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Isolation and Phenotyping of Adult Mouse Microglial Cells

Methods in Molecular Biology Book Chapter

Kathleen Grabert¹, Barry W. McColl^{1,2}¹The Roslin Institute and R(D)SVS, University of Edinburgh, Edinburgh, UK;²Dementia Research Institute, Edinburgh Medical School, University of Edinburgh, Edinburgh, UK

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satyavati Kharde

ABSTRACT

Microglia are the resident macrophages of the central nervous system parenchyma and fulfill crucial roles in brain development, homeostasis, and inflammation. The isolation of a pure microglia population from brain tissue enables the examination of microglial phenotypes without the interference of other cell populations. Microglial extractions from the neonatal brain have been described in various protocols, yet the more established and complex adult mouse brain poses a greater challenge. Here we describe a refined protocol including enzymatic and mechanical dissociation of adult mouse brain tissue and removal of myelin by Percoll density gradient. Microglial cells were subsequently extracted by an immunomagnetic approach. This isolation procedure enables the use of functionally viable cells for various applications such as cell culture, flow cytometry, functional assays including bacteria- or bead-based phagocytosis, stimulation assays, and transcriptome profiling techniques such as qRT-PCR and microarray/RNA sequencing.

ATTACHMENTS

Isolation and Phenotyping
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KEYWORDS

Microglia, Enzyme digestion, Percoll gradient, CD11b immunomagnetic beads

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Oct 21, 2020  Lenny Teytelman protocols.io

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GUIDELINES

1 Introduction

Microglia, specially adapted tissue-resident macrophages of the central nervous system (CNS), are highly dynamic and vital players in CNS physiology and neuroimmune function. Their multifunctional role begins during embryonic and early postnatal development sculpting the developing CNS [1] by supporting synaptic maturation of active neurons through synaptic pruning and remodeling [2–4]. In adulthood under steady-state conditions microglia function further extends to include maintenance of brain homeostasis by continuous surveillance of their local environment and neural parenchyma, examining synaptic activity [5], and phagocytic removal of cell debris [6]. As one of the earliest responders to sterile and microbial inflammatory stimuli, activated microglia migrate to sites of injury/infection and initiate neuroimmune and inflammatory responses involving the internalization of damaged cells or invading pathogens, and the synthesis of a variety of immunoregulatory and inflammatory components [7–9] for the protection and repair of the brain [10, 11]. Dysregulation of microglial activity is also increasingly implicated in a range of neurological conditions [12, 13].

Yolk sac-derived microglia migrate into the brain during early development and are evident from embryonic day (E) 9.5 in mice [14] and soon after birth these cells comprise 5–12% of the total number of cells [15]. The investigation of the phenotypes and crucial functions of microglia in response to distinct physiological and pathological conditions, including ageing, has been constrained by the considerable challenge to directly isolate pure populations of microglia from the adult brain. Over the last 20 years, a range of methods have evolved from initial in vitro culture systems of microglia purification from rodent neonates [16, 17] and early postnatal brains [18] to adult microglia in rats [19, 20] and humans [21, 22] as well as more recently the generation of microglia derived from induced pluripotent stem cells (iPSC) [23, 24]. Due to the low abundance of these cells the strategy of microglial isolation has become crucial to achieve sufficient numbers and with minimal deviation from the microglial in vivo signature. The latter is crucial as it has been reported that a few hours after isolation and culture the unique signature of microglia is downregulated and ultimately their functional phenotype altered [25, 26]. However, efforts are made to identify key factors, which promote an in vivo phenotype in culture [27, 28] after the isolation as described in this protocol. In addition to the investigation of whole brain microglia, the isolation of cells was furthermore applied to study discrete brain regions [29] and single cells [30] to identify discrete microglia phenotypes and their contribution to age-related neurodegenerative diseases.

