Published in final edited form as:

Curr Protoc Immunol. ; 104: Unit-14.35.. doi:10.1002/0471142735.im1435s104.

Isolation and Analysis of Mouse Microglial Cells

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Abstract

Microglia are mononuclear phagocytes that make up about 10% of the central nervous system (CNS). They are known for their surveillant behavior which comprises continuously monitoring neural tissue by extending and retracting their processes. Microglial cells are derived from myeloid progenitor cells and play important roles in homeostasis, inflammatory and immune responses in the brain. This Unit describes several microglial cell isolation protocols (Basic Protocol 1, Alternate Protocol, and Basic Protocol 2) that can be easily adapted for projects requiring a rapid and efficient analysis of mouse microglial cells by flow cytometry (Support Protocol 1). Methods for visualizing microglial cells using *in situ* immunohistochemistry (Basic Protocol 3) and immunochemistry in free-floating sections (Basic Protocol 4) are also included.

Keywords

mononuclear cells; microglial isolation; immunostaining; CX3CR1/GFP; CNS; myeloid cells; microglia; percoll gradients; flow cytometry; histochemistry

INTRODUCTION

Microglia, mononuclear phagocytes, are specialized, surveillant phagocytic cells (Prinz & mildner, 2011) that colonize the central nervous system (CNS) during embryonic development and are known for their ability to scan the environment with their extensive, mobile processes. Microglia are known for their rapid response to injury and inflammation. CNS tissues, the adult brain in particular, contain a significant amount of myelin which can make it difficult to isolate microglia in a clean cell suspension. Myelin is a membrane that insulates axons in the peripheral and central nervous system. Myelin formation continues throughout life, therefore brains from older mice will contain higher levels of myelin. It is

imperative to remove the myelin from cell suspensions prior to cell sorting or staining to obtain clean cell fractions.

In order to fully understand the functions and expression patterns of certain cell markers and cytokines that characterize microglia, it is necessary to employ cell isolation and immunostaining techniques. These protocols make it possible to isolate microglia and label a vast variety of cell markers of interest. In addition to the ability to identify cell populations, immunohistochemistry has the advantage of analysis of localization within the tissue as well as co-localization with other cell types.

Basic *PROTOCOL 1. ISOLATION OF LEUKOCYTES FROM ADULT MOUSE CNS TISSUES*

Murine leukocytes containing microglial cells can be isolated from CNS tissues for the purpose of analyzing different cell populations within a given sample. Depending on the neuroinflammatory model employed, it might be advantageous to pool brain tissues as needed to obtain adequate cell numbers. Cell yield is affected by the homogenization procedure and the usage of digestive enzymes must be contemplated as they could alter cell surface expression. The following protocol describes the isolation of cells using a Percoll gradient and is beneficial for procedures aiming to identify cell surface markers by flow cytometry since it does not include treatment with digestive enzymes.

Materials

Donor mice: C57BL6 7–9 weeks old, male or female (Jackson Laboratory, cat. No. 000664)

1xHBSS free of calcium, magnesium chloride and magnesium sulfate (Gibco, cat. No. 14175)

200mL beakers

Sterile 1x PBS

70% ethanol

Absorbent pads, paper towels, plastic bags to collect carcass.

RPMI 1640 supplemented with L-glutamine without phenol red (Gibco, cat. No. 11835).

10x HBSS (Gibco, cat. No. 14185)

1M HEPES (Gibco, cat. No. 15630)

1x HBSS/ 10mM HEPES (see Reagents and Solutions)

Percoll (GE Healthcare, cat. No. 17-0891-02)

100% and 70% Percoll (see Reagents and Solutions)

7 mL dounce homogenizers with A (loose) and B (tight) pestles (Pyrex, Wheaton).

S1 pipet filler (Thermo Scientific #9531) or pipet aid.

Isoflurane induction chamber

Refrigerated centrifuge (Eppendorf, model no. 5810 R) or similar

Additional reagents and equipment for anaesthesia (Unit 1.4)

1. Anesthetize mouse using isoflurane in the presence of oxygen in induction chamber for 3–4 minutes. Perfuse with 1x HBSS by cutting the thoracic cavity open and making a small incision in the right atrium of the heart allowing blood to flow out. Inject 15–20mL of ice cold 1x HBSS into the left ventricle to completely remove blood from tissues.

