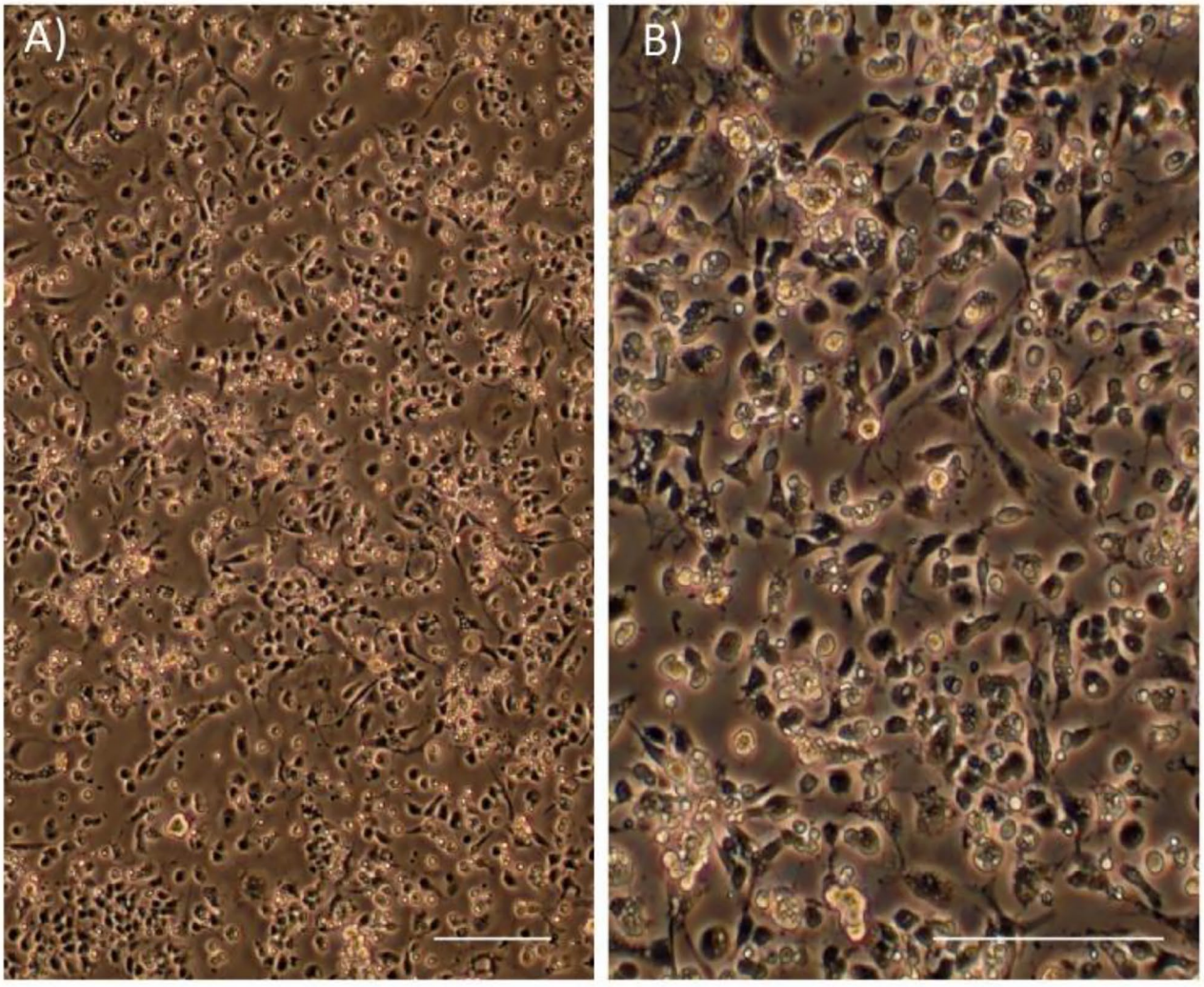

Figure 5. Cultured microglia from novel method. (A–B) Cells isolated using the novel isolation method described in this article. Images taken at (A) 10x and (B) 20x. Images were taken with a Motic AE2000 inverted light microscope outfitted with a Canon EOS DSLR camera. Scale bars = 50 µm. (A color version of this figure is available in the online journal.)