In general, the isolation of microglia from the adult mouse brain as described here is composed of three steps. The brain tissue is first dissociated with enzymes and subsequently homogenized by a Dounce homogenizer. To facilitate the separation of microglia, myelin is removed by a two-layer density gradient, followed by the labeling of

microglia with CD11b immunomagnetic beads and magnetic separation of CD11b-positive microglia from the remaining brain cell suspension. Once isolated and cell purities and yield are confirmed, extracted microglial cells are suitable for a range of downstream phenotyping and functional applications including flow cytometry, cell culture, stimulation and phagocytosis assays, and -omics profiling.

4 Notes

1. All procedures should be performed under the tissue culture hood, particularly if intending to use isolated cells for subsequent cell culture.
2. By performing perfusions blood is removed from the circulation and minimizes the contamination with red blood cells, which has been observed to reduce the efficiency of the bead-based microglia separation from brain cell suspension, and other immune cells such as CD11b+ leukocytes.
3. To shorten the time frame of the overall procedure the brain tissue digest was performed without and with different durations of enzyme mix. Our results (see Fig. 2) demonstrated that an enzymatic digestion improved yield of microglia.
4. Rotating movement is not necessarily required; the pieces of brain tissue should be under gentle movement to avoid pelleting and insufficient enzymatic digestion.
5. Brain cell suspension should have a homogenous and milky appearance after Dounce homogenization.
6. To achieve a good separation of mixed brain cells from the myelin the ratio between tissue and Percoll volume is crucial. A surplus of tissue in the gradient will impair the yield and purity of microglial cells.
7. Resting the samples enables the settling and strengthening of the different layers.
8. The application of CD11b MicroBeads (clone: M1/70) will lead to the reduced binding capacity of any CD11b flow cytometric antibodies if the clone of both antibodies is the same and therefore competing for binding positions. Difference in a lower CD11b staining of positive selected microglia is evident when compared to pre-sorted microglia within the mixed brain cell suspension after myelin removal (see Fig. 3). In view of recent data [27, 31, 32] other antibodies (e.g., TMEM119 or FCRLs) could be applied for microglia isolation as CD11b does not necessarily label microglia only, particularly in inflamed conditions.
9. Flow-through containing depleted fraction can be collected to confirm negligible loss of microglia or to collect information from remaining cells contained in the brain cell suspension.
10. Let wash buffer run through completely each time for maximum washing efficiency.
11. Nonselected and pre-sorted samples can be used for flow cytometry to compare to purified microglia and for the validation of specificity.
12. Depending on the protein of interest, intracellular staining or a secondary antibody may be required.

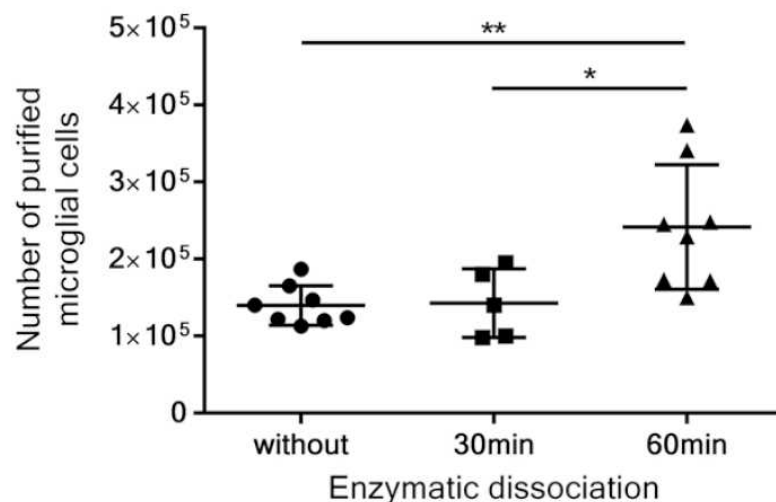


Fig. 2 Effect of enzymatic treatment. The application and duration of the enzymatic digestion of whole-brain tissue resulted in variance in the yield of extracted microglial cells