Additional information regarding how to perfuse mice is described in Unit 15.1 where PBS is used in lieu of HBSS.

2. Carefully remove brain from skull using scissors and forceps and obtain spinal cord by flushing with 1x HBSS. Transfer tissues to 6-well plate containing RPMI and keep on ice until all tissues are collected.
3. Fill dounce homogenizers with 3.5mL RPMI without phenol red, label them accordingly, and keep on ice. You can choose to homogenize up to 2 brains in one 7mL homogenizer
4. Transfer tissues to their respective homogenizer and grind the tissue using the “A” or “Loose” plunger of pestle until the suspension is confluent, then use the “B” or “Tight” plunger. Once the suspension is homogeneous, transfer the suspension to a 15 mL conical tube and top off with RPMI to 7mL. Continue this step with all samples and keep on ice before moving to the next step.

Typically 2 brains or 2 spinal cords are pooled to achieve the desired total number of cells for enrichment and staining in subsequent protocols.

Be sure to use a new, sterile homogenizer for each sample especially if there are genotypical differences.

5. Add 3 mL of 100% Percoll solution to the cell suspension and mix by inversion or gentle pipetting to ensure confluency. Slowly layer the homogenized suspension onto a new 15 mL conical tube containing 2 mL of 70% Percoll. The idea is to set up a density gradient in which the CNS mononuclear cells including microglia will layer atop the 70% Percoll.

It is ideal to use the pipette’s gravity setting when layering cells to ensure the cells are suspended appropriately.

6. Centrifuge 30 minutes at 550 x g at 18°C.
7. First remove debris located at the upper portion of the tube by vacuum or pipetting. Locate the interphase which contains the mononuclear cells just above the 2mL graduation on the 15mL tube. It is easily seen as a cloudy halo when held up to the light. Using a transfer pipette, carefully remove the interphase, approximately 2 mL, and transfer to a 15mL tube containing 8mL of 1x HBSS/10mM HEPES to wash cells of Percoll. Mix by inversion.
8. Centrifuge 7 minutes at 900 g at 4°C. Aspirate supernatant and suspend pellet in 1mL cell staining buffer.

ALTERNATE PROTOCOL. Enzymatic Neural Tissue Dissociation

The enzymatic dissociation of CNS tissue is beneficial in that cell suspensions will contain less debris. This facilitates the recovery of cells when compared to the homogenization protocol due to the fact that the cells are not masked by the debris and are easily isolated in subsequent steps. Neural tissue dissociation with papain in conjunction with the gentleMACS dissociator allows for the gentle isolation of CNS leukocytes while recovering as many cells as possible. This protocol is ideal for creating cell cultures due to the clean isolation of cells recovered. A similar method is described in Unit 15.1. One disadvantage of

using the enzymatic method is the potential to cleave cell surface markers, therefore even when flow cytometry is the main end point, it should be used to confirm that the desired markers were not affected by the treatment. If flow cytometry is an end point, all antibodies should be titrated and tested with the use of the enzymatic dissociation to ensure all desired markers are still intact.

Materials

Donor mice: C57BL6 7–9 weeks old, male or female (Jackson Laboratory, cat. No. 000664)

1xHBSS free of calcium, magnesium chloride and magnesium sulfate (Gibco, cat no. 14175)

200mL beakers

Sterile 1x PBS

70% ethanol

Forceps, dissection scissors

Absorbent pads, paper towels, and plastic bags to collect carcass.

RPMI 1640 supplemented with L-glutamine without phenol red (Gibco, cat. No. 11835).

6-well plate to store tissues

Razor blade

Neural Tissue Dissociation Kit (Papain) (Miltenyi Biotec, cat. No. 130-092-628)

C tubes (Miltenyi Biotec, cat. No. 130-093-237)

gentleMACS Dissociator (Miltenyi Biotec, cat. No 130-093-235) or similar

Refrigerated centrifuge (Eppendorf, model no. 5810 R) or similar

Oven with rotating tube holders

Preparation of enzyme mixes and tissue harvesting

- 1 Prepare enzyme mix 1 and 2 from neural tissue dissociation kit. The volume listed below is sufficient for one sample.
 - Mix 1: 50uL Solution 1 + 1900uL Solution 2
 - Mix 2: 20uL Solution 3 + 10uL Solution 4
 - Preheat mix 1 to 37°C for 10 minutes prior to use.
- 2 Isolate CNS tissues as stated in steps 1–2 from Protocol 1: Isolation of leukocytes from CNS tissues.
- 3 Cut tissue into small pieces using razor blade to facilitate enzyme dissociation.
- 4 Transfer each sample into its respective C tube containing the preheated enzyme mix 1. A maximum of 1600mg CNS tissue can be processed in each C tube. Volume should not exceed 10mL

Dissociation of CNS tissues

- 5 Close C tube and place on gentleMACS dissociator. Run program m_brain_01.

