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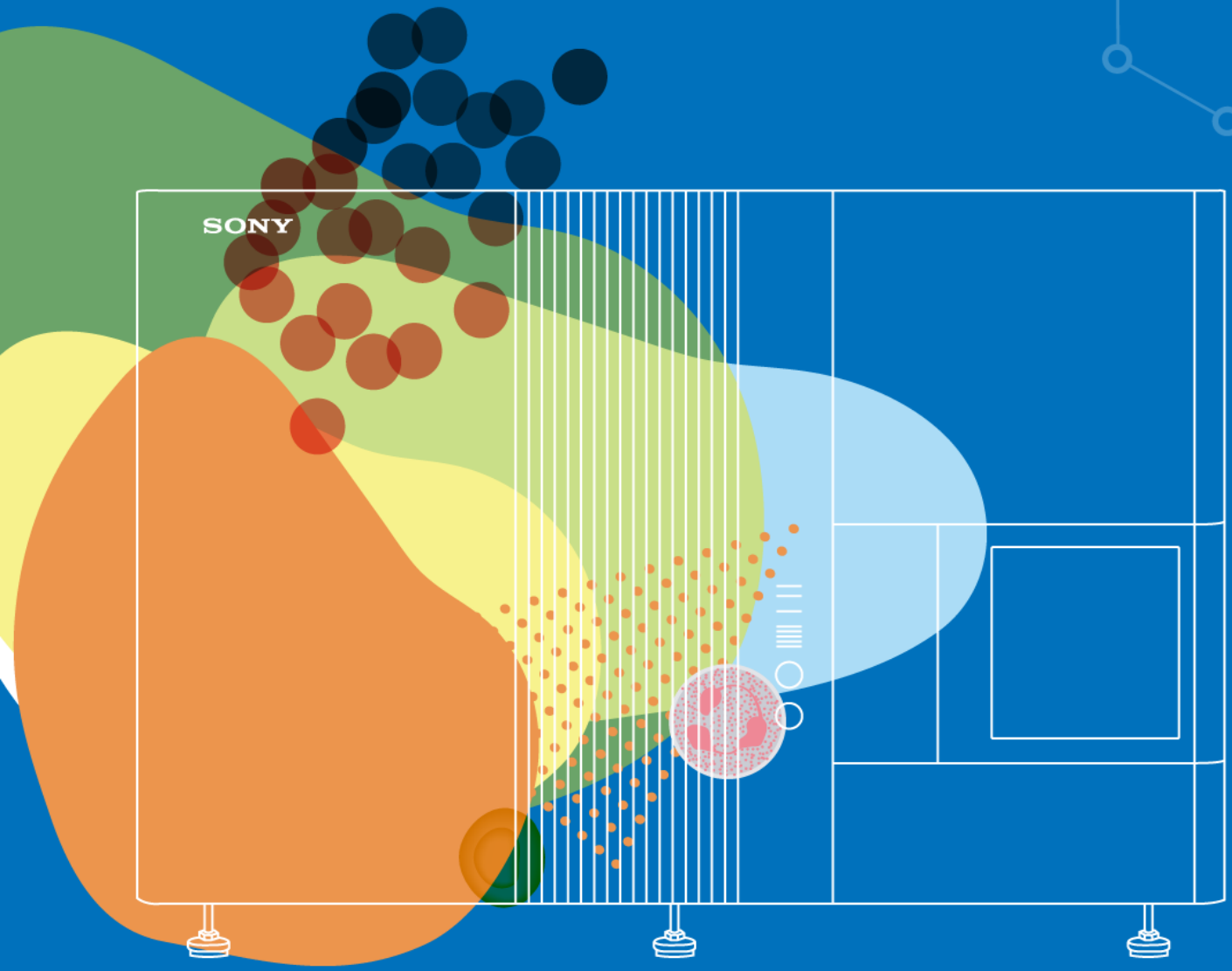

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Isolation and Culture of Microglia

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Microglia represent 5-10% of cells in the central nervous system and contribute to the development, homeostasis, injury, and repair of neural tissues. As the tissue-resident macrophages of the central nervous system, 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 article describes approaches for purification of microglia by fluorescence-activated cell sorting using microglia-specific surface markers and for enrichment of microglia by magnetic sorting and immunopanning. Detailed information about culturing primary microglia at various developmental stages is also provided. Throughout, we focus on special considerations for handling microglia and compare the relative strengths or disadvantages of different protocols. © 2018 by John Wiley & Sons, Inc.

Keywords: culture • FACS • fluorescence-activated cell sorting • immunopanning • MACS • magnetic-activated cell sorting • microglia • tissue-resident macrophage

How to cite this article:
Bohlen, C. J., Bennett, F. C., & Bennett, M. L. (2019). Isolation and culture of microglia. *Current Protocols in Immunology*, *125,* e70. doi: 10.1002/cpim.70

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 central nervous system (CNS) environment, microglia possess features that distinguish them from other tissue-resident macrophages (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 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

CURRENT PROTOCOLS
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Current Protocols in Immunology e70, Volume 125
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpim.70

blood, bone marrow, or the 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 parenchyma, separation of CNS microglia from neighboring cells and the tissue matrix requires extensive tissue damage. As highly sensitive first-responders to injury, microglia have properties that can rapidly change during the isolation procedure unless pointed efforts to mitigate such transformation are employed (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, meningeal, 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 fluorescence-activated cell sorting (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 $CD11b^{+}$ 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.

