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Isolation and culture of microglia

Christopher J. Bohlen^{1,*}, F. Chris Bennett², and Mariko L. Bennett³

¹Department of Neuroscience, Genentech, South San Francisco, CA, USA

²Department of Neurobiology, Stanford University, Stanford, CA, USA

³Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA.

Abstract

Microglia represent 5–10% of cells in the central nervous system (CNS) and contribute to the development, homeostasis, injury, and repair of neural tissues. As the tissue-resident macrophages of the CNS, microglia execute core innate immune functions such as detection of pathogens/damage, cytokine secretion, and phagocytosis. However, additional properties that are specific to microglia and their neural environment are beginning to be appreciated. This unit describes approaches for purification of microglia by Fluorescence-Activated Cell Sorting (FACS) using microglia-specific surface markers, or for enrichment of microglia by magnetic sorting and immunopanning. We also provide detailed information about culturing primary microglia from various developmental stages. Throughout, we focus on special considerations for handling microglia and compare the relative strengths or disadvantages of different protocols.

Keywords

Microglia; culture; FACS (Fluorescence-Activated Cell Sorting); MACS (Magnetic-Activated Cell Sorting); immunopanning; tissue-resident macrophage

Introduction

Microglia are atypical among macrophages in that they derive from early embryonic progenitor cells and self-maintain throughout life without renewal from the bone marrow (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; Ginhoux et al., 2010). In part due to their unusual ontogeny and in part due to cues from the CNS environment, microglia possess features that collectively distinguish them from other tissue-resident macrophages (F. C. Bennett et al., 2018; Gautier et al., 2012; Gosselin et al., 2014; Lavin et al., 2014). For example, microglia possess a highly ramified morphology, exhibit substantial motility during tissue homeostasis, and express signature genes not found in other macrophages including surface receptors such as TMEM119 (Kierdorf & Prinz, 2017; Li & Barres, 2018). The full functional impact of these distinguishing properties have proven difficult to study, as microglia are highly sensitive to manipulations and rapidly change their properties after

*Correspondence to bohlenc@gene.com.

tissue damage, cell culture, or various other experimental manipulations (Bohlen et al., 2017; Butovsky et al., 2014; Gosselin et al., 2017; Krasemann et al., 2017).

The isolation of microglia presents several challenges, some common to all tissue macrophages and some unique to CNS cells. Many studies of innate immunity take advantage of highly accessible macrophages or macrophage progenitors isolated from blood, bone marrow, or lining of the peritoneal cavity (Zhang, Goncalves, & Mosser, 2008), but these systems fail to capture key aspects of the CNS that influence microglial function. Because they are embedded within the CNS parenchyma, separation of microglia from neighboring cells and the tissue matrix requires extensive tissue damage. As highly-sensitive first-responders to injury, microglial properties can rapidly change during the isolation procedure unless pointed efforts to mitigate such a transformation are employed (M. L. Bennett et al., 2016; Haimon et al., 2018; Srinivasan et al., 2016). As an added complication, a substantial proportion of a mature brain or spinal cord is comprised of myelin, a lipid-rich sheet-like structure that interferes with purification of cells from the CNS.

Although microglia are the most abundant macrophage in the CNS, neural tissues also harbor perivascular macrophages, meningeal macrophages, and choroid plexus macrophages (Aguzzi, Barres, & Bennett, 2013). Many macrophage markers fail to discriminate these populations from each other or from circulating monocytes and neutrophils from the blood.

Here, we discuss protocols for isolation and culture of microglia with attention to pitfalls related to microglial sensitivity to experimental manipulations, complications associated with the CNS environment, and potential impurities introduced from related cell types. Basic protocol 1 and Alternate Protocol 1 describe isolation of highly pure microglia by FACS from mouse and human tissue, respectively. Basic protocol 2 and Alternate Protocol 2 describe Magnetic-Activated Cell Sorting (MACS) and immunopanning protocols for isolating CD11b-positive cells from CNS tissue, a population highly enriched for microglia. Basic protocol 3 describes how to establish and maintain microglial cultures under serum-free conditions.

BASIC PROTOCOL 1

Isolation of Mouse Microglia by FACS

Purification of microglia from other major CNS cell types has long been enabled by core immune cell markers such as CD45 and CD11b, which are not present on the surface of other glia or neurons. Microglia present low surface levels of CD45 relative to circulating monocytes (Sedgwick et al., 1991), but separation of CD45^{Hi} and CD45^{Lo} populations still does not cleanly separate microglia from all other myeloid populations such as neutrophils or choroid plexus macrophages. Furthermore, microglial CD45 levels may change in various injury/disease states. Additional markers that distinguish microglia from other CNS macrophages and circulating leukocytes have recently been identified (M. L. Bennett et al., 2016; Butovsky et al., 2014; Haynes et al., 2006). Here we present a FACS protocol for efficient purification of a uniform population of microglia from adolescent to adult CNS tissue based on immunoreactivity to an antibody raised against TMEM119, a transmembrane

protein that is highly specific to microglia as compared to neurons, glia, and other CNS macrophage populations (M. L. Bennett et al., 2016).

To minimize *ex vivo* changes in microglial gene expression, work on ice with cold buffers whenever possible. Complete the cell dissociation/staining as rapidly as possible, and time the prep in order to begin sorting immediately after cells have been stained. We aim to begin sorting cells in under 2.5 hours after euthanizing mice.

Materials—Donor mice: e.g. 6–8 week old C57BL/6J (The Jackson Laboratory, cat # 000664)

Standard CO₂ gas tank and euthanasia chamber

Dissection tools: scissors, spring scissors, forceps

Ice and a large ice bucket

Tube Rocker (e.g. VWR, cat # 10159–756)

50 mL conical tubes (e.g. Falcon)

2 mL microcentrifuge tubes (e.g. Eppendorf)

PBS (from 10x stock: Gibco, cat # 70011–044)

Glass tissue grinder (Wheaton, cat # 357424)

Myelin removal beads (Miltenyi, cat # 130–096–733)

LD columns (Miltenyi, cat # 130–042–901)

MACS magnets (Miltenyi, cat # 130–090–976)

Compensation beads (BD cat # 552845, Invitrogen cat # A10628)

Anti-Tmem119 (Rb anti-ms, Abcam 210405)

Anti-CD45 PE-Cy7, 0.2 mg/mL (Rt anti-ms, Ebiosciences 25–0451–82)

Anti-CD11b PerCP/Cy5.5 (Rt anti-ms/hu, Biolegend 101228)

Anti-rabbit BV421 secondary, 0.2 mg/mL (Dk anti-rb, Biolegend 406410)

Mouse BD Fc block (BD Biosciences 553141)

Live/Dead Green (Life Technologies, cat # L34969)

70-µm cell strainer (Sigma, cat # CLS431751)

RNase-free DNase (Qiagen, cat # 79254)

RNasin (Promega, cat # N2615)

RLT lysis buffer supplemented with β -mercaptoethanol (optional, Qiagen, cat # 79216)

FACS Homogenization Buffer*

MACS Buffer*

FACS Buffer*

*see Reagents and Solutions section below

Dissociation and Generation of Single Cell Suspension

Note: This protocol uses 1 whole brain from mice P10 (postnatal day 10) or up to 3 pooled whole brains from mice <P10 for each “sample”. Additional mice can be processed in parallel using separate tubes, separate rounds of tissue homogenization, and additional magnetic columns.