If a gentleMACS dissociator is not available, the tissues can be dissociated manually by pipetting. To do this, collect all minced tissue pieces in 1mL of 1x HBSS and centrifuge at 300 g for 2 minutes at 4°C.

- 6 Once program is complete, remove tube and incubate at 37°C for 15 minutes under slow continuous rotation
- 7 Place C tube on gentleMACS dissociator and run program m_brain_02.
(Optional) Manual method: Further dissociate tissues by pipetting with a wide-tipped glass pipette followed by a narrow-tipped pipette until a cell suspension is formed.
- 8 Add 30uL of enzyme mix 2 to each sample. Mix by inversion. Do not vortex. Incubate at 37°C for 10 minutes under slow continuous rotation.
- 9 Place C tube on gentleMACS dissociator and run program m_brain_03.
- 10 Incubate at 37°C for 10 minutes under slow continuous rotation.
- 11 Rinse a 40µM nylon mesh strainer with 10mL of 1x HBSS containing Ca+, Mg +. Apply cell suspension to 40µM strainer and collect in a 50mL tube.
- 12 Centrifuge cell suspension 10 minutes at 300 x g at room temperature. Aspirate supernatant completely and suspend cells in 1800uL of myelin removal buffer per 500mg of brain tissue. Continue with Myelin Removal and microglia enrichment protocol.

Scale up volume of buffer used accordingly.

Basic PROTOCOL 2. CD11B ENRICHMENT of Microglial Cells

Myelin debris from the dissociation of neural tissue can impose on the capability of cell isolation and immunostaining. Myelin Removal Beads II are antibodies that produce high purity cell suspensions and recovery of target cells by removing myelin. The removal beads positively select for myelin using a magnetically labeled antibody. As the column is attached to the magnetic separator the myelin will be retained within the column allowing a pure cell suspension to flow through. Cell suspensions are then subjected to a CD11b positive selection. Unlike the myelin removal protocol, we will capture what is retained within the column and flush out the wanted cells with a plunger after removing from the magnetic field. Antibodies will stain target cells more affectively once myelin has been removed, therefore, myelin removal is recommended prior to cell sorting or antibody staining. Cells can be stained for further desired analysis or culture. It is beneficial to use this protocol in conjunction with either isolation of mononuclear cells protocol in addition to myelin removal.

Materials

Myelin removal Buffer: Cold PBS containing 0.5% BSA (*see Reagents and Solutions*)

Myelin Removal Beads II (Miltenyi Biotec, cat. No. 130-096-733)

LS columns (Miltenyi Biotec, cat. No. 130-042-401)

MS columns (Miltenyi Biotec, cat. No. 130-402-201)

Sterile Transfer pipette

5mL polystyrene tubes

15 mL conical tubes

MidiMACS Separator (Miltenyi Biotec, cat. No. 130-402-302)

CD11b enrichment buffer (PBS, 0.5% BSA, 2mM EDTA) (*see Reagents and Solutions*)

CD11b (Microglia) MicroBeads (Miltenyi Biotec, cat. No. 130-093-634)

OctoMACS Separator (Miltenyi Biotec, cat. No. 130-042-109)

Myelin depletion

- 1 Add 200uL of Myelin Removal Beads II to cell suspension in 5mL polystyrene tube, mix well and incubate 15 minutes at 4°C.

Amount listed is for up to 500mg of tissue. Scale volume accordingly.

- 2 After incubation with myelin removal beads, place 3 LS columns in the magnetic field of the MACS separator and rinse with 3mL of PBS +0.5% BSA buffer.

Always wait until the column reservoir is empty before proceeding to the next step. Failure to do so may lead to a clogged column.