from other brain cells, such as neurons and other glia, but can be very hard to differentiate from other types of macrophages. Thus, the isolation and purification of microglia is a challenging task for the scientific community. Rigorous identification criteria must be developed to ensure the proper identity of the microglial populations in normal and pathological brains. The CNS houses two broad types of tissue-specific resident macrophages: microglia (parenchyma – an immune privilege compartment of the brain) and non-microglia macrophages called border associated macrophages (BMS). Microglia are the specialized brain-resident macrophages which are seeded into the brain during embryogenesis.[28–30] Bone marrow-derived blood leukocytes are recruited from the periphery and travel to the CNS through the vascular system, and can be labeled by CD45 markers. Microglia, however, are found in all regions of the brain and spinal cord and comprise 10–15% of all CNS cells.[1] Of the CNS leukocytes, 70–80% are microglia, while other populations of note consist of BMS (8.5–10.5%), neutrophils (2.4–4.0%), and monocytes (2%), with lymphocytes making up the remainder of the leukocytes.[23] There have been several protocols developed over the past decade to isolate primary microglia.[8,10,11,14,15,18,31–33] Protocols can be found to isolate microglia from neonatal or adult mice and utilize a variety of methods, including the use of proprietary kits, magnetic bead separation, and Percoll gradients. None of the protocols we found in the literature determine the purity of their microglial population using the latest microglial-specific markers, which can distinguish them from other types of macrophages.

Here, we describe a novel purification protocol to isolate and enrich for primary microglia and have described the protocol in a stepwise fashion, including methods of brain harvesting and microglial cell isolation. We also describe methods for cell culture, cell labeling, and importantly, use of more precise microglial markers for flow cytometry. We sought to develop a protocol to isolate brain microglia in a manner that maintained their normal immune function, such as phagocytosis, so these cells could be cultured for downstream experiments. We tried many different combinations