1. For each sample, prepare 5 mL FACS Homogenization Buffer and 2 mL MACS Buffer with 4 μ L RNasin.
2. Sacrifice 1–2 mice at a time using a CO₂ euthanasia chamber and compressed gas canister, following established institutional guidelines. Allow 4–5 minutes and ensure each mouse is non-responsive before proceeding.

We typically do not perform endovascular perfusion prior to brain dissection since TMEM119 is only expressed on parenchymal brain macrophages, but it can be performed to suit downstream applications. Expected cell yields from mice of various ages are listed in Table 1 to help determine the number of mice required for your downstream application. The following procedure can be followed for mice of various ages, but mice P10 and younger cannot be euthanized by CO₂ and must be euthanized by rapid decapitation with sharpened scissors. Additionally, TMEM119 surface expression is not detectable in microglia from embryonic and neonatal brains, but this protocol can be used to isolate CD45/CD11b double-positive cells from these immature tissues.

3. Using scissors, decapitate the animal, remove the skin from the scalp, then cut the skull starting from the spinal canal and proceeding around the lateral edge of the brain to the rostral end. Use forceps to peel back the skull and remove the brain, transferring it to 5 mL FACS Homogenization Buffer as quickly as possible.

Please refer to previous protocols for additional details on brain tissue extraction (Collins & Bohlen, 2018).

4. Homogenize the tissue in a glass tissue grinder on ice. Use 2–3 strokes, then transfer the suspension through a 70 μ m cell strainer into a 50 mL conical on ice.

If tissue chunks remain after the first round of homogenization, leave the incompletely homogenized tissue in the tissue grinder, add 2 mL FACS Homogenization Buffer, and repeat homogenization with another 2–3 strokes.

5. Rinse strainer twice with 1 mL FACS Homogenization Buffer.

To maximize yield, we typically use the plunger end of a 1 mL syringe to homogenize any remaining tissue chunks against the strainer mesh.
6. Aliquot filtered cells into 2 mL microcentrifuge tubes.

You will fill several tubes, depending on final volume of homogenate.
7. Centrifuge the cells for 30 s at $9,300 \times g$ and 4°C .
8. Discard supernatant and resuspend all cells for a given sample in 1.8 mL MACS buffer with 4 μL RNasin.

Myelin Removal

9. Mix the myelin removal bead stock slurry, then add 200 μL slurry to each cell suspension.
10. Mix well with gentle pipetting and divide the sample into two separate 2 mL microcentrifuge tubes (1 mL for each tube).
11. Incubate at 4°C for 10 min.
12. Meanwhile, place 2 LD columns per sample into the MACS magnet and rinse with 2 mL MACS buffer.
13. After incubation, dilute each cell suspension to 2 mL with MACS buffer.
14. Centrifuge the cells for 30 s at $9,300 \times g$ and 4°C .
15. Repeat Steps 13 and 14
16. Resuspend cells in 1 mL MACS buffer per tube.
17. Apply cells from one tube to one LD column, and collect flow-through in 50 mL conical tubes on ice.

Allow sample to run completely into column bed before adding washes.

18. Rinse tube with 2 mL more MACS buffer and apply to column, collecting the flow-through in the same 50 mL conicals.
19. Wash the column with 2 mL MACS buffer, collecting the flow-through.

Samples flow more slowly during myelin removal than during typical positive selection or blood sorting, especially if the columns are overloaded. We sometimes agitate the settled contents above the column bed using a pipette, to reduce clogging of the column.

20. Aliquot the cells collected from the flow-through into 2 mL tubes. Centrifuge the cells for 30 s at $9,300 \times g$ and 4°C , then resuspend the pellets in PBS and combine to a final volume of 1 mL PBS per sample.

The pellet will be red due to red blood cells, which are removed during cell sorting. There should be on the order of 1 million cells per brain at this stage, depending on the age.

Live/Dead and Cell Staining:

21. Remove 100 μL of the cell suspension for negative controls. Dilute this suspension by adding 1500 μL FACS buffer. Then, make $5 \times 300 \mu\text{L}$ aliquots and label the 5 tubes as “CD11b SC”, “CD45 SC”, “Tmem119 SC”, “secondary only”, and “unstained”. SC refers to single-color control.

Alternatively, we frequently use beads for compensation, especially if input sample size is limiting. Hold all tubes on ice while not being used.

22. To the remaining 900 μL sample in PBS from Step 20, add 1 μL Live/Dead Green and incubate in dark at 4°C for 5 min.
23. Dilute the sample to 2 mL with FACS buffer.
24. Centrifuge the Live/Dead treated cells for 30 s at $9,300 \times g$ and 4°C . Discard the supernatant.
25. Resuspend the Live/Dead treated cells in 320 μL FACS buffer.
26. Remove 20 μL for Live/Dead only and FMO (Full Minus One) controls and dilute with 580 μL FACS buffer. Split the diluted cell suspension into two tubes labeled “Live/Dead SC” or “FMO”. Label the tube containing the remaining 300 μL of the original Live/Dead treated cell suspension “All” for staining with all markers.

At this point you will have eight tubes containing 300 μL FACS buffer and varying concentrations of cells. The “All” tube will contain 30 to 45-fold higher cell density than negative control, SC, and FMO tubes.

27. Add 5 μL mouse Fc receptor block to each of seven tubes labeled in steps 21 and 26.
28. To the tubes labeled “All” and “Tmem119 SC”, add anti-Tmem119 primary ab
- Titrate antibody concentration empirically – we observe small fluctuations between lots in effective concentration, but typically stain at 0.1–0.5 $\mu\text{g/mL}$ final antibody concentration
29. Incubate 15–20 minutes at 4°C on a tube rocker ($\sim 18 \text{ rpm}$).
30. Wash “All” and “Tmem119 SC” tubes by raising the volume to 2 mL with FACS buffer and centrifugation for 30 s at $9,300 \times g$ and 4°C . Discard the supernatant and resuspend the pellet in 300 μL FACS Buffer.

31. Add 1 μ L CD11b-PerCP/Cy5.5, 1 μ L CD45-PE-Cy7, and 1 μ L anti-rabbit BV421 secondary to “All” and “FMO” tubes, and as appropriate to single color or secondary only control tubes.
32. Incubate 15 minutes at 4 °C on a tube rocker (~18 rpm).
33. Wash all cells by raising the volume to 2 mL with FACS buffer and centrifugation for 30 s at $9,300 \times g$ and 4 °C. Discard the supernatant and repeat this step for a second wash.
34. Resuspend the cells for sorting in 300 μ L FACS buffer containing 3 μ L RNase free DNase and 0.6 μ L RNasin.

At this point you will have eight tubes containing 300 μ L FACS buffer and varying concentrations of cells. The “All” tube contains the cells that will be sorted, which are stained with TMEM119, CD11b, CD45, and Live/Dead. The remaining tubes are stained with none, one, or some of these markers and will be used to calibrate the instrument, set gates, and serve as controls.

FACS Protocol

35. On a BD FACSaria II, prepare the 100 μ m nozzle, and tube chillers (set to 4 °C).
We typically set the flow rate to 1 (~10 μ L/min) to maximize sort quality.

36. After compensation using single color controls or beads, set gates for singlet live cells, using FSC/SSC/Live-Dead stain properties. Run all control samples to set gates as shown in Figure 1.