NOTE: All protocols involving live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations for the care and use of laboratory animals.

NOTE: All protocols involving human samples must conform to appropriate institutional and governmental regulations and require informed consent. In addition, we practice Biosafety Level 2 (BSL-2) precautions with all human samples.

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 (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 (Bennett et al., 2016).

This protocol uses one whole brain from a ≥P10 (postnatal day 10) mouse or up to three pooled whole brains from <P10 mice for each sample. Additional mice can be processed in parallel using separate tubes, separate rounds of tissue homogenization, and additional magnetic columns. Expected cell yields from mice of various ages are listed in Table 1 to help determine the number of mice required for the downstream

Table 1 Estimated Microglial Yields from Various Protocols, Ages, and Species

FACS (Basic Protocol 1)				
Species	**Age**	**Weight per brain**	**Expected yield of TMEM119$^+$ cells**	**Notes on TMEM119 surface immunoreactivity**
Mouse	<10 days	~0.3–0.5 g	5–10 × 10^4/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 × 10^4/brain (double-sort)	Most microglia have high TMEM119 surface-expression
Mouse	>21 days	~0.9–1 g	5–10 × 10^4/brain (double-sort)	High TMEM119 expression in all or almost all parenchymal microglia
MACS/immunopanning (Basic Protocol 2 and Alternate Protocol 2)				
Species	**Age**	**Weight per brain**	**Expected yield of CD11b$^+$ cells**	**Notes on viability and quality of cultures**
Mouse	<10 days	~0.3–0.5 g	1–4 × 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	1–4 × 10^5/brain	Moderate viability cultures, less robust than rat cultures
Mouse	>21 days	~0.9–1 g	1–2 × 10^5/brain	Cultures not advised due to poor or variable viability
Rat	<10 days	~0.3–1 g	5–20 × 10^5/brain	Highest viability cultures, but cells have not reached maturity
Rat	10-21 days	~1–1.5 g	5–20 × 10^5/brain	High viability cultures with more ramified morphology and mature marker expression than younger cells
Rat	>21 days	~1.5–2 g	3–5 × 10^5/brain	Moderate viability cultures

application. The following procedure can be followed for mice of various ages, but mice P10 and younger cannot be euthanized by CO_2 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$^+$ cells from these immature tissues. We typically do not perform endovascular perfusion prior to brain dissection because TMEM119 is expressed only on parenchymal brain macrophages, but it can be performed to suit downstream applications.

To minimize *ex vivo* changes in microglial gene expression, work should be performed on ice using cold buffers whenever possible. Cell dissociation/staining should be completed as rapidly as possible, and the prep should be timed so that sorting can begin immediately after the cells have been stained. Sorting should be performed <2.5 hr after euthanizing mice.

Materials

FACS homogenization buffer (see recipe)
MACS buffer (see recipe)
4 mg/ml DNase I stock (see recipe)
RNasin (Promega, cat. no. N2615)
Donor mice: e.g., 6- to 8-week-old C57BL/6J (Jackson Laboratory, cat. no. 000664)
Myelin removal beads (Miltenyi, cat. no. 130-096-733)
PBS (from 10× stock, Gibco, cat. no. 70011-044)
FACS buffer (see recipe)
Compensation beads (BD Biosciences, cat. no. 552845 or Invitrogen, cat. no. A10628; *optional*)
Live/Dead Green (Life Technologies, cat. no. L34969)
Mouse BD Fc Block (BD Biosciences, cat. no. 553141)
Anti-Tmem119 primary (0.205 mg/ml, Rb anti-ms, Abcam, cat. no. 210405)
Anti-CD45 PE-Cy7 (0.2 mg/ml, Rt anti-ms, Ebiosciences, cat. no. 25-0451-82)
Anti-CD11b PerCP/Cy5.5 (Rt anti-ms/hu, Biolegend, cat. no. 101228)
Anti-rabbit BV421 secondary (0.2 mg/ml, Dk anti-rb, Biolegend, cat. no. 406410)
RNase-free DNase (Qiagen, cat. no. 79254)

Standard CO_2 gas tank and euthanasia chamber
Dissection tools: scissors, spring scissors, forceps
Glass tissue grinder (Wheaton, cat. no. 357424)
70-μm cell strainer (Sigma, cat. no. CLS431751)
50-ml conical tubes (e.g., Falcon)
2-ml microcentrifuge tubes (e.g., Eppendorf)
1-ml syringe
LD columns (Miltenyi, cat. no. 130-042-901)
MACS magnet (Miltenyi, cat. no. 130-090-976)
Tube rocker (e.g., VWR, cat. no. 10159-756)
BD FACSAria II (BD Biosciences)
Low-adhesion microcentrifuge tubes

Prepare single-cell suspension

1. For each sample, prepare the following buffers on ice:

   10 ml FACS homogenization buffer with 400 μl DNase I and 20 μl RNasin
   2 ml MACS buffer with 4 μl RNasin

2. Sacrifice one or two mice at a time, following established institutional guidelines.

   For mice ≥P10: Use a CO_2 euthanasia chamber and compressed gas canister, allowing 4-5 min and ensuring that each mouse is non-responsive before proceeding. After euthanasia, use scissors to decapitate the animals.