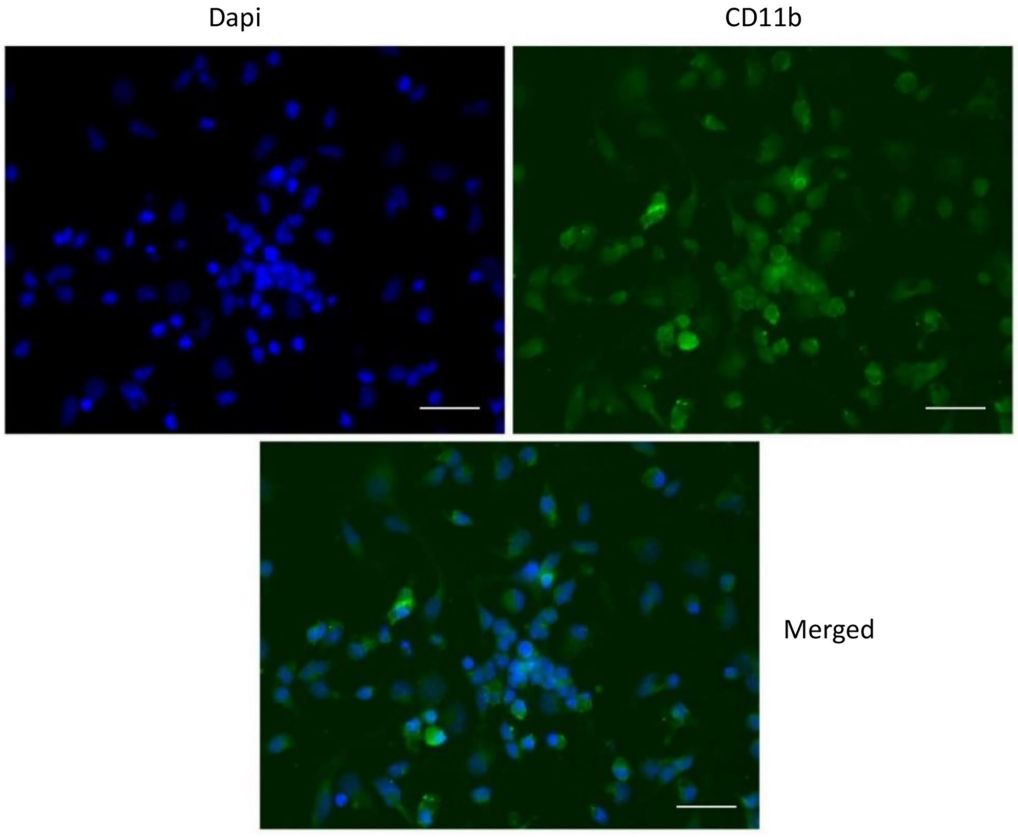

Figure 6. Immunofluorescent staining of isolated microglial cells.
Isolated microglia were grown in cell culture and stained with CD11b antibody and mounted with DAPI Prolong Gold. Scale bars = 50 µm. (A color version of this figure is available in the online journal.)

