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Primary Microglia Isolation from Postnatal Mouse Brains

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TITLE:

Primary Microglia Isolation from Postnatal Mouse Brains

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KEY WORDS:

microglia, primary microglia, cell culture, live-cell imaging, primary cell culture

SUMMARY:

Primary cell culture is one of the primarily used approaches for studying microglial biology in vitro. Here, we developed a method for simple and rapid microglia isolation from the mouse postnatal day 1 (P1) to P4.

ABSTRACT:

Microglia are the mononuclear phagocytes in the central nervous system (CNS), which play key roles in maintaining homeostasis and regulating the inflammatory process in the CNS. To study the microglial biology in vitro, primary microglia show great advantages compared to immortalized microglial cell lines. However, microglia isolation from the postnatal mouse brain is relatively less efficient and time-consuming. In this protocol, we provide a quick and easy-to-follow method to isolate primary microglia from the neonatal mouse brain. The overall steps of

this protocol include brain dissection, primary brain cell culture, and microglia isolation. Using this approach, researchers can obtain primary microglia with high purity. In addition, the harvested primary microglia were able to respond to the lipopolysaccharides challenge, indicating they retained their immune function. Collectively, we developed a simplified approach to efficiently isolate primary microglia with high purity, which facilitates a wide range of microglial biology investigations in vitro.

INTRODUCTION:

Microglia, the resident immune cells in the central nervous system (CNS), play pivotal roles in the maintenance of homeostasis, which respond to the neuropathological challenges[1]. Recently, intensive investigations have been conducted to figure out the physiological functions of microglia, e.g., in Alzheimer's disease[2]. Currently, the transcriptional profile of microglia at the single-cell resolution obtained during the CNS development, aging, and disease provides a better understanding of microglial function in healthy and diseased brains[3]. Previous studies identified a disease-associated microglial subtype in AD and other neurodegenerative diseases[4–6]. This sub-population is proximal to the amyloid β (Aβ) deposition. Genes associated with phagocytosis and lipid metabolism (e.g., *Apoe*, *Tyrobp*) were found to be up-regulated in these populations[6,7]. However, the subcellular processes, extracellular and intracellular signaling pathways that regulate the dynamic molecular profile changes in microglia are not fully understood. Particularly, the mechanism underlying chronic microglial activation in neurodegenerative diseases remains elusive. Therefore, it is crucial to understand microglia-involved cellular mechanisms since microglia responses clearly contribute to brain development and the progression of neurodegeneration.

Although there are a couple of in vivo and in vitro tools to study microglia, there are still some limitations. It is technically difficult to obtain a large enough number of microglial cells with high purity for routinely carrying out experiments, such as western blot, to elucidate intracellular signaling pathways. Primary microglia culture provides an alternative means to study the biology of microglia. The cultured primary microglia can be applied to analyze microglial phagocytic capacity after gene manipulation, and evaluate pro- and anti-inflammatory cytokines production in response to inflammatory stimuli, and other biological aspects to understand their roles in the brain[8]. Here, we present a new protocol and describe a step-by-step instruction for the isolation and culture of primary microglia from neonatal mouse brain, with emphasis on the steps that are critical for obtaining healthy and pure primary microglial cells.

PROTOCOL:

All the animal procedures were approved by the committee on Animal Research and the Institutional administration panel of laboratory animal care at Shanghai Jiao Tong University. Every effort has been made to minimize animal suffering.

1. Preparation of culture buffer, flasks, and coverslips

1.1. Culture medium and microglia culture medium: Supplement DMEM with 10% FBS to make the culture medium. The microglia culture medium is made by adding 10% FBS and 20%

LADMAC conditioned medium to the DMEM. Add 1% Penicillin/Streptomycin to both the medium.

1.1.1. To prepare 20% LADMAC conditioned medium, collect the culture medium from the LADMAC cells, centrifuge at 200 x *g* for 10 min, filter using 0.22 µm cell strainer, and then store the aliquots at -80 ˚C.

1.2. Digestion buffer: Prepare digestion buffer by adding 8 U/mL papain and 125 U/mL DNase to DMEM.

NOTE: For best results, the digestion buffer should be prepared freshly. Calculate the desired amount based on the number of pups (1 mL solution for every 2 pups).