- 3 Apply cell suspension evenly across the LS columns collecting unlabeled cells that pass through the column. Wash the column twice with 1mL buffer.
- 4 Centrifuge 10 minutes at 300 x g for at 4°C. Aspirate supernatant and suspend cells in 1mL PBS, 0.5% BSA, 2mM EDTA. Cell suspension is now depleted of myelin and is now ready for CD11b isolation.

CD11b positive selection

- 5 Count cells to determine the volume of CD11b MicroBeads antibody needed.

- 6 Centrifuge 10 minutes 300 x g for at 4°C. Aspirate supernatant and suspend pellet in 90uL of buffer per 10^7 total cells.

For total cell numbers up to 2×10^8 use MS columns. LS columns should be used with total cell counts up to 2×10^9 .

- 7 Add 10uL of CD11b MicroBeads per 10^7 cells. Mix well and incubate for 15 minutes at 4°C.
- 9 Wash cells by adding 1–2mL of CD11b buffer per 10^7 cells and centrifuge at 300 x g for 10 minutes. Aspirate supernatant completely and resuspend up to 10^8 cells in 500uL of buffer.

- 10 Place column in the magnetic field of a suitable MACS separator. For details see chart above.

Prepare column by rinsing with the appropriate amount of buffer:

MS: 500uL	LS: 3mL
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- 11 Apply cell suspension to the column. Collect total effluent; this is the unlabeled cell fraction and discard. Perform washing steps by adding appropriate volume of buffer 3 times.

- 13 Remove column from the separator and place it on a 15mL conical tube.

- 14 Pipette the appropriate amount of buffer into the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

MS: 1 mL LS: 5 mL

- 15 Centrifuge for 10 minutes at 300xg at 4°C. Resuspend in 1 mL cell staining buffer.
- 16 Count cells and continue with flow cytometry staining (Support Protocol 1)

Support *PROTOCOL*. CELL SURFACE STAINING AND FLOW CYTOMETRIC ANALYSIS

The staining of cells with fluorescent antibodies for the purpose of flow cytometric analysis enables the researcher to determine cell populations within a given sample using different fluorochromes. If cells are counted prior to staining, it is possible to determine absolute numbers of cell populations present in any given samples. Cell surface staining does not however answer any questions about cell populations in proximity to each other or localization within the tissue. For this analysis we utilize the immunostaining protocol.

Materials

Double distilled sterile water

1xHBSS free of calcium, magnesium chloride and magnesium sulfate (Gibco, cat. No. 14175)

10x HBSS (Gibco, cat. No. 14185)

1M HEPES (Gibco, cat. No. 15630)

1x HBSS/ 10mM HEPES (*see Reagents and Solutions*)

15mL conical tubes

1.5mL micro centrifuge tubes

Sterile 5 mm Goldenrod Animal Lancet 5mm to draw blood for single stain controls

FC block: rat anti-mouse CD16/CD32 clone 2.4G2 in cell staining buffer (BD Pharmingen)

Cell staining buffer (Biolegend, cat. no. 420201)

Fluorescent antibodies (*see Table 1.1*)

4% paraformaldehyde (*see Reagents and Solutions*)

LSR II flow cytometer (BD Biosciences) or similar

FlowJo analysis software

Red blood cell lysis

- 1 Collect blood from each mouse using 10 µl Heparin per each 100 µl blood (see Unit 1.7).
- 2 Lyse RBCs by mixing 100µL of peripheral blood with 1700µL distilled water and mix by inversion for 20 seconds. Add 200µL of 10x HBSS, invert and add 5mL of 1x HBSS/10mM HEPES.

All solutions must be added quickly to prevent the lysis of white blood cells.

Hypotonic lysis is a fast and consistent method but more conventional alternatives for RBC depletions such as ficoll separation or similar methods can be utilized.

- 3 Centrifuge 7 minutes at 900 g at 4°C. Aspirate supernatant.
- 4 Wash lysed blood as well as CD11b enriched samples with cell staining buffer and transfer to 1.5mL micro centrifuge tubes.

Blocking and staining

- 5 Centrifuge for 1 minute at 9000 g. Aspirate supernatant and suspend cells in FC block solution. Incubate for 5–10 minutes on ice.