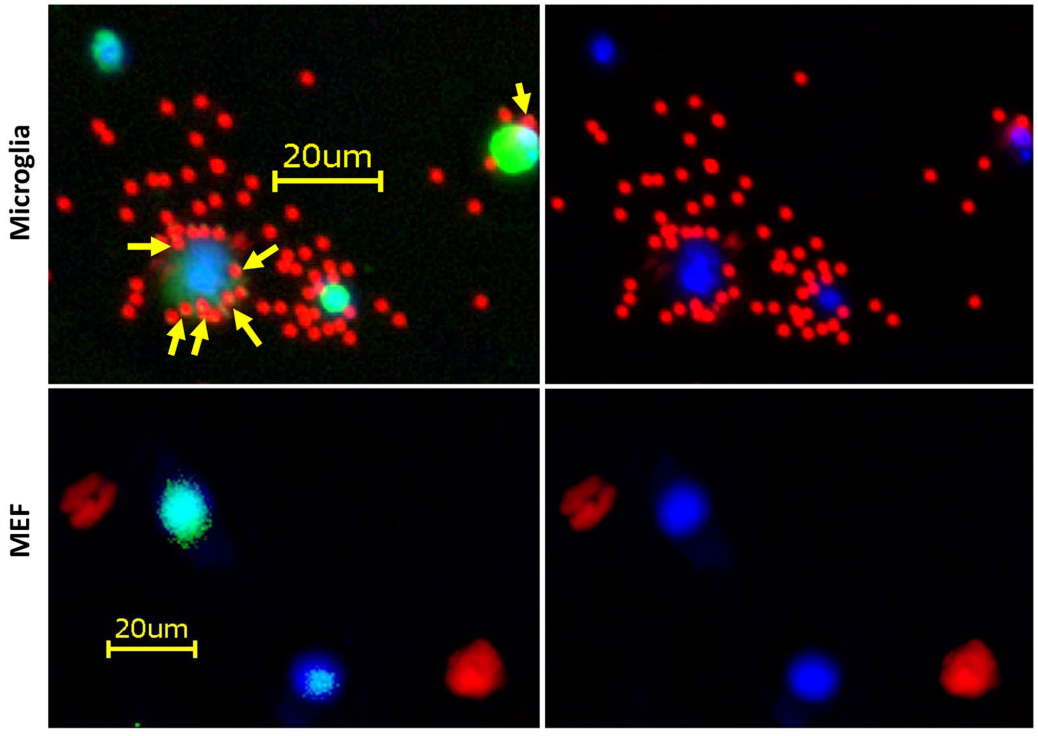

Figure 7. Phagocytosis assay using isolated microglial cells.
Isolated microglia (top panels) and mouse embryonic fibroblasts (bottom panels) were grown in cell culture and incubated with fluorescent particles for 3 h following LPS treatment. Yellow arrows point to examples of engulfed red-fluorescent particles. Images were obtained using Z-stack mode (10–12 images from above to below the cells) and reconstructed using two to three images through the central part of the cells. Left panel shows cytoplasm (CSFE staining, fluorescein), micro beads (red), and nucleus (DAPI). Right panel shows only beads and nucleus. Scale bars = 20 µm. (A color version of this figure is available in the online journal.)

of manual and enzymatic homogenization steps, with and without commonly used proprietary cell separation kits and magnetic bead separation kits. In our hands, cell isolation kits and magnetic beads kits yielded few cells, most of which were unable to robustly survive in culture, and/or did not yield a homogeneous population of microglia (Figures 1 and 2). Much of these effects may be due to inadvertent activation of the cells during the isolation process. The protocol we established uses the Miltenyi Neural Tissue Dissociation Kit (P), followed by manual tissue dissociation and Percoll density gradient separation. We found that use of a kit containing needed enzymes and buffers reduced the amount of reagent preparation often associated with other published protocols. Our protocol also does not require animal perfusion. These two aspects are particularly welcome in a laboratory, such as ours, which has a large number of undergraduate researchers. This protocol can be used with juvenile and adult mice, although younger mice did yield larger numbers of cells (between 6.5×10^5 and 2.3×10^6 microglia from juvenile mouse brains (<1 month old), and between 4×10^5 and 8×10^5 microglia from adult brains.

We first stained our isolated cells with commonly used markers, such as CD11b and CD45. These two markers have been used extensively in the literature to identity microglia, but more recent studies have highlighted a growing number of issues with using only these two cell surface markers for flow cytometry, such as expression changes due to activation status.[16,23] Our variable results with CD11b suggest that protocols isolating or identifying microglia using only CD11b magnetic beads, for example, may be excluding a large portion of the microglial population or pulling out other types of macrophages, which are also $CD11b^+$. More recently, it has been described that CX3CR1 and Siglec-H cell surface markers are more specific to microglia and expressed to a lesser extent in other types of macrophages.[27,26] CX3CR1 is the receptor for the chemokine fractalkine and plays a role in microglia adhesion and migration.[34,35] Our study revealed 83.9–92.5% of our isolated microglia express

Table 4. Microglia marker expression intensity levels.

	Expression intensity
CX3CR1	+++
Siglec-H	+++
F4/80	++
CD11b	+
CD45	Low/–

Microglia have high expression of CX3CR1 and Siglec-H, medium expression of F4/80, low-medium expression of CD11b, and very low to no expression of CD45.[6,24–28]

Table 5. Flow cytometry results of isolated brain cells (first round).

	Sample 1 (%)	Sample 2 (%)
$CX3CR1^+$	85.8	83.9
$CD11b^+$	25.3	19.1
$CD45^+$	2	11.2
$CD49d^+$	0.6	0.3
$CX3CR1^+CD11b^+$	25.2	19.1
$CX3CR1^+CD45^{-/low}$	80.1	68.2
$CD11b^+CD49d^-$	22.1	17.2
$CD11b^+CD45^{-/low}$	28	21.5

Microglia isolated with our novel method were stained with various markers for flow cytometry. Samples consisted of cells isolated from three pooled brains. Data displayed as a percentage of 7AAD PerCP-Cy5 negative cells.