   For mice <P10: Perform euthanasia by rapid decapitation with sharpened scissors.

3. 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 to 5 ml FACS homogenization buffer.

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

4. Homogenize tissue in a glass tissue grinder on ice, using two to three strokes. Transfer the suspension through a 70-μm cell strainer to a 50-ml conical tube 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 two to three strokes.

5. Rinse strainer twice with 1 ml FACS homogenization buffer. To maximize yield, 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.

   This will fill several tubes, depending on the final volume of homogenate.

7. Centrifuge cells for 30 sec at 9,300 × *g*, 4°C. Discard supernatant.

8. Resuspend all cells for a given sample in 1.8 ml MACS buffer with 4 μl RNasin.

Remove myelin

9. Mix the myelin removal bead stock slurry, then add 200 μl slurry to the cell suspension. Mix well by gentle pipetting.

10. Divide sample into two separate 2-ml microcentrifuge tubes (1 ml per tube), then incubate at 4°C for 10 min.

11. Meanwhile, place two LD columns per sample into the MACS magnet and rinse with 2 ml MACS buffer.

12. After incubation, dilute suspension in each tube to 2 ml with MACS buffer. Centrifuge cells for 30 sec at 9,300 × *g*, 4°C, and discard the supernatant. Repeat.

13. Resuspend cells in 1 ml MACS buffer per tube.

14. Apply cells from each tube to a separate LD column and collect the flowthrough in 50-ml conical tubes on ice.

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

15. Rinse tubes with 2 ml MACS buffer and apply to columns, collecting the flowthrough in the same 50-ml tubes. Repeat.

    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 pipet to reduce clogging of the column.

16. Aliquot cells collected from the flowthrough into 2-ml tubes. Centrifuge for 30 sec at 9,300 × *g*, 4°C. Discard supernatant.

17. Resuspend pellets in PBS, combining all cells (from both columns) in a final volume of 1 ml PBS per sample.

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

Perform live/dead and cell staining

18. Remove 100 μl cell suspension for negative controls. Dilute by adding 1500 μl FACS buffer, then make five 300-μl aliquots. Label tubes as "CD11b SC", "CD45 SC", "Tmem119 SC", "secondary only", and "unstained" (SC refers to single-color control). Keep on ice.

    Alternatively, we frequently use beads for compensation, especially if the input sample size is limiting

19. To the remaining 900 μl sample, add 1 μl Live/Dead Green and incubate in the dark at 4°C for 5 min.
20. Dilute sample to 2 ml with FACS buffer.
21. Centrifuge the Live/Dead–treated cells for 30 sec at 9,300 × *g*, 4°C. Discard supernatant.
22. Resuspend cells in 320 μl FACS buffer.
23. Remove 20 μl and dilute with 580 μl FACS buffer. Split into two tubes labeled "Live/Dead SC" (Live/Dead only control) and "FMO" (full minus one control).
24. Label the tube containing the remaining 300 μl of Live/Dead–treated cells "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 the negative control, SC, and FMO tubes.

25. Add 5 μl mouse Fc receptor block to each tube.
26. To the tubes labeled "All" and "Tmem119 SC", add anti-Tmem119 primary antibody.

    Titrate antibody concentration empirically. We observe small fluctuations between lots in effective concentration, but typically stain at 0.1-0.5 μg/ml final antibody concentration.

27. Incubate 15-20 min at 4°C on a tube rocker (~18 rpm).
28. Wash the "All" and "Tmem119 SC" tubes by bringing the volume to 2 ml with FACS buffer and centrifuging for 30 sec at 9,300 × *g*, 4°C. Discard supernatant and resuspend pellets in 300 μl FACS buffer.
29. Add 1 μl CD11b-PerCP/Cy5.5, 1 μl CD45-PE-Cy7, and 1 μl anti-rabbit BV421 secondary antibody to the "All" and "FMO" tubes and, as appropriate, to the SC and secondary only control tubes.
30. Incubate 15 min at 4°C on a tube rocker (~18 rpm).
31. Wash cells by bringing the volume to 2 ml with FACS buffer and centrifuging for 30 sec at 9,300 × *g*, 4°C. Discard supernatant and repeat wash.
32. Resuspend cells for sorting in 300 μl FACS buffer containing 3 μl RNase-free DNase and 0.6 μl RNasin.

    At this point you will have eight tubes containing 300 μl 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.

Perform FACS

33. On a BD FACSAria II, prepare the 100-μm nozzle and tube chillers (set to 4°C).

    We typically set the flow rate to 1 (~10 μl/min) to maximize sort quality.