1.3. Coating flasks: Use T25 or T75 flasks for pre-plating the isolated brain cells. Before the brain dissection, coat T25 flasks with 2 mL (or 4 mL for a T75 flask) of 0.1 mg/mL Poly-D-lysine (PDL) and incubate at 37 °C for at least 1 h. Aspirate the PDL solution, wash twice with 1x PBS, and then allow the flask to air-dry in the cell culture hood.

NOTE: The size and number of flasks used in the experiment were dependent on the number of postnatal pups. To let the cells reach the confluent astrocyte cell layer in a short time, prepare one T25 flask for 3–5 brains, or one T75 flask for 5 brains. Unused coated flasks can be stored at 4 °C for up to a month. To save time, 10x (1 mg/mL) stock solution of PDL can be prepared before the start of the experiment and stored at -20 ˚C as single-use aliquots. Dilute the aliquots in sterile 1x PBS right before use.

1.4. Prepare coverslips: Wash commercial glass coverslips with 0.1 M HCl for 1 h, rinse twice with autoclave water, then store in 100% ethanol. Before use, flame the coverslips using fine forceps with a burner lamp to remove residual ethanol. 8 mm glass coverslips can be placed in a 48-well plate, and 12 mm glass coverslips can be placed in a 24-well plate.

1.5. Prepare dissection tools: Soak fine scissors, forceps, and stainless steel micro-spoons in 75% ethanol for at least 20 min. Then place these tools under ultraviolet radiation in the tissue culture hood for 30 min.

2. Dissection of mouse brain

NOTE: To evaluate the efficacy of the protocol, here we used CX3CR1^{GFP} knock-in mice for tracing microglia[9]. CX3CR1$^{GFP/GFP}$ mice and wild type C57BL/6J females at 2- to 8-month were crossed to generate CX3CR1$^{+/GFP}$ mice, both were originally obtained from commercial sources. Mice were housed under a 12–12 h light-dark cycle in a specific pathogen-free (SPF) environment at Shanghai Jiao Tong University. Pups from postnatal day 1 to day 4 can be used for this protocol.

2.1. Decapitate all pups with surgical scissors. To minimize contamination, rinse all heads with 75% ethanol and quickly put in the ice-cold 1x PBS. Meanwhile, warm the digestion buffer and

co-culture medium in a 37 ˚C water bath.

NOTE: Decapitate and isolate brain tissue from one pup at a time to preserve structural integrity. To prevent contamination, all surgical tools should be placed in 75% ethanol between decapitation.

2.2. Use fine dissecting scissors to cut the skull along with the medulla oblongata. Place a stainless steel micro-spoon beneath the brain tissue from the cutting site to squeeze out the brain from the head cavity. Transfer it to a new chilled 6-well plate containing 2 mL of pre-cold 1x PBS per well.

2.3. Cut off and discard the cerebellum and olfactory bulbs. Gently transfer the remaining brain tissue to a chilled 6-well plate containing 2 mL of pre-cold 1x PBS.

NOTE: Place 4–5 brain tissues in one well.

2.4. Repeat the same procedure for the remaining heads until all tissue is collected.

3. Seed the mixed primary cells

3.1. Aspirate 1.5 mL of 1x PBS in the 6-well plate, and fully mince the brain tissue into small pieces (approximate 1 mm^2) with the help of spring scissors.

NOTE: To reduce the amount of fine debris, do not over mince the tissue.

3.2. Add the desired amount of digestion buffer to the well (0.5 mL of digestion buffer per brain), then place the plate in the 5% CO_2, 37 °C humidified incubator for 20 min. Swirl the plate every 10 min.

NOTE: During the incubation time, wet a 70 µm cell strainer with culture medium (10% FBS in DMEM), and then place the cell strainer onto a fresh 50 mL collection tube.

3.3. Take the plate out of the incubator and terminate papain digestion by adding 3 mL of culture medium to each well. Mildly pipette the cells up and down 10 times using a 1 mL pipette. Transfer the mixture to a 15 mL tube, and let it settle for 1 min. A cloudy cell pellet can be seen at the bottom. To remove big clumps and cell debris, carefully pass the cell suspension through a 70 µm cell strainer and collect the flow-through in the 50 mL collection tube.

3.4. Add 3 mL of warmed culture medium (10% FBS in DMEM) to the undissociated cell pellet in the 15 mL tube. Triturate 10 times as in steps 3.3 to continue the dissociation. Let the tissue settle for 1 min and then pass the cell suspension through the strainer to the 50 mL collection tube. Repeat this step, and discard the remaining cell debris.