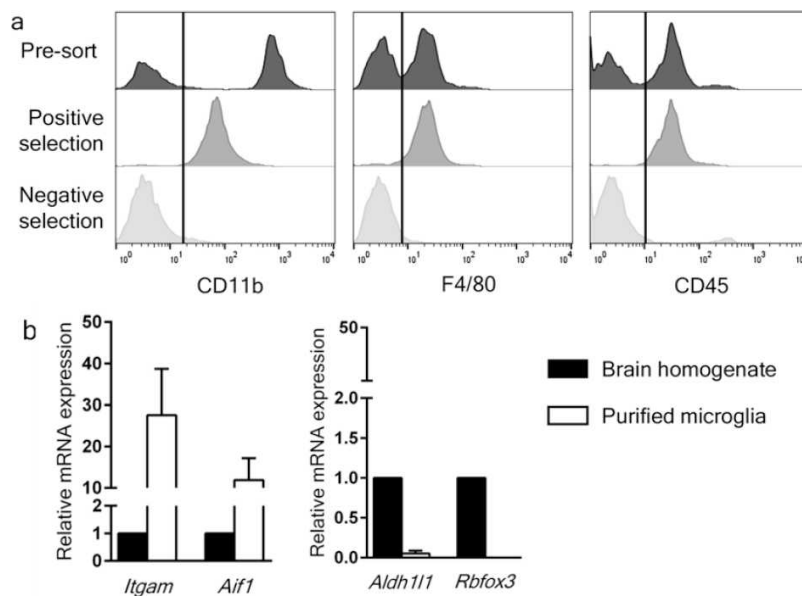


Fig. 3 Purity of adult microglia isolated from mouse whole brain. **(a)** Flow cytometric comparison of positive selected microglia with pre-sorted and nonselected samples demonstrates highly pure CD11b⁺ F4/80⁺ and CD45^{low} positive selected microglia and 1 log higher CD11b positivity in microglia before bead separation. Nonselected samples display a negligible loss. **(b)** Relative mRNA expression of microglial (*Itgam*, *Aif1*), astrocyte (*Aldh1l1*), and neuronal (*Rbfox3*) marker in whole-brain homogenates versus isolated microglia

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2 Materials

1. Perfusion pump
2. Tissue culture hood
3. Incubating oven
4. 15 mL Dounce homogenizer
5. Falcon tubes and microtubes
6. 5, 10, and 20 mL stripettes
7. Centrifuge
8. LS columns (Miltenyi Biotec)
9. MACSmix Tube Rotator
10. MidiMACS separator and MultiStand

2.1 Reagents

1. 1× Hanks' balanced salt solution (HBSS) without calcium and magnesium.
2. 10× HBSS
3. Percoll
4. CD11b human/mouse MicroBeads
5. Purified anti-mouse CD16/CD32 (Fc block)
6. Anti-mouse antibodies against CD_{11b}, CD₄₅, and F_{4/80}

2.2 Solutions

1. Physiological saline: **[M]0.9 % Saline** treated with **[M]0.1 % diethyl pyrocarbonate (DEPC)** , store at **4 °C**
2. Enzyme cocktail: **10 mL enzyme cocktail** contains the following ingredients and stock concentrations:
50 µl 50 U/mL collagenase D ,
10 µl 100 µg/mL Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride ,
50 µl 5 U/mL DNase I , and **250 µl 340 U/mL dispase** in **9.64 mL HBSS** . Prepare stock concentration of enzymes according to the manufacturer's instruction. Enzyme mix was prepared fresh or stored in appropriate aliquots at **-20 °C** .
3. **[M]10 % Fetal bovine serum (FBS)** : Make up **[M]10 % FBS** in HBSS. Prepare fresh on the day or freeze in aliquots at **-20 °C** .
4. Isotonic Percoll: Prepare isotonic Percoll by adding **1 volume 10× HBSS** to **9 volumes Percoll** . Prepare fresh each time.
5. **[M]35 % Percoll** : **16 mL** is required per whole brain. Add **5.6 mL isotonic Percoll** to **10.4 mL 1× HBSS** . Prepare fresh each time.
6. **[M]0.5 Molarity (M) Ethylenediaminetetraacetic acid (EDTA) stock** : Add **56 g NaOH** to **800 mL water** . Weigh **186.12 g EDTA disodium salt** (or **146.2 g EDTA anhydrous**) and add to NaOH solution. Let dissolve, adjust pH to **pH7.5** , and top up to **1 L** with water.
7. Separation buffer: **[M]1 X PBS** , **pH7.2** , **[M]0.5 % bovine serum albumin (BSA, low endotoxin)** , **[M]2 Milimolar (mM) EDTA** . Add **0.5 g BSA** to **100 mL PBS (pH 7.2)** and add **400 µl 0.5 M EDTA stock solution** . Store at **4 °C** .
8. Flow cytometry buffer: Add **0.1 g BSA (low endotoxin)** to **100 mL PBS (without calcium and magnesium)** . Store at **4 °C** .