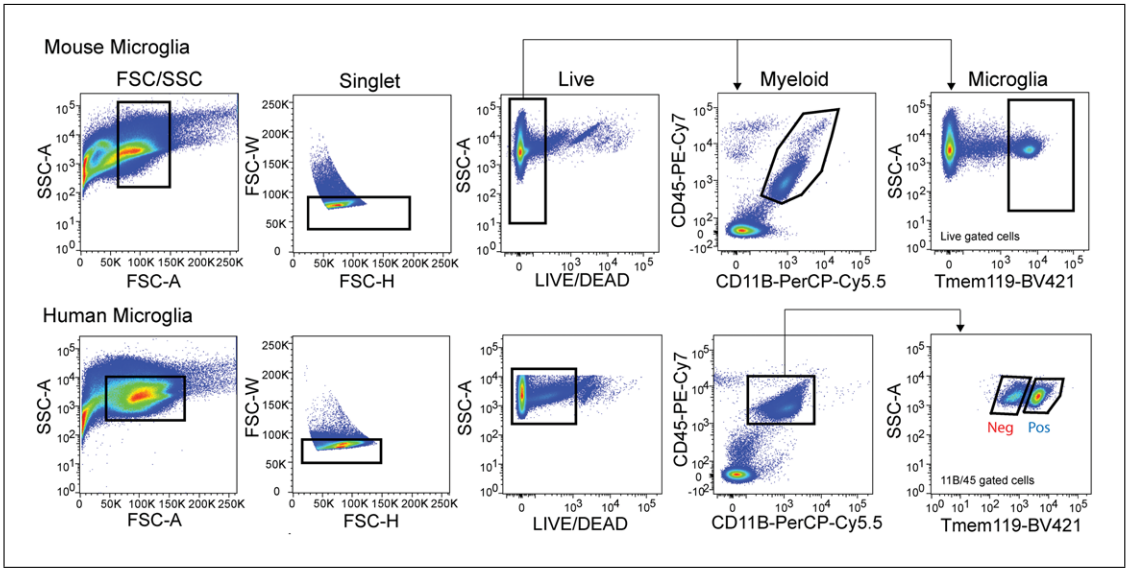

Figure 1 Representative gating strategy for mouse (top row) and human (bottom row) microglia sorting. We typically gate on FSC/SSC, then single cells, then live cells for both mouse and human. Depending on the goals of a given sort, we then either gate on $CD45^+/CD11B^+$ populations and individually sort $TMEM119^+$ and $TMEM119^-$ cells within this gate (as shown for human microglia), or gate directly on $TMEM119^+$ cells (as shown for mouse microglia, where a $CD45^+/CD11B^+$ gate is shown, but not used in the gating hierarchy, as evidenced by the very large $TMEM119^-$ population in the right-most column).

34. After compensation using SC 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.

35. Load the "All" tube into the instrument and sort into low-adhesion microcentrifuge tubes containing FACS buffer and RNasin (2 µl per 1 ml FACS buffer) using four-way sorting. Prepare two collection tubes: one for $Tmem119^+$ cells (microglia) and one for $Tmem119^-/CD45^{Hi}/CD11b^+$ cells (myeloid cells), which represent non-microglial immune cells that may be useful for comparison.

    If high purity is needed, the cells can be resorted. 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 cells for RNA analyses, sort directly into lysis buffer (e.g., Qiagen Buffer RLT [cat. no. 79216] supplemented with β-mercaptoethanol) to halt any further transcriptional changes from the cells.

    Although we have successfully used FACS-sorted microglia for in vivo transplantation and cell culture, we find that FACS sorting significantly reduces microglial viability.

ISOLATION OF HUMAN MICROGLIA BY FACS

ALTERNATE PROTOCOL 1

Microglia from human tissue also exhibit surface expression of TMEM119 (Bennett et al., 2016; Bennett et al., 2018). Here we provide a FACS protocol for efficient isolation of high-purity microglia from human tissue using a monoclonal antibody that we recently developed to recognize a surface-exposed epitope in the N-terminal domain of TMEM119 (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 the number of myelin depletion columns to use based on input tissue, and gating for $TMEM119^+$ cells for sorting, as this staining is dimmer in human tissue than in mouse.

For this protocol, each sample refers to up to 750 mg 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.

Additional Materials *(also see Basic Protocol 1)*

Human tissue
Human BD Fc Block (BD Biosciences, cat. no. 564220)
Anti-Tmem119 primary (0.5 mg/ml, Ms anti-hu, Biolegend, cat. no. A16075D)
Anti-CD45 PE-Cy7 (0.2 mg/ml, Ms anti-hu, BD Biosciences, cat. no. 557748)
Anti-mouse BV421 secondary (0.2 mg/ml, Gt anti-ms, Biolegend, cat. no. 405317)
Isotype control (IgG2b, k, Biolegend, cat. no. 401201)

1. For each sample, prepare the following buffers on ice:

   10 ml FACS homogenization buffer with 400 μl DNase I and 20 μl RNasin
   2 ml MACS buffer with 4 μl RNasin

2. Homogenize the tissue, perform myelin depletion, and perform Live/Dead staining as described above for mouse tissue (see Basic Protocol 1, steps 4-24), with the following exceptions:

   In step 10, distribute the sample equally to three separate tubes (667 μl per tube).
   In step 11, use three LD columns per sample instead of two.
   In step 18, change the label of the "secondary only" tube to "isotype".

   You should have eight tubes containing 300 μl FACS buffer and varying amounts of cells.

3. Add 30 μl human Fc receptor block to each tube and incubate for 5 min at room temperature.

4. To the "All" and "Tmem119 SC" tubes, add anti-Tmem119 primary antibody to reach a final concentration of 0.3 μg/ml. To the "FMO" and "isotype" tubes, add a matched concentration of IgG2b isotype control antibody.

   Titrate antibody concentration empirically. We have observed decreased signals at higher final concentrations of primary antibody, with maximal signal typically observed at 0.3 μg/ml or lower.