3.5. Transfer the cell suspension from the 50 mL collection tube to 15 mL tubes and centrifuge

the cells at 200 x *g* for 10 min at room temperature.

3.6. Aspirate the supernatant, and resuspend the cells in 5 mL of culture medium. Seed the primary cells in a T25 or T75 flask at a cell density of about 5 x 10^6 and culture in the humidified incubator (5% CO_2, 37 °C). This is Day 0 in this culture schedule.

NOTE: If cells are seeded in a T25 flask, a final volume of 5 mL medium per flask is sufficient. Add an extra 5 mL of culture medium to each flask when seeded in a T75 flask.

4. Collection of primary microglia

4.1. Evaluate the confluency of the mixed cells on the next day (Day 1). Since the mixed cells were seeded at a pretty high density, it takes 3–4 days for the astrocytes to reach confluency, allowing the microglia to be collected 1–2 days later.

NOTE: Be cautious if cells are not attached to the bottom of the flask on the next day; it might be due to cell contamination or cell death caused by over digestion or harsh handling.

4.2. On Day 2, wash the flasks with 3 mL of warm 1x PBS twice and change the medium by adding 3–5 mL of fresh culture medium to each T25 flask (7–10 mL for a T75 flask). The discarded culture medium contains cell debris and various types of cells, including neurons, oligodendrocytes and microglia.

4.3. On Day 4, ensure that astrocytes attached to the bottom of the flask are at 100% confluency. Meanwhile, a subpopulation of microglia is loosely attached to the mixed cell surface layer which can easily detach from the surface and float in the culture medium by handshaking. Transfer the culture medium to a fresh 6-well plate, and primary microglia enriched in the medium are collected during this process. Next, provide the cells on the original 6-well plate with fresh culture medium for further cell collection.

NOTE: Tapping the flasks on the table or shaking the flasks on a laboratory shaker at 180 rpm for 30 min before medium transferring is appropriate to yield more cells.

4.4. Carefully place the 6-well to the incubator to allow cell attachment. About 2 h after incubation, all the cells will be attached to the plate. When the cells are attached, gently wash them with warm 1x PBS twice to remove cell aggregates and cell debris; then, supply the cells with 3 mL of fresh microglia culture medium. The isolated microglia cells can be maintained for more than a month in vitro by refreshing the microglia culture medium every 2 days.

NOTE: Colony-stimulating factor 1 (CSF1) is an important growth factor to support microglia survival in vitro. Previous studies report that CSF1 can be secreted from the LADMAC cell line, which is a transformed cell line originated from mouse bone marrow cells. The LADMAC conditioned medium can be collected by centrifuging the LADMAC culture at 200 x *g* for 10 min;

then, pass the supernatant through a 0.22 µm cell strainer. Microglia culture medium was prepared by adding 20% LADMAC conditioned medium to the culture medium (10% FBS, DMEM).

4.5. Every 2–3 days, continue to collect the microglia from the mixed cell culture medium as in step 4.4. Culture the remaining mixed cells in the flask and use them for consistently harvesting microglia every 2–3 days for up to 1 month.

NOTE: Microglia can be successfully harvested by collecting the medium form mixed culture cells for up to 50 days. Each time, the collected co-culture medium will yield about ~1-3 x 10^5 microglia cells per T75 flask. Combine microglial cells, if needed.

4.6. Use primary microglial cells for the desired functional assay.

REPRESENTATIVE RESULTS:

Primary microglia collected from CX3CR1$^{+/GFP}$ at Day 7 and Day 35 after cell seeding were demonstrated in **Figure 1.** As shown in the immunofluorescence staining of microglia/macrophage cell marker IBA1, all IBA1 positive cells are positive for the GFP signals, and negative for S100β (astrocyte marker) and CC1(oligodendrocyte marker), suggesting that the purified GFP positive cells are indeed microglia. Next, it was found over 95% of isolated cells are GFP positive cells, indicating the isolated microglia are highly pure[10]. Besides, to further characterize the function of the primary microglia cell in vitro, the isolated microglia were treated with 10 ng/mL and 100 ng/mL LPS for 24 h (as shown in **Figure 2**). Compared to control, LPS exposure induced an activated phenotype, including upregulating expression of CD68 and transforming to an amoeboid morphology with long and branched processes. The findings suggested the isolated primary microglia cells in this protocol allow for at least successful inflammation investigation.