We observe some basal signal in the LIVE/DEAD channel in live macrophages, as shown in Figure 1.

37. Load the “All” tube from step 34 into the instrument and sort into low-adhesion microcentrifuge tubes containing FACS buffer and RNasin using four-way sorting. Prepare two collection tubes: one for TMEM119-positive cells (microglia) and a second for Tmem119-negative/CD45^{Hi}/CD11b-positive cells (“myeloid cells”), which represent non-microglial immune cells that may be useful for comparison.

38. If high purity is needed for your application, you may re-sort cells.

There is a tradeoff between yield and purity between single and double sorting, and the relative importance of each should be weighed depending on the specifics of the experiment. If sorting the cells for RNA analyses, sort the cells directly into lysis buffer (e.g. RLT buffer supplemented with β -mercaptoethanol) to halt any further transcriptional changes from the cells. Although we have successfully used FACS-sorted microglia for in vivo transplantations and cell culture, we find that FACS sorting significantly reduces microglial viability.

ALTERNATE PROTOCOL 1

Isolation of Human Microglia by FACS

Microglia from human tissue also exhibit surface expression of TMEM119 (F. C. Bennett et al., 2018; M. L. Bennett et al., 2016). Here we provide a FACS protocol for efficient isolation of high-purity microglia from human tissue using a monoclonal antibody that we have recently developed to recognize a surface-exposed epitope in the N-terminal domain of TMEM119 (F. C. Bennett et al., 2018). This protocol is highly similar to the one used for isolation of mouse cells (Basic Protocol 1) – differences relate to determining number of myelin depletion columns to use based on input tissue, and gating for TMEM119-positive cells for sorting, which is dimmer than in mouse.

Materials—Ice and a large ice bucket

Tube Rocker (e.g. VWR, cat # 10159–756)

50 mL conical tubes (e.g. Falcon)

2 mL microcentrifuge tubes (e.g. Eppendorf)

PBS (from 10x stock: Gibco, cat # 70011–044)

Glass tissue grinder (Wheaton, cat # 357424)

Myelin removal beads (Miltenyi, cat # 130–096–733)

LD columns (Miltenyi, cat # 130–042–901)

MACS magnets (Miltenyi, cat # 130–090–976)

Compensation beads (BD cat # 552845, Invitrogen cat # A10628)

Anti-Tmem119, 0.5 mg/mL (Ms anti-hu, Biolegend A16075D)

Anti-CD45 PE-Cy7, 0.2 mg/mL (Ms anti-hu, BD Biosciences 557748)

Anti-CD11b PerCP/Cy5.5 (Rt anti-ms/hu, Biolegend 101228)

Anti-mouse BV421 secondary, 0.2 mg/mL (Gt anti-ms, Biolegend 405317)

Isotype control (IgG2b, k, Biolegend 401201)

Human BD Fc block (BD Biosciences 564220)

Live/Dead Green (Life Technologies, cat # L34969)

70-µm cell strainer (Sigma, cat # CLS431751)

RNase-free DNase (Qiagen, cat # 79254)

RNasin (Promega, cat # N2615)

RLT lysis buffer supplemented with β -mercaptoethanol (optional, Qiagen, cat # 79216)

FACS Homogenization Buffer*

MACS Buffer*

FACS Buffer*

*see Reagents and Solutions section below

Preparation and Staining of Cells

Note: Each “sample” refers to up to 750 mg of fresh human tissue. This protocol was developed using surgical resection tissue obtained from clinical collaborations. Surface markers (particularly TMEM119) may become degraded in fixed tissue or postmortem samples. We practice BSL-2 precautions with all human samples.

1. For each sample, prepare 5 mL FACS Homogenization Buffer and 2 mL MACS Buffer with 4 μ L RNasin.
2. Homogenize the tissue, perform myelin depletion, and perform Live/Dead staining as described above for mouse tissue in Basic Protocol 1 steps 4–26. In step 10, distribute the sample equally into three separate tubes (667 μ L per tube), and in step 12, use three LD columns per sample instead of two.
3. You should have eight tubes containing 300 μ L FACS buffer and varying amounts of cells. Change the label of the “secondary only” tube to “isotype”. Add 30 μ L human Fc receptor block to each tube and incubate for 5 min at room temperature (RT).
4. To “All” and “Tmem119 SC” tubes, add anti-Tmem119 primary ab to reach a final concentration of 0.3 μ g/mL. To “FMO” and “isotype” tubes add matched concentration of IgG2b isotype control antibody.

Titrate antibody concentration empirically. We have observed decreased signal at higher final concentration of primary antibody, with maximal signal typically observed at 0.3 μ g/mL or lower.

5. Incubate 10–15 minutes at RT on a tube rocker (~18 rpm).
6. Wash “All”, “Tmem119 SC”, “FMO”, and “isotype” tubes by raising the volume to 2 mL with FACS buffer and centrifugation for 30 s at $9,300 \times g$ and 4 °C. Discard the supernatant and resuspend the pellet in 300 μ L FACS Buffer.
7. Add 1 μ L CD11b-PerCP/Cy5.5, 1 μ L CD45-PE-Cy7, and 1 μ L anti-mouse BV421 secondary to “All” and “FMO” tubes, and as appropriate to single color or isotype only control tubes.
8. Incubate 10 minutes at RT on a tube rocker (~18 rpm).

9. Wash all cells by raising the volume to 2 mL with FACS buffer and centrifugation for 30 s at $9,300 \times g$ and 4 °C. Discard the supernatant and repeat this step for a second wash.
10. Resuspend the cells for sorting in 300 μ L FACS buffer containing 3 μ L RNase free DNase and 0.6 μ L RNasin.

FACS Protocol

11. On BD Aria II sorter, prepare the 100 μ m nozzle, and tube chillers (set to 4 °C).
We typically set the flow rate to 1 (~10 μ L/min) to maximize sort quality.
12. After compensation using single color controls or beads, set gates for singlet live cells, using FSC/SSC/Live-Dead stain properties. Run all control samples to set gates as shown in Figure 1.
13. Load the “All” tube from step 34 into the instrument and sort into low-adhesion microcentrifuge tubes containing FACS buffer and RNasin using four-way sorting. Prepare two collection tubes: one for TMEM119-positive cells (microglia) and a second for Tmem119-negative/CD45^{Hi}/CD11b-positive cells (“myeloid cells”), which represent non-microglial immune cells that may be useful for comparison.

We observe significant nonspecific staining of human CD45/CD11b double-positive human cells with secondary antibody for TMEM119. It is critical to set TMEM119-positive gates based on isotype control staining, which is nearly identical to secondary only staining, or occasionally slightly higher
14. If high purity is needed for your application, you may re-sort cells.

BASIC PROTOCOL 2

Enrichment of Microglia by Magnetic CD11b Selection

While FACS is the gold-standard for isolation of pure microglia, it requires specialized instrumentation, sorts that can be time-consuming at large scale, and hydrodynamic stresses that can damage cells targeted for continued study *ex vivo*. Several alternative protocols have been described for enrichment of microglia that are more amenable to high-throughput applications (Garcia, Cardona, & Cardona, 2014; Joseph & Venero, 2013). We have had reliable yields and purity from both magnetic and immunopanning protocols, which we provide below.