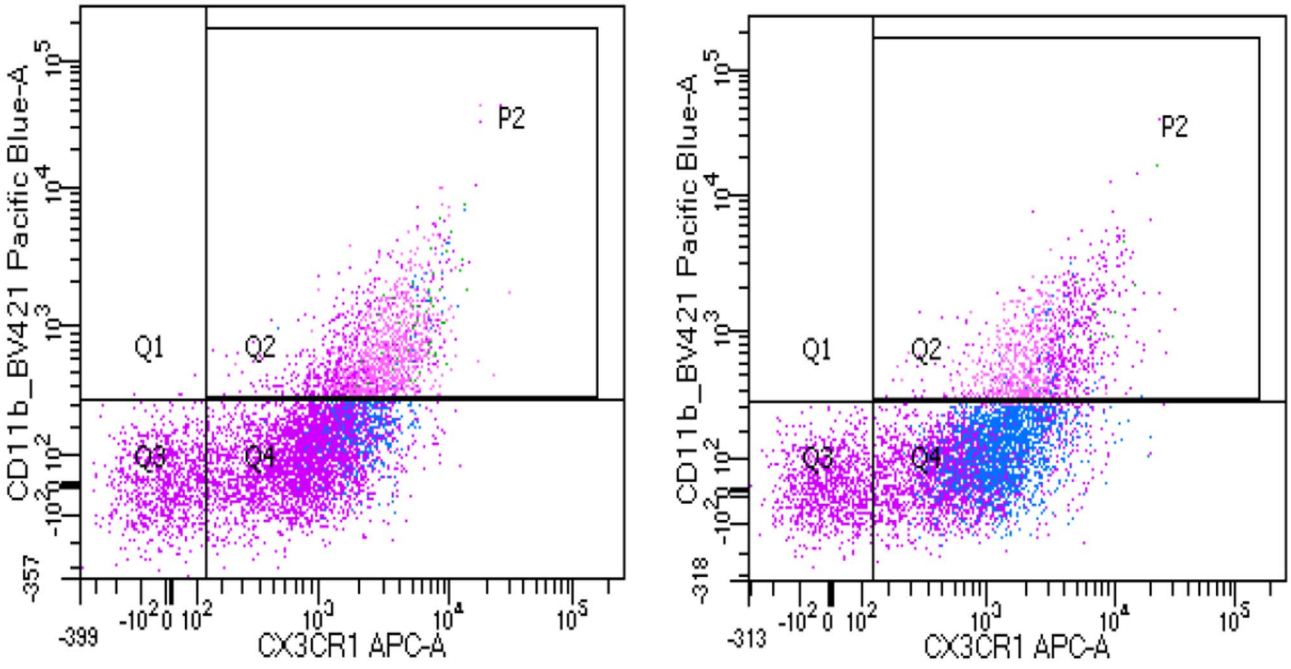

Figure 8. Flow cytometry data of isolated microglial cells.
Isolated cells were stained and evaluated for CX3CR1 and CD11b markers. Left histogram displays data from Sample 1 and right histogram displays data from Sample 2, as indicated in Table 5. (A color version of this figure is available in the online journal.)