5. Incubate 10-15 min at room temperature on a tube rocker (~18 rpm).

6. Wash the "All", "Tmem119 SC", "FMO", and "isotype" tubes by bringing the volume to 2 ml with FACS buffer and centrifuging for 30 sec at 9,300 × *g*, 4°C. Discard supernatant and resuspend pellets in 300 μl FACS buffer.

7. Add 1 μl CD11b-PerCP/Cy5.5, 1 μl CD45-PE-Cy7, and 1 μl anti-mouse BV421 secondary antibody to the "All" and "FMO" tubes and, as appropriate, to the SC and isotype only control tubes.

8. Incubate 10 min at room temperature on a tube rocker (~18 rpm).

9. Wash cells by bringing the volume to 2 ml with FACS buffer and centrifuging for 30 sec at 9,300 × *g*, 4°C. Discard supernatant and repeat wash.

10. Resuspend cells for sorting in 300 μl FACS buffer containing 3 μl RNase-free DNase and 0.6 μl RNasin.

11. Perform FACS as described (see Basic Protocol 1, steps 33-35).

We observe significant nonspecific staining of human CD45$^+$/CD11b$^+$ human cells with secondary antibody for TMEM119. It is critical to set TMEM119$^+$ gates based on isotype control staining, which is nearly identical to secondary only staining, or occasionally slightly higher.

ENRICHMENT OF MICROGLIA BY MAGNETIC CD11B SELECTION

BASIC PROTOCOL 2

Although 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 the magnetic protocol (provided here) and the immunopanning protocol (see Alternate Protocol 2).

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$^+$ cells over other major CNS cell types, and the large majority of CD11b$^+$ 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. Magnetic isolation can be considered for routine culture applications (where survival of non-microglial myeloid cells can be selected against; Collins & Bohlen, 2018) or for applications in which demands for throughput outweigh the importance of purity in the initial population.

Both magnetic and immunopanning protocols have been optimized for isolation of CD11b$^+$ cells out of whole brain from P6-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.

This protocol uses two whole rat brains for each sample. Up to five samples can be pooled on a single LD or LS column. We typically process a full litter of eight to ten P14 rats in each preparation, but this can be adjusted as necessary for downstream assays using the guidelines in Table 1. With practice, the yield 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.

Materials

Donor rats (recommended P7-P21, Charles River, strain code 400)
Ketamine
Xylazine
Perfusion buffer (see recipe)
Dissociation buffer (see recipe)
Myelin separation buffer (see recipe)
PBS (from 10× stock, Gibco, cat. no. 70011-044)
Myelin removal beads (Miltenyi, cat. no. 130-096-733)
Rat CD11b/c (Microglia) MicroBeads (Miltenyi, cat. no. 130-105-643)
MGM (see recipe; *optional*)

Standard CO_2 gas tank and euthanasia chamber
1-ml syringe with 5/8-in., 25-G needle (Becton Dickinson, cat. no. 309626)
Dissection tools: scissors, spring scissors, forceps
20-ml syringe with 21-G needle
Razor blade (VWR, cat. no. 55411-050)
Glass tissue grinder (Wheaton, cat. no. 357424)
50-ml conical tubes (e.g., Falcon)
2-ml microcentrifuge tubes (e.g., Eppendorf)
LD columns (Miltenyi, cat. no. 130-042-901)
LS columns (Miltenyi, cat. no. 130-042-401)
MACS magnet (Miltenyi, cat. no. 130-090-976)

NOTE: All solutions and instruments should be ice-cold before beginning the protocol.

Collect tissue

1. Euthanize rats following established institutional guidelines. If processing tissue from multiple animals, stagger the CO_2 or anesthetic administration such that tissue is harvested from each animal as quickly as possible after euthanasia.

   For rats ≥P10: Use a CO_2 euthanasia chamber and compressed gas canister, allowing 4-5 min and ensuring that each rat is non-responsive before proceeding.

   For rats <P10: Perform euthanasia with an intraperitoneal injection of ketamine/xylazine (100-200 µl of 24 mg/ml ketamine, 2.4 mg/ml xylazine) using a 1-ml syringe with a 5/8-in., 25-G needle.

2. Transcardially perfuse rat with 10-30 ml ice-cold perfusion buffer using a 20-ml syringe with a 21-G needle.

   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.

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 as quickly as possible to 10 ml chilled dissociation buffer on ice.

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

4. Repeat steps 1-3 as needed for remaining animals.

Dissociate tissue

5. Transfer each sample (two brains) to a Petri dish lid with ∼1 ml dissociation buffer and chop into ∼1-mm^3 chunks using a razor blade.

   Only two brains should be processed at a time in the homogenizer.

6. Transfer chunks to a glass tissue grinder and add 4.5 ml dissociation buffer. Dissociate 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.

8. Repeat steps 5-7 for remaining samples.

   This mechanical homogenization kills a substantial portion of other CNS cell types, but microglia are preferentially spared.

Remove myelin and debris

9. Add cold dissociation buffer to each sample to bring the total volume to 33.5 ml.
10. Add 10 ml cold myelin separation buffer to each sample 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.