For the magnetic protocol, antibodies that recognize the microglial surface antigen CD11b are conjugated to tiny superparamagnetic particles that allow retention of labelled cells in a magnetic field. We have streamlined effective protocols described by other groups and the manufacturer (Garcia et al., 2014) to maximize throughput, yield, and purity. These protocols efficiently select CD11b-positive cells over other major CNS cell types, and the large majority of CD11b-positive cells from the uninjured CNS are microglia. However,

these protocols suffer from the shortcoming that they do not separate microglia from barrier associated macrophages, monocytes, neutrophils, or certain B cells also present in the tissue. As such, magnetic isolation can be considered for routine culture applications (where survival of non-microglial myeloid cells can be selected against (Collins & Bohlen, 2018)) or applications for which demands for throughput outweigh the importance of purity in the initial population.

Both magnetic and immunopanning protocols were optimized for isolation of CD11b-positive cells out of whole brain from P14-P21 rats. The general protocols can be applied (albeit with reduced yields) to mouse, human, or rat tissue of varying ages; where applicable, we have provided some guidelines for modifications relevant to different types of input material. We have introduced small differences in sample processing compared to the FACS protocols above to streamline the cell isolation procedure.

Materials—Donor rats (recommended P7–P21, Charles River, strain code # 400)

Standard CO₂ gas tank and euthanasia chamber

Dissection tools: scissors, spring scissors, forceps

Ice and a large ice bucket

20 mL syringe

21-gauge needle

Razor blade (VWR, cat # 55411–050)

Glass tissue grinder (Wheaton, cat # 357424)

50 mL and 15 mL conical tubes (e.g. Falcon)

2 mL microcentrifuge tubes (e.g. Eppendorf)

Refrigerated centrifuge

70-µm cell strainer (Sigma, cat # CLS431751)

PBS (from 10x stock: Gibco, cat # 70011–044)

Myelin removal beads (Miltenyi, cat # 130–096–733)

MACS magnets (Miltenyi, cat # 130–090–976)

LD columns (Miltenyi, cat # 130–042–901)

LS columns (Miltenyi, cat # 130–042–401)

CD11b/c (Microglia) MicroBeads, rat (Miltenyi, cat # 130–105–643)

Basic light microscope (e.g. Zeiss Axiovert 40 C)

Hemocytometer

Perfusion buffer*

Dissociation buffer*

Myelin separation buffer*

Microglia growth medium (MGM)*

*see Reagents and Solutions section below

Tissue Collection

Note: This protocol uses 2 whole rat brains for each “sample”. Up to 5 samples can be pooled at step 12 onto a single LD and MS column. We typically process a full litter of 8–10 P14 rats in each preparation, but this can be adjusted as necessary for downstream assays using the guidelines in Table 1.

1. Chill instruments and solutions on ice.
2. Sacrifice 1–2 rats at a time using a CO₂ euthanasia chamber and compressed gas canister, following established institutional guidelines. Allow 4–5 minutes and ensure each rat is non-responsive before proceeding.

Animals younger than P10 should be sacrificed with an intraperitoneal injection of ketamine/xylazine (100 – 200 μ L of 24 mg/mL ketamine, 2.4 mg/mL xylazine). If using multiple animals, extract the tissue from one animal before proceeding to subsequent animals.

3. Transcardially perfuse the rat with a 20 mL syringe, 21-gauge needle and 10 to 30 mL of ice-cold perfusion buffer.

Volume of perfusion buffer will vary depending on the age of the animal. Perfusions can be performed on anesthetized or euthanized adult animals; we routinely obtain clean perfusions from euthanized animals and favor that option when possible to avoid unnecessary exposure of the cells to anesthetics.

4. Using scissors, decapitate the animal, remove the skin from the scalp, then cut the skull starting from the spinal canal and proceeding around the lateral edge of the brain to the rostral end. Use forceps to peel back the skull and remove the brain, transferring it as quickly as possible into 10 mL chilled dissociation buffer on ice.

Please refer to previous protocols for additional details on brain tissue extraction (Collins & Bohlen, 2018).

Tissue Dissociation

5. Transfer each sample (2 brains) into a petri dish lid with ~1 mL of dissociation buffer and chop into ~1 mm³ chunks using a razor blade.
6. Transfer the chunks to the homogenizer and add 4.5 mL of dissociation buffer. Dissociate the tissue using 10–15 gentle and incomplete strokes followed by 3 complete strokes.

Don't crush the tissue at the bottom of the homogenizer but impel the tissue through the space between the sides of the piston and the reservoir. Be careful not to introduce air bubbles.

7. Carefully remove the piston and transfer the suspension to a chilled 50 mL conical tube. Repeat steps 5–7 for each sample.

Only process 2 brains at a time in the homogenizer and repeat to process all samples. This mechanical homogenization kills a substantial portion of other CNS cell types, but microglia are preferentially spared.

Myelin and Debris Removal

8. Add cold dissociation buffer to each sample to adjust the total volume to 33.5 mL.
9. Add 10 mL of myelin separation buffer to each sample cell suspension and mix.

Myelin separation buffer is a high-density solution that is used to exclude the majority of cellular debris (mostly low-density myelin) from a pellet of viable cells.

10. Centrifuge cells for 15 min at 500 × g at 4 °C with slow braking.

This spin will generate an upper layer of myelin and debris, a murky supernatant, and a small pellet that is enriched for live cells.

11. Remove the top layer of myelin/debris and the supernatant with a pipette.
12. Resuspend the cell pellet in 2.7 mL of PBS per ~10 g of starting tissue (this represents 8–10 juvenile rat brains or 4–5 pooled samples).

Smaller volumes can be used for smaller amounts of input material. For larger preparations, use multiple tubes and multiple LD/LS columns in steps 15–28.

13. Add 300 µL myelin removal beads for every 2.7 mL PBS used in step 12. Mix well and split the sample to 2 × 2 mL microcentrifuge tubes.
14. Incubate at 4 °C for 10 min.
15. Meanwhile, place 1 LD column into the MACS magnet and rinse with 2 mL PBS.
16. Centrifuge the cell suspension at 5,000 × g for 30 s at 4 °C.

17. Discard the supernatant and resuspend each pellet in 2 mL PBS, then centrifuge the cell suspension again at $5,000 \times g$ for 30 s at 4 °C.
18. Remove the PBS and, using only 0.5 mL of PBS, pool the pellets and apply the suspension to the LD column.
19. Collect the flow-through and 2×1 mL washes in a 50 mL conical tube on ice.
Allow sample to run completely into column bed before adding washes
20. Redistribute the flow-through into 2×2 mL chilled microcentrifuge tubes, then centrifuge the cell suspension at $5,000 \times g$ for 30 s at 4 °C.

CD11b Selection (Day 1)

21. Resuspend all cells and pool into 180 μ L PBS per 10 juvenile rat brains or equivalent amount of tissue.
22. Add 20 μ L rat CD11b microbeads and mix well.
Mouse/human CD11b microbeads are also effective for isolating CD11b-positive cells from those species.
23. Incubate at 4 °C for 10 min.
24. Meanwhile, place 1 LS column into the MACS magnet and rinse with 2 mL PBS.
25. Dilute the cell suspension with 1 mL PBS and centrifuge for 30 s at $5,000 \times g$ and 4 °C.
26. Resuspend the pellet in 0.5 mL PBS and apply the suspension to the LS column.
27. Add 2 mL PBS to wash away CD11b-negative cells and discard the flow-through. After the full 2 mL has passed through the column, repeat two additional times for a total of three 2 mL washes.
28. Remove the LS column from the magnet, add 2 mL of PBS or MGM, and use the plunger to elute the CD11b-positive cells.