FIGURE AND TABLE LEGENDS:

Figure 1: Cells isolated from p2 mouse pups are of high purity. Representative images showed the purity of primary microglia collected from Day 7 (**A**) and Day 35 (**B**) after mixed cell culture. Cells are stained with IBA1 (red), S100β (purple), CC1 (purple). Scale bar 50 µm.

Figure 2: Isolated primary microglia responds well to LPS exposure. Representative confocal images showed the expression of CD68 (gray) and IBA1 (red) at isolated microglia cells after LPS treatment. Scale bar 50 µm.

DISCUSSION:

This protocol is based on the methods described previously with some modifications[11]. Tips for improving the viability and purity of isolated microglia are listed as follows. First, take care to avoid contamination when preparing buffers used for tissue isolation and cell culture. Make sure the surgery tools, containers, and plastic equipment are sterile. Usually, we perform the brain dissection in a separate tissue culture hood aside from the cell culture hood for general cell culture to avoid cross-contamination. Second, the tissue dissection and seeding steps should take

an experienced researcher about 1.5–2 h for 10–12 pups. Large-scale preparation is feasible but definitely extends the time, especially the time for tissue dissection. However, it is better to finish the cell isolation in a timely fashion to reduce cell exposure to damage signals, so as to minimize hypoxic and ischemic induced cell activation. Third, it is necessary to gently handle the mixed cell culture to avoid disruption of astrocytic monolayers. Additionally, do not vigorously shake the flask during the isolation process to minimize excessive microglial activation.

In the subsequent collection steps, previous protocols highlight that mechanical dissociation can increase the yield of microglia. For instance, Tamashiro et al. recommended shaking the culture flasks for 1 h at 100 rpm to enhance microglial detachment from the astrocytic layer[12]. Lian et al. suggested that it was necessary to tap the flasks when harvesting the cells[11]. However, consideration should be taken into account that although the yield of microglia could be increased by introducing mechanical force, additional mechanical manipulation also increases the dissociation of oligodendrocytes and astrocytes, leading to a reduction of cell purity. Moreover, shaking and vigorously tapping often generate lots of air-bubbles to the culture medium, resulting in a higher risk of contamination. Also, additional time will be needed to remove the bubbles and minimize cell contamination.

In this protocol, we were able to isolate healthy microglia from Day 40 mixed cultured cells, which were still suitable for live-cell images to record microglial morphology and function as we showed in the video. However, It should be noted that the properties of microglia isolated from different time points might differ. Caldeira et al. found that primary microglia show an irresponsive and senescent phenotype when cultured in vitro for 16 days[13]. Therefore, it is important to assess the cell state of primary microglia to minimize the artifact introduced by in vitro culture system. It is highly recommended that sequential experiments should be conducted with primary microglial cells that are isolated at the same timepoint, and triplicate experiments should be performed on microglia derived from independent isolation.

ACKNOWLEDGMENTS:

The authors would like to show their respects to the heroes combating the COVID-19 outbreak. The authors thank Fang Lei (Fudan University) for the excellent laboratory management, Dr. Zikai Zhou, Dr. Jing Li, and Dr. Guiqing He (Shanghai Mental Health Center) for the discussion of microglia isolation. Last but not least, the authors show their gratitude and respect to all animals sacrificed in this study. This study was supported by the National Key R&D Program of China (Grant No. 2017YFC0111202) (B.P.), National Natural Science Foundation of China (Grant No. 31922027) (B.P.) and (Grant No. 32000678) (Y.R.), and Shenzhen Science and Technology Research Program (Grant No. JCYJ20180507182033219 and JCYJ20170818163320865) (B.P.) and (Grant No. JCYJ20170818161734072) (S.X.).