11. Centrifuge cells for 15 min at 500 × *g*, 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.

12. Remove the top layer and supernatant using a pipet.
13. Resuspend cell pellets and pool into 2.7 ml PBS per ∼10 g starting tissue (eight to ten juvenile rat brains or four to five pooled samples).

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

14. Add 300 µl myelin removal beads for every 2.7 ml PBS in step 13, mix well, and split the sample to two 2-ml microcentrifuge tubes. Incubate at 4°C for 10 min.
15. Meanwhile, place one LD column into the MACS magnet and rinse with 2 ml PBS.
16. Centrifuge cell suspension for 30 sec at 5,000 × *g*, 4°C. Discard supernatant.
17. Resuspend each pellet in 2 ml PBS, repeat centrifugation, and remove the supernatant.
18. Using only 0.5 ml PBS, resuspend the pellets and apply suspension to the LD column. Collect flowthrough in a 50-ml conical tube on ice.

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

19. Wash column twice with 1 ml PBS, collecting the flowthrough in the same 50-ml tube.
20. Divide the flowthrough into two 2-ml chilled microcentrifuge tubes, then centrifuge 30 sec at 5,000 × *g*, 4°C.

Perform CD11b selection

21. Resuspend all cells and pool into 180 µl PBS per ten juvenile rat brains or equivalent amount of tissue.
22. Add 20 µl rat CD11b microbeads, mix well, and incubate at 4°C for 10 min.

    Mouse/human CD11b microbeads are also effective for isolating CD11b$^+$ cells from those species.

23. Meanwhile, place one LS column into the MACS magnet and rinse with 2 ml PBS.
24. Dilute cell suspension with 1 ml PBS and centrifuge for 30 sec at 5,000 × *g*, 4°C. Discard supernatant.
25. Resuspend pellet in 0.5 ml PBS and apply suspension to the LS column. Collect flowthrough in a 50-ml tube.
26. Add 2 ml PBS to wash away CD11b$^-$ cells, allowing the full 2 ml to pass through the column. Repeat for a total of three washes, then discard the flowthrough.

27. Remove the LS column from the magnet, add 2 ml PBS or MGM, and use the plunger to elute the $CD11b^+$ cells.

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.

In this protocol, one immunopanning dish can accommodate two juvenile rat brains or up to six neonatal rat brains. See Table 1 for estimated brain weights and yields from rats and mice of various ages. With practice, cell yields should be comparable to those described for magnetic separation (see Basic Protocol 2).

Additional Materials *(also see Basic Protocol 2)*

50 mM Tris, pH 9.5
Goat anti-mouse IgG (H+L chains, Jackson ImmunoResearch, cat. no. 115-005-003)
Panning buffer (see recipe)
OX42 monoclonal mouse anti-ratCD11b antibody (Bio-Rad, cat. no. MCA275G)
M1/70 monoclonal rat anti-mouse/human CD11b antibody (Thermo Scientific, cat. no. 14-0112-81; *optional*)
Dulbecco's phosphate-buffered saline with calcium and magnesium ($DPBS^{++}$, Gibco, cat. no. 14040182)
TrypLE Express Enzyme (no phenol red, Gibco, cat. no. 12604013)
MGM (see recipe)

15-cm untreated plastic Petri dish (Falcon, cat. no. 351058)
70-μm cell strainer (Sigma, cat. no. CLS431751)
10-ml pipet with pipet controller
Basic light microscope (e.g., Zeiss Axiovert 40 C)
15-ml conical tubes (e.g., Falcon)
Hemocytometer

Prepare panning dish

1. Add 25 ml of 50 mM Tris, pH 9.5, to a 15-cm untreated plastic Petri dish.
2. Add goat anti-mouse IgG (H+L chains) at a final concentration of 6 μg/ml and incubate at 37°C for 1-3 hr.
3. Rinse dishes three times with panning buffer.
4. Replace final rinse with panning buffer containing 1 μg/ml OX42 antibody. Leave dishes on a 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.

Prepare cell suspension and deplete myelin

5. The next day, generate a single-cell suspension and deplete myelin as described for magnetic separation (see Basic Protocol 2, steps 1-12).

6. Resuspend cell pellets from up to 5 mg starting tissue (approx. two to six rat brains, depending on age) in 1 ml panning buffer, then dilute to 12 ml with panning buffer.

Perform immunopanning

7. Pass cell suspension through a 70-μm cell strainer.

8. Rinse the OX42-coated panning dish three times with $DPBS^{++}$.

   Do not allow the plate to dry between washes.

9. Remove the last wash and apply the cell suspension to the panning dish. 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 dish ten times with PBS to remove non-adherent cells. Swirl the plate with each rinse to ensure removal of non-adherent cells.

    The microglia will remain firmly attached to the panning dish.

11. Replace the last wash with 15 ml TrypLE Express Enzyme and incubate 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.

12. Pour off TrypLE and gently wash twice with 15 ml PBS.

13. Replace the last wash with 12 ml ice-cold MGM and place the dish on ice for 2 min to help weaken cell/substrate interactions.

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

14. Using a 10-ml pipet and pipet controller on high speed, pipet vigorously to dislodge cells from the dish.

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

15. Use a microscope to mark spots on the dish where cells are still attached, then repeat pipetting in those areas.

16. Collect cell suspension and divide into four 15-ml conical tubes.

17. Centrifuge 15 min at 500 × *g*, 4°C with slow braking.

18. Aspirate the supernatant, leaving 0.5 ml MGM with the cell pellet. Resuspend cells in the remaining MGM, then pool all cells, and count with a hemocytometer.