With practice, cell yields should approach 2×10^6 cells per juvenile rat brain.

Maximum cell yields are achieved from animals in the range of P6–P14. Tissue from younger or older rats will generate lower yields down to $\sim 3 \times 10^5$ cells from adults. Expected yields from mouse brains are three to four times lower than the same number of rat brains. Refer to Table 1 for guidelines.

ALTERNATE PROTOCOL 2

Enrichment of Microglia by CD11b Immunopanning

Magnetic selection is highly effective but requires significant upfront investment in reagents and equipment. Additionally, the magnetic isolation protocol attaches iron-oxide-conjugated

antibodies to the cell surface, although we have not observed major functional consequences of superparamagnetic antibodies in any of our commonly used assays. Here we provide an alternative immunopanning strategy that requires minimal upfront reagent investment or specialized equipment. Briefly, antibodies recognizing CD11b are immobilized on a petri dish and used to retain microglia from brain single-cell suspensions. Further separation of different myeloid populations is unlikely to be achievable using immunopanning due to the propensity of various myeloid cell populations to adhere to the panning dish, even dishes not coated with antibody. This protocol is slightly more laborious than magnetic separation and requires trypsinization of cells, but avoids introduction of magnetic particles in downstream applications.

Materials—Donor rats (recommended P7–P21, Charles River, strain code # 400)

Standard CO₂ gas tank and euthanasia chamber

Dissection tools: scissors, spring scissors, forceps

Ice and a large ice bucket

50 mM Tris pH 9.5 solution

15 cm untreated plastic Petri dish (Falcon cat # 351058)

Goat anti-mouse IgG (H+L chains), (Jackson ImmunoResearch, cat # 115–005–003)

DPBS⁺⁺: Dulbecco's phosphate-buffered saline with calcium and magnesium (Gibco, cat # 14040182)

OX42 monoclonal mouse anti-ratCD11b antibody (Bio-Rad, cat # MCA275G)

M1/70 monoclonal rat anti-mouse/human CD11b antibody (optional, Thermo Scientific, cat # 14–0112–81)

20 mL syringe

21-gauge needle

Razor blade (VWR, cat # 55411–050)

Glass tissue grinder (Wheaton, cat # 357424)

50 mL and 15 mL conical tubes (e.g. Falcon)

2 mL microcentrifuge tubes (e.g. Eppendorf)

Refrigerated centrifuge

70-µm cell strainer (Sigma, cat # CLS431751)

PBS (from 10x stock: Gibco, cat # 70011–044)

TrypLE Express Enzyme, no phenol red (Gibco, cat # 12604013)

Basic light microscope (e.g. Zeiss Axiovert 40 C)

Hemocytometer

Perfusion buffer*

Dissociation buffer*

Myelin separation buffer*

Microglia growth medium (MGM)*

*see Reagents and Solutions section below

Panning Dish Preparation

1. Add 25 mL of 50 mM Tris pH 9.5 solution to a 15-cm untreated plastic Petri dish.

One immunopanning dish can accommodate 2 juvenile rat brains or up to 6 neonatal rat brains.
2. Add goat anti-mouse IgG (H+L chains) to the dish for a final concentration of 6 µg/mL and incubate at 37 °C for 1–3 hours.
3. Rinse dishes three times with panning buffer, then replace with a solution of panning buffer containing 1 µg/mL OX42 antibody. Leave the dishes on flat surface overnight at room temperature.

The OX42 monoclonal antibody is specific for rat CD11b. If using tissue from other species, the M1/70 monoclonal can be used with goat anti-rat IgG at the same concentrations to recognize either mouse or human CD11b.

Cell Suspension and Myelin Depletion

4. The next day, follow steps 1–11 in the magnetic separation Basic Protocol 2 above to generate a single-cell suspension and deplete myelin through centrifugation.
5. Resuspend the cell pellets from up to 5 mg starting tissue (~2–6 rat brains, depending on age) in 1 mL of panning buffer, then dilute to 12 mL with panning buffer.

See Table 1 for estimated brain weights and yields from rat and mice from various ages.

Immunopanning

6. Pass the cell suspension through a 70-µm cell strainer.
7. Rinse the OX42-coated panning dish three times with DPBS⁺⁺.

Don't allow the plate to dry between washes.

8. Remove the last wash and apply the cell suspension to the panning dish.
9. Incubate on a flat surface at room temperature for 20 min to allow cells to adhere.

Incubating longer than 20 min will make recovery of cells very difficult.

10. Rinse the panning dish with PBS 10 times to remove non-adherent cells. Microglia will be firmly attached to the plate so swirl the plate with each rinse to ensure removal of non-adherent cells.
11. Remove the last wash and replace with 15 mL TrypLE express enzyme solution.
12. Incubate the dish for 10 min at 37 °C.

Microglia will be strongly adhered to the panning dish. TrypLE protease treatment will weaken the interaction, but will not cause them to detach. Longer incubations will not improve recovery.

13. Pour off the TrypLE solution and gently wash twice with 15 mL of PBS.
14. Remove the last wash and add 12 mL of ice-cold microglia growth medium (MGM). Place the panning dish on ice for 2 min to help weaken cell/substrate interaction.

Ensure that the dish is flat to prevent areas of the panning dish from drying out.

15. Using a 10 mL pipette and pipette controller on high speed, pipette vigorously to dislodge cells from the panning dish.

Cells will only be dislodged when hit directly with the pipette stream. Trace a pattern of vertical then horizontal lines with the pipette stream to cover the full area of the dish.

16. Use the microscope to mark spots on the dish where cells are still attached and repeat pipetting in those areas.
17. Collect cell suspension and divide into four 15 mL conical tubes. Spin 15 min at 500xg at 4°C with slow braking.
18. Aspirate the supernatant, leaving 0.5 mL of MGM with the cell pellet. Resuspend each pellet in the 0.5 mL remaining MGM, pool the cells, and count with a hemocytometer.

With practice, cell yields should be comparable to those described in the magnetic separation Basic Protocol 2 above.

BASIC PROTOCOL 3

Culture of Rodent Microglia

The earliest described method for culturing purified microglia takes advantage of the loosely-adherent layer of microglia that forms over the course of weeks in mixed cultures of perinatal brain cells (Giulian & Baker, 1986). CD11b-positive cells isolated by immunopanning or MACS can be sustained in culture for weeks if provided with the necessary nutrients and growth factors, providing a number of advantages over classical ‘shake-off’ cultures. First, cells can be isolated from various developmental stages, not just perinatal brains. Second, relatively pure cultures can be established in several hours rather than several weeks. Third, potential variables (including variable levels of contaminating cell types) introduced from prolonged growth in mixed culture are avoided. Finally, freshly isolated cells can be maintained in fully-defined medium and without serum, which we have shown to have lasting impact on microglial properties *in vitro* (Bohlen et al., 2017). Here we provide protocols for sustaining cultures of purified microglia in culture under serum-free conditions optimized to promote a ramified morphology.

STRATEGIC PLANNING

Cell culture experiments aspire to accurately model *in vivo* processes in a simplified and tightly-controlled system, preferably under conditions that are compatible with high-throughput chemical or genetic screening. Many variables that influence these ideal properties need to be considered, including species of origin, age of the donor, time in culture, and media/substrate that make up the culture environment.