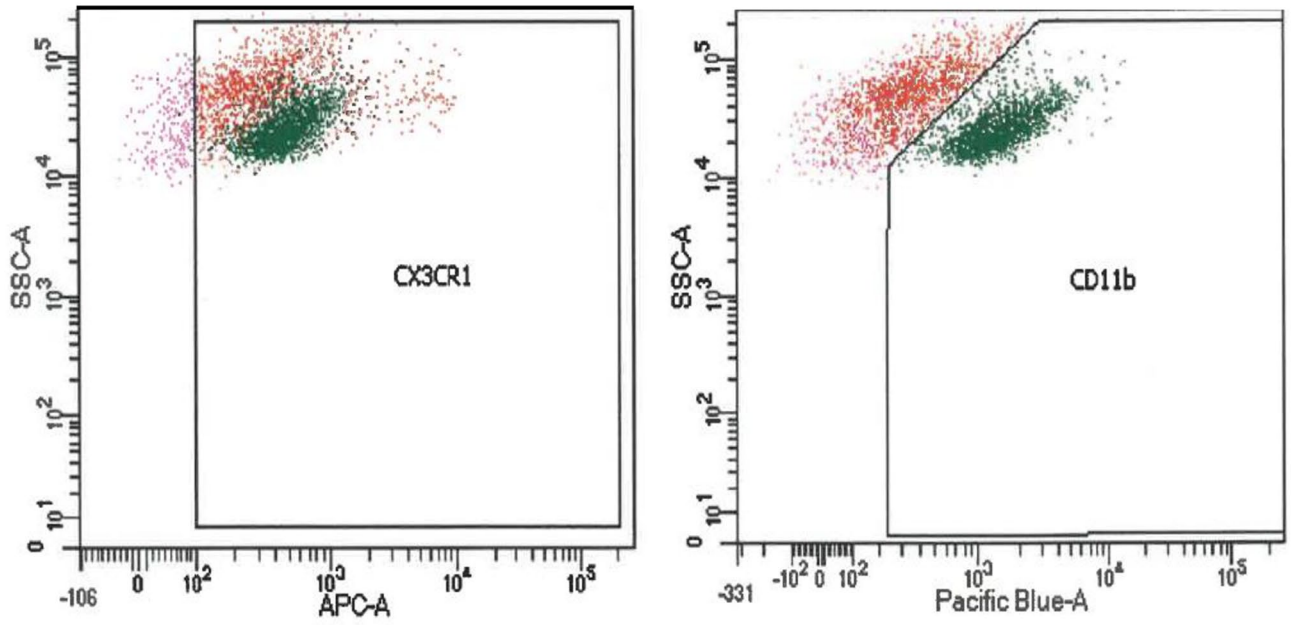

Figure 9. Flow cytometry data of isolated microglial cells.
Isolated cells were stained and evaluated for CX3CR1 and CD11b markers. Left histogram displays data from CX3CR1 stained cells and right histogram displays data from CD11b stained cells, as indicated in Table 6. (A color version of this figure is available in the online journal.)

Table 6. Flow cytometry results of isolated brain cells (second round).

	Sample (%)
CX3CR1 $^{+}$	92.5
CD11b $^{+}$	57

A second round of microglia isolated with our novel method were stained with CX3CR1 and CD11b for flow cytometry. Sample consisted of cells isolated from three pooled brains. Data displayed as a percentage of 7AAD PerCP-Cy5 negative cells.