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

Prepare Solutions as described in section '[Materials](#)'.

Carry out all procedures at **4 °C** and use **ice-cold** reagents unless otherwise specified (*see* **Note 1**). The isolation procedure is summarized in Fig. 1.

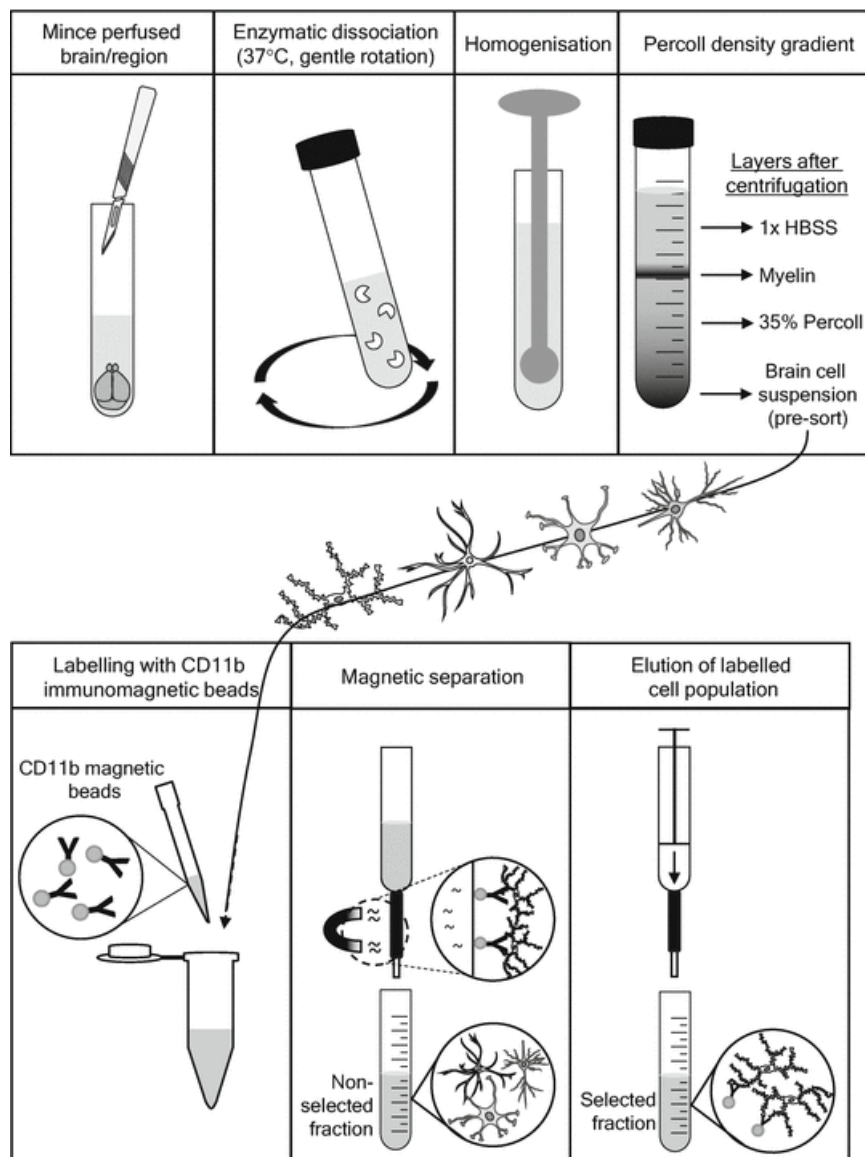


Fig. 1 Schematic workflow of the microglia isolation process

Perfusion and Isolation of Mouse Brain

- 1 Perfuse animals transcardially with physiological saline (10 mL/min) until exudate runs clear (*see* **Note 2**).
- 2 Remove brain and transfer into 50 mL Falcon tube containing **10 mL HBSS** either as whole brain, hemisphere, or brain region.