This protocol has been optimized for primary microglial cultures from juvenile rats. Rat tissue provides high yields of cells that show robust survival. CD11b-positive cells from human brain tissue also survive under these culture conditions, but such experiments suffer from difficulty in obtaining tissue and heterogeneity of tissue samples. Cultures from young mice (<P14) are also viable, although with somewhat lower yields and survival rates than cultures from rat tissue. Cultures can be established from developing or adult rat brains, but cell yields and viability drop with increasing animal age. Due to the combined decrement in yields/viability from adult animals as well as from mice relative to rats, we recommend against attempting cultures from adult mice using these procedures. With these factors in mind, we focus our experiments on microglia from P14-P21 rat brains, which enable high yields of microglia from an age at which these cells exhibit an essentially fully matured transcriptional profile *in vivo* (M. L. Bennett et al., 2016; Matcovitch-Natan et al., 2016).

This protocol provides several factors meant to mimic key features of the CNS environment. Microglial survival *in vivo* is fully dependent on constitutive CSF1R activation by CSF-1 or IL-34 (Blevins & Fedoroff, 1995; Elmore et al., 2014; Erbllich, Zhu, Etgen, Dobrenis, & Pollard, 2011; Greter et al., 2012; Nandi et al., 2012; Wang et al., 2012), and constitutive TGFBR2 activation in the CNS has a major impact on cellular morphology and gene expression patterns (Butovsky et al., 2014; Buttgerit et al., 2016). CSF1R and TGFBR2 signaling similarly impact microglial survival and morphology in serum-free medium *ex vivo* (Bohlen et al., 2017) and should typically be included in microglial culture

experiments. Other additives that are crucial to microglia survival in serum-free conditions are cholesterol and selenite (Bohlen et al., 2017). Two monounsaturated fatty acids (oleic acid and gondoic acid) and matrix molecules (heparan sulfate and collagen IV) are also provided to facilitate process extension.

Microglia in culture rapidly lose defining features that distinguish them from other tissue macrophages. Many microglia signature genes such as *Tmem119* and *P2ry12* are downregulated by 10–100 fold within hours of entering the culture environment, resulting in greatly reduced protein expression (Bohlen et al., 2017; Gosselin et al., 2017). Additionally, cultures exhibit upregulation of other genes typically only observed *in vivo* in the context of disease or injury (Bohlen et al., 2017; Butovsky et al., 2014; Gosselin et al., 2017). Thus, cultured microglia have substantial limitations and cannot be expected to fully predict the behavior of *in vivo* microglia across all circumstances. However, the culture protocol described here does retain some microglia-like properties such as ramified morphology, rapid extension/retraction of processes, and detectable (albeit very low) levels of expression of microglia signature genes (Bohlen et al., 2017). Thus, these cultures serve as an imperfect model with advantages over other non-microglial macrophage cultures or cell lines.

Materials—Collagen IV (Corning, cat # 354233)

Poly-D-lysine (PDL)-coated coverslips (optional, Fisher Scientific, cat # NC0343705)

Phenol red free DMEM/F-12 (Gibco, cat # 21041–02)

Tissue culture plates (e.g. Corning Primaria TC-treated 24-well plate, cat # 734–0078, or Falcon TC-treated 384-well plate, cat # 353961)

Humidified cell culture incubator

Microglia growth medium (MGM), see Reagents and Solutions section below

Protocol steps

1. Dilute collagen IV to 2 µg/mL in DMEM/F-12 and add sufficient volume to fully coat tissue culture plates or dishes.

Use of Primaria plastic maximizes extended morphologies. Cells can survive in a range of tissue culture ware, from 384-well plates to 15-cm dishes even without collagen or other coating; glass should be coated with PDL (Poly-D-Lysine) prior to additional coating with collagen to facilitate adhesion.

2. Incubate the plates 1–2 hr at 37°C, aspirate off the collagen solution, and allow the plates to dry for >10 min at room temperature.
3. Prepare Microglia Growth Medium (MGM) as described in the reagents and solutions section and purify microglia following Basic Protocol 2 or Alternate Protocol 2. Dilute cells to a concentration of 2×10^5 cells/mL in MGM. Add the

appropriate volume to each well of the culture plate and place in a humidified 37°C incubator with 5–10% CO₂.

For typical experiments, we plate 40 µL (8,000 cells) per well of a 384-well plate, 100 µL (20,000 cells) per well of a 96-well plate, 500 µL (100,000 cells) per well of a 24-well plate, or 12 mL (2.4 million cells) per 10 cm dish.

4. Every 2 days, feed the cells by removing 50% of the growth medium and adding an equal volume of fresh MGM.

Removal of 100% of the growth medium is damaging to the cells, even when fresh medium is provided immediately. CSF-1 is a critical growth factor present in MGM. Cells rapidly consume CSF-1 and will begin dying within 2–3 days if fresh CSF-1 is not provided by regular 50% medium changes. Figure 2 illustrates how cells should look over the first six days in culture.

5. Perform functional assays.

Cells show hallmarks of classical activation (such as induction of Tnf and Il1b mRNA expression) during the first several hours after isolation. Classical activation markers return to baseline levels within a few days. We typically perform assays at 5–12 days in vitro (div) to allow cells time to recover from the initial activation and to extend processes. Cells can be used for a variety of assays, and we have had success measuring cell survival, morphology, phagocytosis, chemotaxis, and proliferation in response to various stimuli (Bohlen et al., 2017; Collins & Bohlen, 2018). Cultures can additionally be used to study interactions between microglia and other purified cell types through conditioned-medium or co-culture experiments (Liddelow et al., 2017) or through re-implantation into brains that have an open myeloid niche (F. C. Bennett et al., 2018).

REAGENTS AND SOLUTIONS

Materials—DNaseI (Worthington, cat # DPRFS)

10x HBSS no phenol red (Gibco, cat # 14185–052)

Glucose (Sigma, cat # G8270)

1 M HEPES (Gibco, cat # 15630–080)

RNase Inhibitor RNasin (Promega, cat # N2615)

BSA- Bovine Serum Albumin (Sigma, cat # A4161)

0.5 M EDTA stock (Gibco, cat # 15575)

FCS- Fetal Calf Serum (Gibco, cat # 10437–028)

DPBS⁺⁺: Dulbecco's phosphate-buffered saline with calcium and magnesium (Gibco, cat # 14040182)

1x PBS (from 10x stock: Gibco, cat # 70011-044)

Peptone from milk solids (Sigma, cat # P6838)

Heparin (Sigma, cat # H3149)

Percoll PLUS (GE Healthcare, cat # 17-5445-02)

N-acetyl cysteine (Sigma, cat # A9165)

Sodium selenite (Sigma, cat # S-5261)

Apo-transferrin (Sigma, cat # T1147)

Oleic acid (Cayman Chemicals, cat # 90260)

Gondoic acid (Cayman Chemicals, cat # 20606)

Cholesterol from ovine wool (Avanti Polar Lipids, cat # 700000P)

Ethanol

Glass vials

CSF-1, rat or mouse (Peprotech, cat # 400-28 for rat; cat # 315-02 for mouse)

TGF- β 2, human (Peprotech, cat # 100-35B)

Heparan sulfate (Galen Laboratory Supplies, cat # GAG-HS01)

Pen-strep/glutamine (100X), (Gibco, cat # 10378016)

Phenol red free DMEM/F-12 (Gibco, cat # 21041-02)

Reagents and Solutions

DNaseI Stock—Dissolve DNaseI to 4 mg/mL in 1x PBS. Sterile filter and make 200 μ L aliquots. Store at -20°C for up to 1 year.