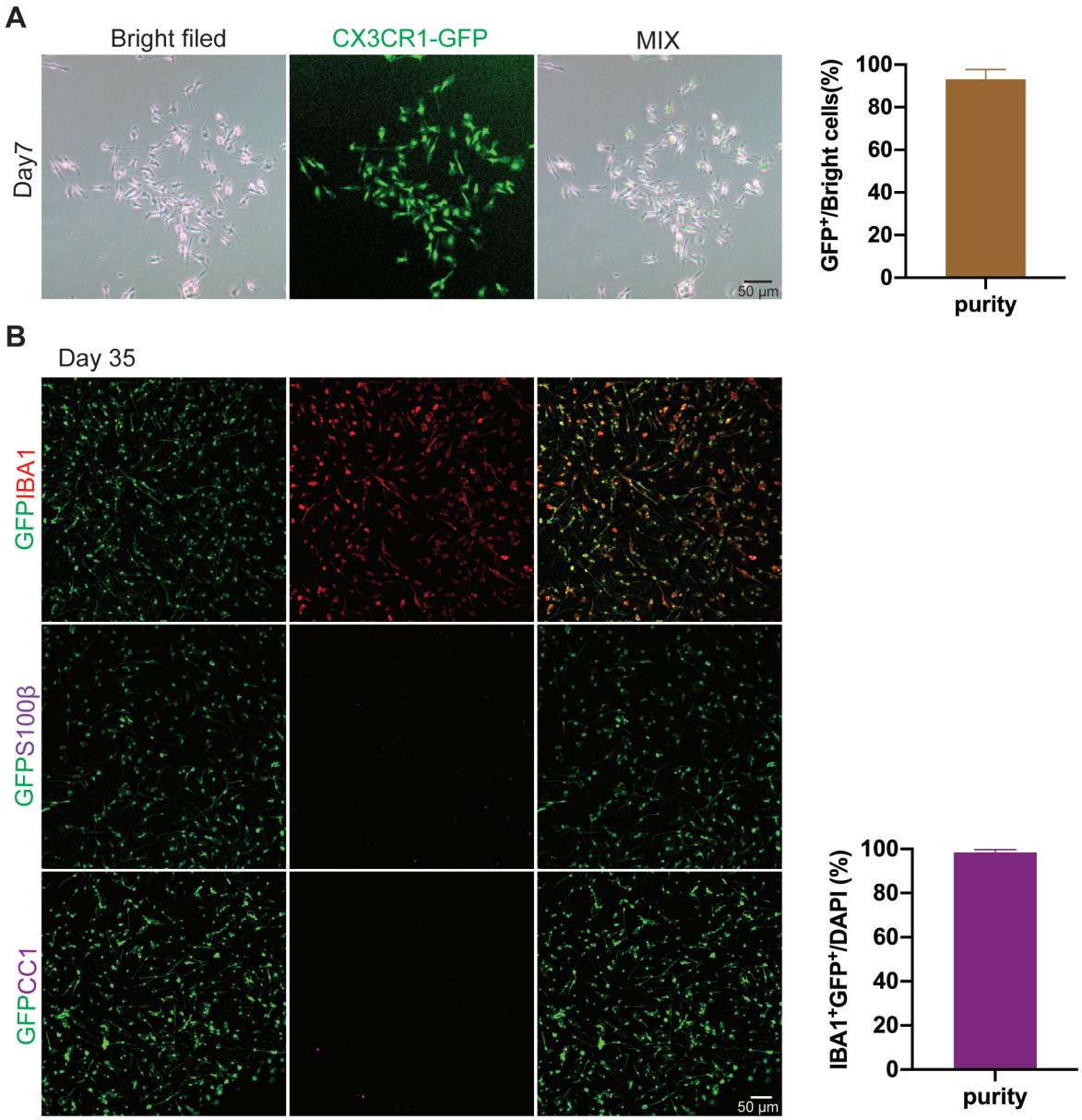
DISCLOSURES:

All the authors disclosed that there are no conflicts of interest. All the authors declare no competing financial interests.

REFERENCES:

1. Prinz, M., Jung, S., Priller, J. Microglia biology: one century of evolving concepts. *Cell*. **179** (2), 292–311 (2019).
2. Perry, V. H., Nicoll, J. A., Holmes, C. Microglia in neurodegenerative disease. *Nature Reviews. Neurology*. **6** (4), 193–201 (2010).
3. Parker, K. R. et al. Single-cell analyses identify brain mural cells expressing CD19 as potential off-tumor targets for CAR-T immunotherapies. *Cell*. 183 (1), 126–142.e17 (2020).
4. Tay, T. L., Sagar, Dautzenberg, J., Grun, D., Prinz, M. Unique microglia recovery population revealed by single-cell RNAseq following neurodegeneration. *Acta Neuropathologica Communications*. **6** (1), 87 (2018).
5. Deczkowska, A. et al. Disease-associated microglia: a universal immune sensor of neurodegeneration. *Cell.* **173** (5), 1073-1081, (2018).
6. Keren-Shaul, H. et al. A unique microglia type associated with restricting development of Alzheimer's disease. *Cell*. **169** (7), 1276–1290.e1217 (2017).
7. Krishnaswami, S. R. et al. Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. *Nature Protocols*. **11** (3), 499–524 (2016).
8. Timmerman, R., Burm, S. M., Bajramovic, J. J. An overview of in vitro methods to study microglia. *Frontiers in Cellular Neuroscience*. **12**, 242 (2018).
9. Huang, Y. et al. Repopulated microglia are solely derived from the proliferation of residual microglia after acute depletion. *Nature Neuroscience*. **21** (4), 530–540 (2018).
10. Garcia, J. A., Cardona, S. M., Cardona, A. E. Analyses of microglia effector function using CX3CR1-GFP knock-in mice. *Methods in Molecular Biology*. **1041**, 307–317 (2013).
11. Lian, H., Roy, E., Zheng, H. Protocol for primary microglial culture preparation. *Bio-protocol*. **6** (21) (2016).
12. Tamashiro, T. T., Dalgard, C. L., Byrnes, K. R. Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue. *Journal of Visualized Experiments: JoVE*. (66), e3814 (2012).
13. Caldeira, C. et al. Microglia change from a reactive to an age-like phenotype with the time in culture. *Frontiers in Cellular Neuroscience*. **8**, 152 (2014).