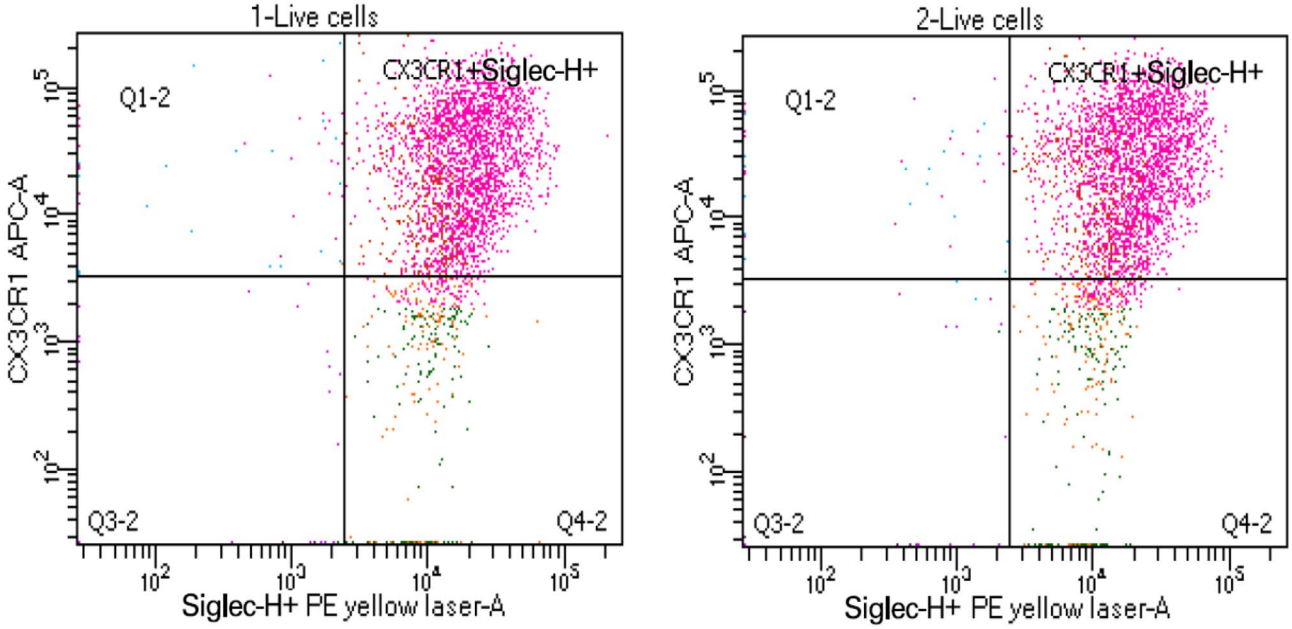

Figure 10. Flow cytometry data of isolated microglial cells.
Isolated cells were stained and evaluated for CX3CR1 and Siglec-H markers. Left histogram displays data from Sample 1 cells stained with CX3CR1 and Siglec-H. Right histogram displays data from Sample 2 cells stained with CX3CR1 and Siglec-H, as indicated in Table 7. (A color version of this figure is available in the online journal.)

Table 7. Flow cytometry results of isolated brain cells (third round).

	Sample 1 (%)	Sample 2 (%)
CX3CR1 $^+$	91.8	92
Siglec-H $^+$	97.8	98.1
F4/80 $^+$	76.6	77.6
CX3CR1 $^+$ Siglec-H $^+$	86.4	85.8
CX3CR1 $^+$ F4/80 $^+$	77.9	78
F4/80 $^+$ Siglec-H $^+$	78.8	79.6
CX3CR1 $^+$ Siglec-H $^+$ F4/80 $^+$	79.8	80.5

A third round of microglia isolated with our novel method were stained with various markers for flow cytometry. Samples consisted of cells isolated from three pooled brains. Data displayed as a percentage of 7AAD PerCP-Cy5 negative cells.

CX3CR1 (Tables 5 to 7). Even more striking is the positivity rate of Siglec-H in our isolated population, which was approximately 98% (Table 7). Siglec-H is a member of the sialic acid-binding immunoglobulin (Ig)-like lectins (Siglec) family[36] and is expressed highly in microglia and has low expression in bone marrow-derived macrophages.[6,23,35] We are confident that our procedure isolates a homogeneous microglial population based on the high-staining percentages of these markers. As such, this procedure should be very useful in better understanding microglia function during both normal and disease conditions.

AUTHOR CONTRIBUTIONS

*Denotes equal contribution. D.D., S.K.R., and J.W.B. conceptualized and planned the study; All authors (D.D., S.K.R., A.T., A.A., A.K.K., B.R.A., A.S.A., and J.W.B.) contributed to the methodology and data collection; D.D., S.K.R., A.S.A., and J.W.B. analyzed the data; D.D., S.K.R., and J.W.B. wrote the article and all authors contributed to article review and editing; J.W.B. and A.S.A. provided supervision for the experiments and funding was acquired by J.W.B. All authors have read and agreed to the final version of the article.

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DECLARATION OF CONFLICTING INTERESTS

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DATA AVAILABILITY

Original data presented in this study can be obtained from the authors on request.

ORCID IDS

Amy Trang https://orcid.org/0000-0003-1500-8585
Ahmet K Korkaya https://orcid.org/0000-0002-1842-1749
Jennifer W Bradford https://orcid.org/0000-0002-1337-8382

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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