FACS Homogenization Buffer—Prepare a 30% glucose stock by dissolving glucose in warmed water. Allow time for glucose to dissolve. Add 1.5 mL 1M HEPES, 1.67 mL 30% Glucose, and 10 mL 10x HBSS to 76.8 mL sterile water (15 mM HEPES, 0.5% Glucose, 1x HBSS final). Sterile filter and store at 4°C for up to 2 weeks. On day of prep, add 200 μ L DNaseI stock and 10 μ L RNasin to 5 mL ice-cold homogenization buffer.

MACS Buffer—Dissolve 1.25 g BSA to in 249 mL 1x PBS. Add 1 mL 0.5 mM EDTA stock (1x PBS, 2 mM EDTA, 0.5% BSA final). Sterile filter and store at 4°C for up to 2 weeks.

FACS Buffer—Add 1 mL FCS, 0.4 mL 0.5M EDTA Stock, and 2.5 mL HEPES to 96.1 mL 1x PBS (% FCS, 2 mM EDTA, 25 mM HEPES final). Sterile filter and store at 4°C for up to 2 weeks.

Panning Buffer—Dissolve milk peptone solids to 2 mg/mL in DPBS⁺⁺ and sterile filter. Store at 4°C for up to 2 weeks.

Perfusion Buffer—Prepare a heparin stock solution by dissolving porcine heparin to 50 mg/mL in DPBS⁺⁺. Sterile filter. Dilute heparin stock 100x to 0.5 mg/mL in DPBS⁺⁺ to get perfusion buffer. Store concentrated stock at 4°C for up to 1 year.

Dissociation Buffer—Dilute 200 µL of DNaseI stock into 50 mL of DPBS⁺⁺. Use within 1 day.

Myelin Separation Buffer—Prepare 1M stock solutions of CaCl₂ and MgCl₂ in water. To 90 mL of Percoll PLUS, add 10 mL of 10x DPBS, 90 µL of 1 M CaCl₂ solution, and 50 µL of 1 M MgCl₂ solution. Mix well to get myelin removal buffer. Store at 4°C for up to 1 year.

TNS Stock—Dissolve N-acetyl cysteine to 50 mg/mL in DMEM/F-12. Dissolve sodium selenite to 10 mg/mL in DMEM/F-12. Dissolve 100 mg apo-transferrin in 9.89 mL of DMEM-F12. Mix 100 µL of N-acetyl cysteine stock, 10 µL of sodium selenite stock, and 9.89 mL apo-transferrin. Mix, sterile filter, make 500 µL aliquots, and store at –20°C for up to 1 year.

COG Stock—Prepare a 10x OG stock by diluting oleic acid to 1 mg/mL and gondoic acid to 0.01 mg/mL in ethanol. Dissolve cholesterol to 1.67 mg/mL in warm ethanol. Warm the cholesterol solution for 20 min at 37°C or until cholesterol is fully dissolved. Add 100 µL 10x OG stock to 900 µL of cholesterol solution to make the COG stock. Store in a glass vial at –20°C for up to 1 month.

Use glass vials for all ethanol solutions to prevent leaching impurities from plastics. Crush the cholesterol into fine powder before adding ethanol to facilitate dissolving. Cholesterol can become oxidized and lose activity. Replace the COG solution every month, and replace cholesterol powder stock every 12 months.

TCH Stock—Dissolve CSF-1 to 12.5 µg/mL in 1x PBS. Dissolve human TGF-β2 to 20 µg/mL in PBS. Dissolve heparan sulfate to 10 mg/mL in 1x PBS. Add 50 µL of TGF-β2 solution and 50 µL of heparan sulfate solution to 400 µL of CSF-1 solution and mix. Make 50 µL aliquots and store at –20°C for up to 1 year.

Repeated freeze-thaws will terminate CSF-1 activity. CSF-1 is only effective on CSF1R from the same species, so use rat CSF-1 for rat cultures, mouse CSF-1 for mouse cultures, etc. Murine IL-34 may be used in place of CSF-1 and is active on rat, mouse, and human CSF1R. Use IL-34 at a final concentration of 100 ng/mL instead of 10 ng/mL for CSF-1.

Microglia Growth Medium (MGM)—Warm 49 mL of phenol red free DMEM/F-12 to room temperature. Add 500 μ L Pen-strep/glutamine stock, add 500 μ L TNS stock, add 50 μ L of COG stock, mix well, and add 50 μ L of TCH stock. Store at 4°C for up to 1 week.

Discard unused MGM after 1 week.

Pen-strep/glutamine stock must be warmed to dissolve glutamine.

Addition of COG stock to cold medium will result in precipitation of cholesterol.

COMMENTARY

Background Information

The earliest descriptions of microglial isolation took advantage of differential adherence of microglia versus other cells in long-term neonatal mixed brain cell cultures (Giulian & Baker, 1986). Since then, many surface markers have been identified that can be used to rapidly isolate microglia with improved purity. A number of well-developed protocols have served as the starting point for the optimized methods described here (Garcia et al., 2014; Joseph & Venero, 2013; Sedgwick et al., 1991; Srinivasan et al., 2016; Zhang et al., 2008). We have presented three separate isolation strategies (FACS, magnetic separation, and immunopanning) that we believe represent the best current approaches for microglial isolation, with each demonstrating advantages in purity, throughput, or cost.

We hope that these protocols will facilitate advances in microglial biology and serve as a starting point for future improvements in isolation and culture methods. For example, improved instrumentation or techniques may improve the speed and convenience of FACS sorting or the long-term viability of sorted cells. Magnetic separation approaches can likely be improved to better separate microglia from other CNS CD11b-positive cells, either through negative selection of contaminating cell types or through positive selection with magnetic antibodies against markers such as TMEM119. Applying such strategies to separate out CD11b-positive subpopulations using immunopanning would require additional technical innovations, as myeloid cells have a general propensity to adhere to IgG-coated or even uncoated petri dishes regardless of the specificity of the panning antibody.

Cultured microglia change substantially from their *in vivo* state, but these alterations can be largely reversed by engrafting the cells back into an intact nervous system (F. C. Bennett et al., 2018; Bohlen et al., 2017). Thus, there exist additional cues that instruct microglial state *in vivo* that, if identified, should facilitate recapitulation of resting properties in cultured cells and improve our ability to study the molecular mechanisms underlying core microglial functions. In all, these approaches that have been optimized for microglia should be instructive (at least to a degree) for the isolation and culture of tissue-resident macrophages from other organs.

Critical Parameters

Microglia are highly responsive to CNS tissue damage, which is inevitable during their isolation. As such, it is essential to chill the cells as quickly as possible and keep them cold

for the duration of their purification to prevent induction of immediate early genes such as *Fos11*, *Jund*, and early-response pro-inflammatory cytokines such as *Tnf*, *Ccl3*, and *Ccl4* (Haimon et al., 2018). Surface protein abundance can also rapidly change, skewing FACS plot representations (Bohlen et al., 2017). Here, we describe a non-enzymatic tissue dissociation technique that generates high yields of viable microglia, although other strategies are also effective, such as use of proteases active at 4°C (Srinivasan et al., 2016). Transcriptional inhibitors such as actinomycin D may also be useful in minimizing microglial changes over the course of their isolation (Wu, Pan, Zuo, Li, & Hong, 2017). In any case, it is of critical importance to treat all samples exactly the same when measuring differential gene expression with freshly isolated microglia, and we caution against over-interpretation of changes that may have arisen during isolation of the cells.