CULTURE OF RODENT MICROGLIA

BASIC PROTOCOL 3

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). $CD11b^{+}$ 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/substrates 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. $CD11b^+$ 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* (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; Erblich, 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; Buttgereit 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 microglial 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 microglial signature genes (Bohlen et al., 2017). Thus,

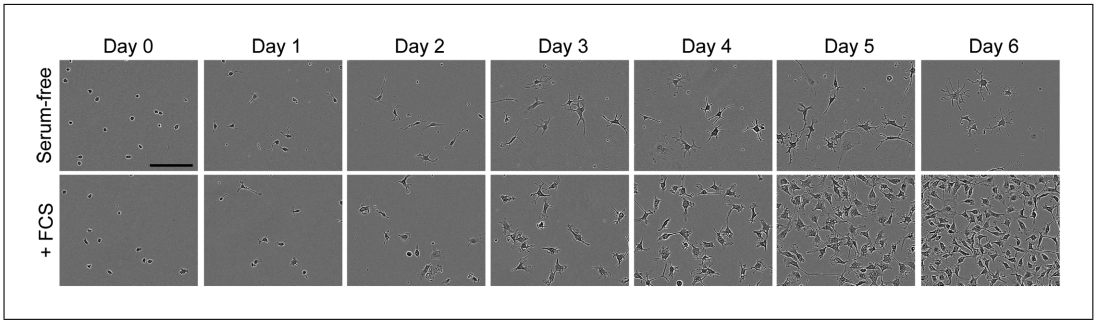

Figure 2 Microglial morphology over six days in culture. Representative phase-contrast images of P14 rat microglial cultures over six days, illustrating morphological and proliferative differences between cells grown with serum-free medium (top) versus medium containing 10% fetal calf serum (FCS, bottom). 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 hr. Scale bar, 100 μm. This figure is duplicated from Collins & Bohlen (2018) and used with permission.

these cultures serve as an imperfect model with advantages over other non-microglial macrophage cultures or cell lines.

Additional Materials *(also see Basic Protocol 2 and Alternate Protocol 2)*

Collagen IV (Corning, cat. no. 354233)
DMEM/F-12 (phenol red free, Gibco, cat. no. 21041-02)
Tissue culture plates (e.g., Corning Primaria TC-treated 24-well plates, cat. no. 734-0078, or Falcon TC-treated 384-well plates, cat. no. 353961)
Poly-D-lysine-coated coverslips (Fisher Scientific, cat. no. NC0343705; *optional*)

1. Dilute collagen IV to 2 μg/ml in DMEM/F-12 and add sufficient volume to fully coat tissue culture plates or dishes. Incubate plates 1-2 hr at 37°C.

   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; 12-mm glass coverslips can be placed in 24-well plates, but should be coated with PDL (poly-D-lysine) prior to additional coating with collagen to facilitate adhesion.

2. Aspirate off collagen solution and allow plates to dry >10 min at room temperature.

3. Prepare fresh MGM and purify microglia as described (see Basic Protocol 2 or Alternate Protocol 2).

4. Dilute cells to a concentration of 2×10^5 cells/ml in MGM.

5. Add the appropriate volume of cell suspension to each well of the coated culture plate and place in a humidified 37°C incubator with 5-10% CO_2.

   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.

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

   CSF-1 is a critical growth factor present in MGM. Cells consume CSF-1 rapidly and will begin dying within 2-3 days if fresh CSF-1 is not provided by regular medium changes. However, removal of 100% of the medium is damaging to cells, even when fresh medium is provided immediately.

   Figure 2 illustrates how cells should look over the first 6 days in culture.

7. 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 (Bennett et al., 2018).

REAGENTS AND SOLUTIONS

COG stock

Prepare a 10× OG stock by diluting oleic acid (Cayman Chemicals, cat. no. 90260) to 1 mg/ml and gondoic acid (Cayman Chemicals, cat. no. 20606) to 0.01 mg/ml in ethanol. Dissolve cholesterol (from ovine wool, Avanti Polar Lipids, cat. no. 700000P) 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 10× OG stock to 900 µl cholesterol solution to make the COG stock. Store in a glass vial up to 1 month at −20°C.

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

DNase I stock, 4 mg/ml

Dissolve DNase I (Worthington, cat. no. DPRFS) to 4 mg/ml in 1× PBS (Gibco, cat. no. 70011-044). Filter sterilize and make 200-µl aliquots. Store up to 1 year at −20°C.

Dissociation buffer

Dilute 200 µl of 4 mg/ml DNase I stock (see recipe) in 50 ml Dulbecco's phosphate-buffered saline with calcium and magnesium ($DPBS^{++}$; Gibco, cat. no. 14040182). Prepare fresh.

FACS buffer

96.1 ml 1× PBS (Gibco, cat. no. 70011-044)
1 ml fetal calf serum (Gibco, cat. no. 10437-028; 1% final)
0.4 ml 0.5 M EDTA (Gibco, cat. no. 15575; 2 mM final)
2.5 ml 1 M HEPES (Gibco, cat. no. 15630-080; 25 mM final)
Filter sterilize
Store up to 2 weeks at 4°C

FACS homogenization buffer

For glucose stock: Prepare a 30% (w/v) stock by dissolving glucose (Sigma, cat. no. G8270) in warm water. Allow time for glucose to dissolve. Store up to 1 year at 4°C.