Figure-1



Figure

Click here to access/download;Figure;LPS treated primary microglia-2.pdf

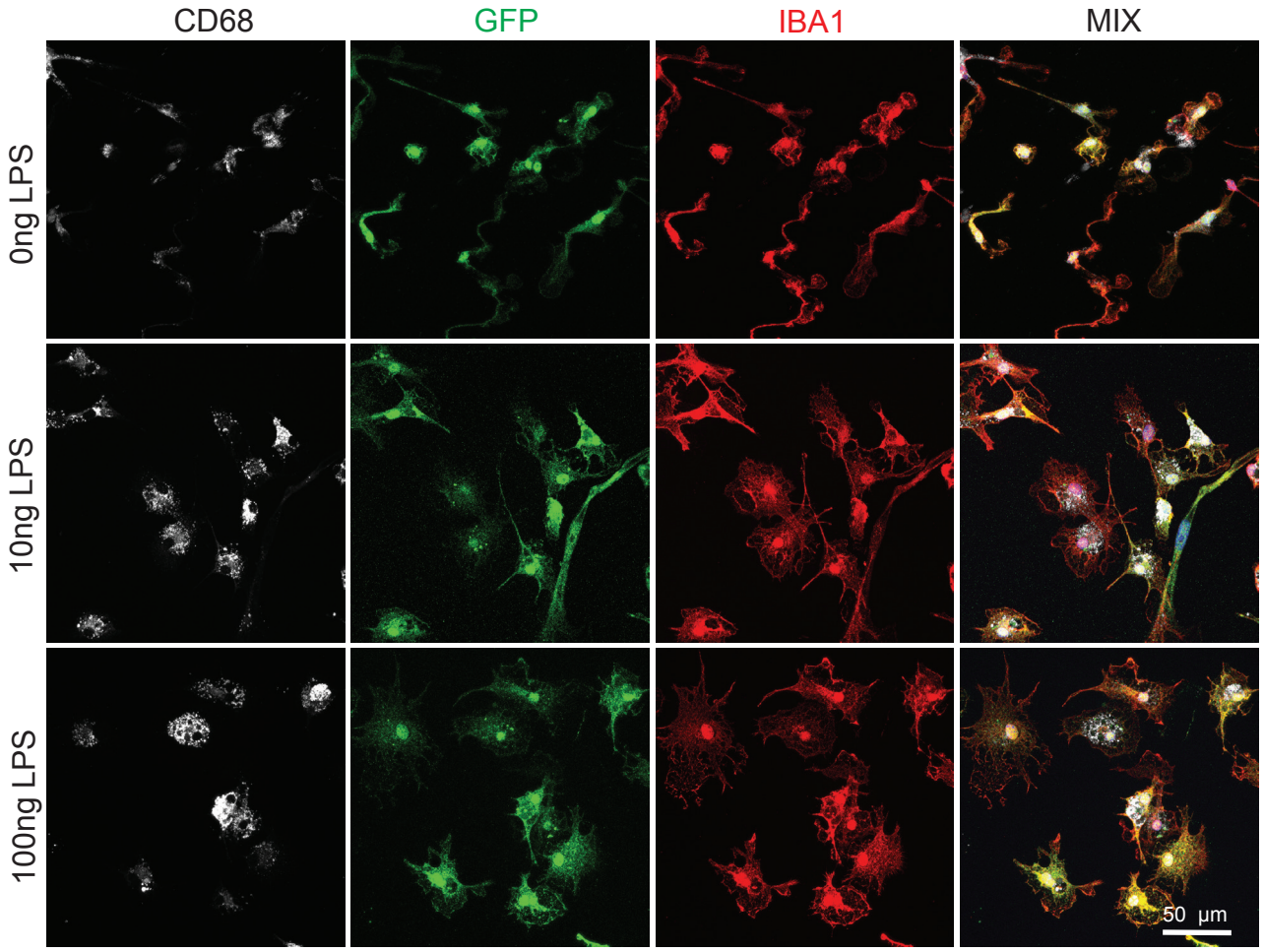


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (3).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Cell strainers, 40 µm	ThermoFisher Scientific	22-363-547	
DNase I	Sigma	11284932001	
Dulbecco's Modified Eagle Medium (DMEM)	Gibco by Life Technologies	C11995500BT	
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco by Life Technologies	14190-144	
Fetal Bovine Serum (FBS)	Gibco by Life Technologies	10099141	
Papain, Suspension	Sangon Biotech	Papain, Suspension	
Penicillin-Streptomycin 100X solution	Hyclone	SV30010	
Poly-D-Lysine	ThermoFisher Scientific	A3890401	

comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Already done.

2. Please revise the title and remove “Simplified protocol for”.
Already modified.

3. Please provide an institutional email address for each author.

1. Siling Du, Siling.du@wustl.edu
2. Xingjuan Du, 20111520007@fudan.edu.cn
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4. Line 130-131: Please define “xx”. What is the specification of the spoon used?
Described in the maintext.

5. Line 140: Please specify the volume of PBS removed.
Already described in the maintex.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

7. Please ensure that each Figure Legend includes a title and a short description of the data presented in the Figure and relevant symbols.

8. Please provide a minimum of 10 references.

9. Figure 1/ Figure 2: Please include scale bars for all the images in the panel. Please provide the details of the magnification in the Figure Legends.

Reviewers' comments:

Reviewer #1:

In this manuscript, Du and colleagues reported a simplified protocol for primary microglia isolation from postnatal days 1-4 mouse brains. The authors claimed to obtain microglia with around 95% purity by using this method. Below, I list more detailed comments regarding this manuscript.

1. As shown in Figure 1, around 95% of isolated cells are microglia. What are the other 5% cells are? How to exclude the effects caused by these cells? The authors need to discuss it.

The 5% of population include astrocytes, neurons, oligodendrocytes. Further culture could remove or reduce some of them. Neurons can be reduced because the culture medium does not contain the essential factors for neuronal growth. Oligodendrocytes are less likely to attach to the petri dish, so they can be reduced by washing the harvested cells by PBS. Astrocytes are relatively hard to remove completely, however, they can be easily distinguished from microglia due to the size. Thus, it might not be problematic in experiments that do not require extreme high purify of microglia. If needed, CD11b beads can be used for further purification.

2. There is a limited description of how to generate LADMAC conditioned medium. To be easily followed by other researchers, more details about LADMAC cell culture and medium collection, etc., are needed.

Thanks for bringing it out! We have included detailed description in Line 197-200.

3. In step 2.1, please clarify how to sacrifice pups by 75% ethanol.
Thanks. It has been clarified.

4. In Step 2.4, the authors mentioned discarding the cerebellum and olfactory bulb. Please advise whether this protocol is not suited for scientists interested in the cerebellum and olfactory bulb microglia isolation.
Thanks for asking! The protocol is not taken the regional heterogeneity of microglia into consideration. Study specifically focuses on microglia in cerebellum and olfactory bulb should focus on the desired tissue when isolating microglia.