Even when care is taken to prevent changes in gene expression during isolation, cultured cells will have been exposed to damage signals and rapidly enter an activated state when returned to physiological temperatures. Expression of classical activation markers is transient and returns to baseline over hours to days, at which point cells are able to respond normally to inflammatory agents (Bohlen et al., 2017). However, this initial response is likely to complicate measurements, particularly those taken shortly after isolation.

Microglial cultures can be sustained under the fully defined, serum-free medium conditions described in Basic Protocol 3, but almost all published studies of microglial properties *in vitro* have been performed in the presence of high concentrations (5–10%) of serum. Even transient serum exposure can have a substantial impact on microglial properties. For instance, microglia cultured in the presence of serum are highly proliferative and exhibit a less ramified morphology (Figure 2). Microglia cultured in the absence of serum are still phagocytically active, but show dramatically reduced phagocytosis relative to their serum-exposed counterparts, beyond what can be explained by the abundance of serum-borne opsonins. Prolonged serum exposure also influences gene expression profiles substantially (Bohlen et al., 2017). In all, serum is a highly complex bioactive additive whose impact should be carefully considered in microglial culture experiments.

As mentioned above, many different myeloid cell populations resemble microglia in their expression of core surface markers such as CD11b. Genes and surface markers unique to the major related populations have been uncovered in mice. For example, neutrophils express high levels of *Camp* and *S100a9* mRNA and can be recognized with Ly6G antibodies. Barrier macrophages express high levels of *Lyve1* and *Clec10a*, and can be recognized by surface presentation of high levels MHCII and CD206. The specificity of these markers may change after experimental manipulations, but can serve as a general guideline for whether measured differences can be explained by altered proportions of these related cell types.

Troubleshooting

For cell isolation procedures, it is important to monitor cell count and viability throughout the procedure to track cell count and survival. The highest cell yields and viability are achieved when the isolation is performed quickly, and both speed and yields will improve with practice.

TMEM119 expression is a valuable marker for microglia in the healthy brain, but protein expression is established relatively late in development, during the second postnatal week (M. L. Bennett et al., 2016; Matcovitch-Natan et al., 2016). Additionally, currently available TMEM119 antibodies for mouse and human are not effective in rat tissue, and we have not identified any surface marker that can perform the equivalent function of separating rat microglia from other rat CD11b-positive cells. Finally, downregulation of *Tmem119* mRNA has been reported in some disease models, suggesting that activated populations of interest may change surface marker expression (Cantoni et al., 2015; Keren-Shaul et al., 2017). Immunohistochemical staining in tissue sections using antibodies against the TMEM119 intracellular domain can help clarify whether TMEM119 protein is present in your targeted cells prior to isolation (M. L. Bennett et al., 2016).

Serum-free microglial cultures require a number of functional reagents, most of which are stored as long-term aliquots. Serum exposure changes the cellular properties, but can also support some level of microglial survival in the absence of most MGM components. Therefore, inclusion of wells cultured with more forgiving serum-containing medium can help to determine whether poor cell vitality is caused by the handling of the cells or problems with medium components.

Understanding Results

Time Considerations—Isolation of microglia from a small number of rodent brains should take a practiced researcher 3–4 hours. Larger-scale preparations are feasible, but will add additional time, particularly to the tissue harvest steps. The more time spent during the isolation, the more gene expression patterns will drift from the initial state.

Microglial cultures require brief but frequent attention, and sustaining cultures will require ~30 min every two days. Culture experiments are typically completed in one or two weeks, but the cells can be maintained for over a month if required.

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Significance statement

As tissue-resident macrophages of the central nervous system, microglia have properties distinct from other macrophage populations that allow them to perform specialized functions. Accordingly, extra considerations should be taken with experimental manipulations and handling of these cells. Here we present best-practice guidelines for multiple approaches of microglial isolation and culture with attention to challenges associated with tissue-resident macrophages generally and microglia specifically.

Figure 1. Representative gating strategy for mouse (top row) and human (bottom row) microglia sorting

We typically gate on FSC/SSC, then single cells, then live cells for both mouse and human. Depending on the goals of a given sort, we then either gate on CD45/CD11B double-positive populations, and individually sort TMEM119-positive and negative cells within this gate (as shown for human microglia), or gate directly on TMEM119-positive cells (as shown for mouse microglia, where a CD45/CD11B double-positive gate is shown, but not used in the gating hierarchy, as evidenced by the very large TMEM119-negative population in the right most column).

Figure 2. Microglial morphology over six days in culture

Representative phase-contrast images of P14 rat microglial cultures over six days in culture illustrating morphological and proliferative differences between cells grown with serum-free medium (top) versus medium containing 10% fetal calf serum (FCS, bottom). CD11b-immunopanned cells were isolated as described in Alternate Protocol 2 and spot-plated as described in Basic Protocol 3 except that IL-34 (100 ng/mL) was used in place of CSF-1. The same field was imaged every 24 hours. Scale bar, 100 μ m. This figure is duplicated from a previous work and used with permission (Collins & Bohlen, 2018).

Table 1.

Estimated microglial yields from various protocols, ages, and species

<i>FACS- Basic Protocol 1</i>				
Species	Age	Weight per brain	Expected FACS yield of TMEM119 ⁺ cells	Notes on TMEM119 surface immunoreactivity
Mouse	<10 days	~0.3–0.5 g	5×10 ⁴ –1×10 ⁵ /brain (double-sort)	Most microglia are immature, showing low or no surface-expression of TMEM119
Mouse	10–21 days	~0.5–0.9 g	5×10 ⁴ –1×10 ⁵ /brain (double-sort)	Most microglia have high TMEM119 surface-expression
Mouse	>21 days	~0.9–1 g	5×10 ⁴ –1×10 ⁵ /brain (double-sort)	High TMEM119 expression in all or almost all parenchymal microglia
<i>MACS/Immunopanning- Basic Protocol 2 and Alternate Protocol 2</i>				
Species	Age	Weight per brain	Expected MACS/IP yield of CD11b ⁺ cells	Notes on viability and quality of cultures
Mouse	<10 days	~0.3–0.5 g	1×10 ⁵ –4×10 ⁵ /brain	High viability cultures, but less robust than rat cultures; cells have not reached maturity
Mouse	10–21 days	~0.5–0.9 g	1×10 ⁵ –4×10 ⁵ /brain	Moderate viability cultures, less robust than rat cultures
Mouse	>21 days	~0.9–1 g	1×10 ⁵ –2×10 ⁵ /brain	Cultures not advised due to poor or variable viability
Rat	<10 days	~0.3–1 g	5×10 ⁵ –2×10 ⁶ /brain	Highest viability cultures, but cells have not reached maturity
Rat	10–21 days	~1–1.5 g	5×10 ⁵ –2×10 ⁶ /brain	High viability cultures with more ramified morphology and mature marker expression than younger cells
Rat	>21 days	~1.5–2 g	3×10 ⁵ –5×10 ⁵ /brain	Moderate viability cultures