3 Finely mince brain tissue with a round-edge blade scalpel to allow fast cooling of the tissue.

4 

Spin minced brain tissue for **400 x g, 00:05:00** and aspirate supernatant.

Enzymatic Dissociation and Homogenization

5 Add enzyme cocktail to the minced whole-brain tissue (**10 mL**) or brain hemisphere/brain region (**5 mL**) (see **Note 3**).

6 

1h

Incubate for **01:00:00** at **37 °C** under gentle rotation (see **Note 4**).

7 Transfer the digested brain tissue to a 15 mL Dounce homogenizer and dissociate **On ice** with 20 passes using the large clearance pestle (see **Note 5**).

8 Transfer the homogenized brain cell suspension to an equal volume of **10 % FBS**.

9 

Centrifuge at **400 x g, 4°C, 00:05:00** (no brake) and remove the supernatant.

Myelin-Free Mixed Brain Cell Suspension by Density Gradient

10 Resuspend the cell suspension from whole brain in **16 mL** or brain hemisphere/region in **8 mL 35% Percoll** (see **Note 6**).

11 Split only the whole brain in 2 x **8 mL**.

12 Carefully overlay each sample with **5 mL 1x HBSS** and leave samples to rest for **00:05:00** **On ice** (see **Note 7**).^{5m}

13 

Spin samples at **800 x g, 4°C, 00:45:00** (no brake) and subsequently different layers can be observed (see Fig. 1).

14 Aspirate the supernatant including the myelin layer carefully, leaving only the pelleted mixed brain cells.

15 

Wash the cell pellet in **1 mL 1× HBSS** and transfer into a new tube containing **4 mL 1× HBSS**.

16 

Spin for **400 x g, 4°C, 00:05:00** and remove supernatant.

Immunomagnetic Bead Separation

17 Resuspend the cell pellet in **90 µl separation buffer** and transfer into a microtube.

18 

15m

Add **10 µl anti-CD11b MicroBeads** and incubate the cell-bead mix for **00:15:00** at **4 °C** under gentle rotation (*see Note 8*).

19 Meanwhile place LS single-use columns in magnet and wash through with **3 mL separation buffer**.

20 Add **500 µl separation buffer** to bead-cell-suspension, apply onto LS column, and collect flow-through (*see Note 9*).

21 


Wash the columns three times with **3 mL separation buffer** (*see Note 10*).

21.1 Wash the columns with **3 mL separation buffer**. (1/3)

21.2 Wash the columns with **3 mL separation buffer**. (2/3)

21.3 Wash the columns with **3 mL separation buffer**. (3/3)

22 Remove LS columns from the magnet and place into a 15 mL tube.

23 Add  **5 mL separation buffer** and flush out bead-bound microglial cells by firmly pushing the plunger.

24 




Pellet the cells at  **400 x g, 4°C, 00:05:00**.

Flow Cytometric Analysis

45m



25 

20m

Resuspend purified microglia in flow cytometry buffer and incubate with  **1 µg/mL anti-mouse CD16/CD32** for  **00:20:00** at  **Room temperature** (see **Note 11**).

26 



Spin at  **400 x g, 00:05:00** and remove supernatant.

27 Resuspend cells in fluorophore-conjugated antibodies for the protein of interest (here CD11b, CD45, F4/80) for  **00:20:00** in the dark at  **Room temperature** (see **Note 12**).

20m

28 

5m

Wash samples for  **00:05:00** at  **400 x g** and resuspend samples in an appropriate volume of flow cytometry buffer.