For buffer:
76.8 ml sterile water
1.5 ml 1 M HEPES (Gibco, cat. no. 15630-080; 15 mM final)
1.67 ml 30% glucose (0.5% final)
10 ml 10× HBSS (no phenol red, Gibco, cat. no. 14185-052; final 1×)
Filter sterilize
Store up to 2 weeks at 4°C

MACS buffer

249 ml 1× PBS (Gibco, cat. no. 70011-044)
1.25 g BSA (Sigma, cat. no. A4161; final 0.5%)
1 ml 0.5 mM EDTA (Gibco, cat. no. 15575; final 2 mM)
Filter sterilize
Store up to 2 weeks at 4°C

Microglial growth medium (MGM)

49 ml DMEM/F-12 (phenol red free, Gibco, cat. no. 21041-02)
500 µl 100× Pen-strep/glutamine stock (Gibco, cat. no. 10378016)
500 µl TNS stock (see recipe)
50 µl COG stock (see recipe)
50 µl TCH stock (see recipe)

Warm all ingredients to room temperature. Combine first four ingredients in order, mix well, then add TCH stock. Store up to 1 week at 4°C

DMEM/F-12 and pen-strep/glutamine must be warmed to ensure that glutamine dissolves completely. Also, addition of COG stock to cold medium will result in precipitation of cholesterol.

Myelin separation buffer

90 ml Percoll PLUS (GE Healthcare, cat. no. 17-5445-02)
10 ml 10× DPBS (no calcium, no magnesium, Gibco, cat. no. 14200075)
90 µl 1 M $CaCl_2$
50 µl 1 M $MgCl_2$
Store up to 1 year at 4°C

Panning buffer

Dissolve milk peptone solids (Sigma, cat. no. P6838) to 2 mg/ml in Dulbecco's phosphate-buffered saline with calcium and magnesium ($DPBS^{++}$; Gibco, cat. no. 14040182). Filter sterilize and store up to 2 weeks at 4°C.

Perfusion buffer

Heparin stock: Prepare a 50 mg/ml heparin stock solution by dissolving porcine heparin (Sigma, cat. no. H3149) in Dulbecco's phosphate-buffered saline with calcium and magnesium ($DPBS^{++}$; Gibco, cat. no. 14040182). Filter sterilize. Store up to 1 year at 4°C.

Perfusion buffer: Dilute heparin stock 100× to 0.5 mg/ml in $DPBS^{++}$. Prepare fresh.

TCH stock

Dissolve rat or mouse CSF-1 (Peprotech, cat. no. 400-28 or 315-02) to 12.5 µg/ml in 1× PBS (Gibco, cat. no. 70011-044). Dissolve human TGF-β2 (Peprotech, cat. no. 100-35B) to 20 µg/ml in 1× PBS. Dissolve heparan sulfate (Galen Laboratory Supplies, cat. no. GAG-HS01) to 10 mg/ml in 1× PBS. Add 50 µl TGF-β2 solution and 50 µl heparan sulfate solution to 400 µl CSF-1 solution. Mix and then make 50-µl. Store up to 1 year at −20°C.

Repeated freeze-thaw cycles 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.

TNS stock

Dissolve *N*-acetyl cysteine (Sigma, cat. no. A9165) to 50 mg/ml in DMEM/F-12 (phenol red free, Gibco, cat. no. 21041-02). Dissolve sodium selenite (Sigma, cat. no. S-5261) to 10 mg/ml in DMEM/F-12. Dissolve 100 mg apo-transferrin (Sigma, cat. no. T1147) in 9.89 ml DMEM-F12. Combine 100 µl *N*-acetyl cysteine stock, 10 µl sodium selenite stock, and 9.89 ml apo-transferrin. Mix, filter sterilize, and make 500-µl aliquots. Store up to 1 year at −20°C.

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 $CD11b^+$ CNS 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 $CD11b^+$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 (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 the purification to prevent induction of immediate early genes such as *Fosl1*, *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 the 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 substantially influences gene expression profiles (Bohlen et al., 2017). In all, serum is a highly complex bioactive additive, the impact of which 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 throughout the procedure to track cell viability and yields. 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 (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$^+$ 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 the targeted cells prior to isolation (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 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.

Anticipated Results

The expected yields, FACS profiles, and cellular morphology in culture are illustrated in Table 1, Figure 1, and Figure 2, respectively.

Time Considerations

Isolation of microglia from a small number of rodent brains should take a practiced researcher 3-4 hr. 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 2 days. Culture experiments are typically completed in 1-2 weeks, but the cells can be maintained for over a month if required.

Acknowledgments

We thank Hannah Collins, Andrew Tucker, and Sara Mulinyawe for assistance in developing various aspects of these protocols, and David Hansen for helpful feedback on the manuscript. This work was supported in part by NIH S10RR025518-01 (Stanford Shared FACS Facility), National Research Service Award (from National Institutes of Health) predoctoral Fellowship F31 NS078813 (to M.L.B.), and training Grants T32MH019938-22 and K08MH112120 (to F.C.B.).

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