5. In Step 3.7, before seeding the isolated cells on flasks, what are the quality controls? For example, the cell number and viability? How many cells are needed to seed in each flask, whether cell count is necessary?
(Similar to the Review #2-major concerns-2)
Normally, the number of cells isolated from one p2-p3 brain (without cerebellum and olfactory bubs) is nearly 1x 10^6. Before seeding the mixture cells, we stain the cell in 0.4% trypan blue to evaluate the cell viability and density, and seed each flask at a density about 5x 10^6

6. The author discussed the detach method is critical and improved in this protocol. In step 4.3, the author mentioned using a shaker to help microglia separate from the dishes. Please give more details, for example, what type of shaker, what is the speed?
Thanks! That has been added.

7. There are some typos, for example, line 150, 'minuet'.
They have been fixed. Thanks!

Reviewer #2:
Manuscript Summary:
This manuscript by Du, et al. described a modified protocol of primary microglia isolation from postnatal mouse brains, which may interest to others.

Major Concerns:
- After dissecting the brain, is brain meninges removed before digestion since meninges includes myeloid cells?
Thanks for asking! In our protocol, we did not remove the meninges in specific step, while the usage of spoon to gouge the brain out reduces the contamination from meninges. We did not observe many myeloid cells while evaluating the purity.

What's the plating cell density in T25/T75 flask in day 0?
About 5x 10^6

After collecting microglia from T25/T75 flasks, an extra step of counting cell number is needed before plating the cells.
Same as above desicried.

- How to get LADMAC conditioned media? It should be described.
Thanks! A more detailed description has been included.

- What's the concentration of CSF1 in the LADMAC conditioned media? Is the concentration consistent in each batch? Can CSF1 replace LADMAC conditioned media to generate more repeatable results?
Thanks for asking! Honestly, we did not measure the concentration of CSF1 in the LADMAC medium. The addition of CSF1 to the culture medium could substitute of usage of LADMAC conditioned medium, but it is less economic.

- Since microglial morphology differed upon collecting time, which time point of the microglia were healthier? Complete functional characterization of those cells at different collecting time is necessary for future studies.
That is a good question. Morphologically, the microglia form earlier culture display more ramified shapes. However, we have tested the microglia collected from Day 40 primary culture medium for lipopolysaccharide

(LPS) experiments and cells are responded well. It depends on different experimental requirement of choosing microglia from different stage.

- It would be beneficial for assessing microglial purity if astrocyte and oligodendrocyte markers were included in immunostaining of Fig.1.
Have already add it in the Fig.1

- here are many other microglial isolation protocols, such as MACS, mild-trypsin digestion, and shaking. Discussion should be expanded to explain the advantages of this protocol compared with previous microglial isolation protocols.
Thanks for suggetion. we expand it in the disscussion part.

Minor Concerns:
- Line 94, collected supernatant "from" instead of "form".
- Line 130, what is "xx"?
- Line 150, "minute", not "minuet"
- Line 230, complete the sentence: "dissect the brain in a timely fashion to reduce tissue exposure to open environment"
- Line 249, complete the sentence.

Reviewer #3:
Manuscript Summary:
The authors provided a simplified protocol for primary microglia isolation from postnatal mouse brains.

Major Concerns:
There are major concerns in manuscripts.
1) There are several previous literatures published previously to describe the protocol for primary microglia isolation, yet, they have not provided the advantages of their protocol compared to theirs. Provide specific advantages about this protocol compare to previously published ones.
(Similar to Reviewer #2, Major concerns- last question)

2) The authors have not provided the details of reagents including LADMAC conditioned medium.
Thanks. It is now added.

3) Methods sacrifice the postnatal pups in the 75% ethanol will not be accepted for IACUC.
Thanks for pointing that out! It is now modified.

Minor Concerns:
In line 143, 4 ml digestion buffer to the well does not match with 0.5 ml for one brain. If 0.5 ml for one brain is right, then they will need to add 1.5-2.5 ml (for 3-5 brain) in T25 flask. If it is 5 brains in T75 flask, it will be 2.5 ml digestion buffer. Clarify this.
Thanks for bring it out! It has been fixed.

In line 148, terminate the digestion by adding 3 mL co-culture medium is not enough to neutralize enzymes.
Thanks. In 3.5, an additional 3 ml medium is added into the cell pellet. The total of 6 ml should be enough for neutralizing the enzymes.

In line 159, Culture the primary cells in where? what vessels need to be used?
Thanks. I have clarified this in the manuscript (culture in T25 or T75 flask).

Provide potential issues for this protocol.
There are typos and lacking details throughout the manuscript, please clarify.
Already modifed and add the details in the maintex.