Blood for single stain controls should be suspended in 100µL per control and enriched cell samples should be suspended in 50µL FC Block. (Alternatively, spleen cells can be used for single stain controls.)

- 6 Prepare dilutions of antibodies in cell staining buffer in a final volume accommodating for 50µL per sample. Add respective antibodies to single stain control samples calculating for a final volume of 100µL.

All antibodies should be titrated prior to conducting the experiment to ensure optimal results.

- 7 Incubate cells in antibody mix for 30 minutes on ice in the dark.
- 8 Wash cells with 1mL cell staining buffer.
- 9 Centrifuge for 1 minute at 9000 g. Aspirate supernatant and suspend cells in 100µL cell staining buffer and add 100µL 4% paraformaldehyde (PFA) for a final concentration of 2% PFA.
- 10 Fluorescently labeled cells can be stored at 4°C and should be read using a flow cytometer within 24 hours.

Analysis

1. Ensure that all fluorochromes are compensated if necessary and proceed to gate the total leukocyte population. (*See figure 1A*) To do this, plot SSC by FSC and gate on leukocyte population as shown. Population close to the y axis is debris, be sure to leave this out of your gating to obtain a clean population. Label the leukocyte population “all”.

Keep in mind that dead cells can give false positive staining due to non-specific antibody binding. Several reagents can be used to exclude dead cells from analysis such as propidium iodide staining solution (eBioscience) or fixable viability dye eFluor780 (eBioscience).

2. From the “all” gating, make a plot that shows CD45 by FSC to view the CD45Hi, CD45lo and CD45 negative populations. CD45Hi population is made up of myeloid cells, whereas the CD45lo population is CD11b+ microglia. Gate each population as shown in figure 1b and label each population accordingly. (*See figure 1B*)

3. From the “CD45^{Hi}” gating, make a plot showing CD11b by CD11c to determine the percentage of infiltrating monocytes (CD11b⁺ CD11c[−]) and monocyte derived dendritic cells (CD11b⁺ CD11c⁺) that make up the CD45^{Hi} population. (*See figure 1C*).
4. From the “CD45^{lo}” gating, make a plot showing CD11b by CD11c to determine the percentage of true resident microglia (CD11b⁺ CD11c[−]) and CD11b⁺ CD11c⁺ populations that make up the CD45^{lo} population. (*See figure 1D*).

Basic *PROTOCOL 3. ISOLATION OF CNS TISSUES FOR IN SITU IMMUNOCYTOCHEMISTRY*

In situ visualization of microglia cells require proper processing of mice and tissues to ensure good signal and specific detection of this cell population. Specificity of the antibody for the designated antigen is as important as the reduction of potential unspecific antigens that can result in the in high background, impairing the visualization of the specific staining. Besides, if immunofluorescence is the desired method to visualize microglia cells, a poor perfusion can lead to the visualization of auto fluorescent molecules found in the CNS, mainly in blood residues which in turn results in underestimated visualization of positive signal.

Materials

Isoflurane
 Dissecting tools
 1x HBSS (Gibco, cat. No. 14175)
 4% PFA
 Cryoprotection solution (*see Reagents and Solutions*)
 Perfusion pump
 Freezing microtome (Leica, model SM 2010R) or similar
 22cm long Profile C Microtome blade (Leica Biosystems, cat. No. 14021607116)
 Absolute ethanol
 Dry ice
 1x PBS
 30% sucrose in PBS
 Cryostorage solution (*see Reagents and Solutions*)
 24-well plates

Perfusion

- 1 Deeply anesthetize a mouse under isoflurane in the presence of oxygen for about 3 – 4 min
 Monitor mice to ensure sedation and respiration before starting perfusion
- 2 Perfuse each mouse with approximately 15–20mL of 1X HBSS, followed by 10 mL of 4% PFA.

- 3 Gently remove skin and then skull to isolate the brain. Post-fix in 4% PFA and incubate overnight at 4°C under constant agitation
 Alternatively, cut head and remove as much skin as possible and place in 4% PFA. Remove skull the next day and store brain in cryoprotection solution.
- 4 Remove skin from the back of the mouse and gently cut along the sides of the spinal column all the way down to the legs. Remove as much extra tissue as possible and post-fix in same tube with the brain and incubate overnight.
- 5 Using dull scissors, carefully dissect the spinal cord by cutting the dorsal vertebrae one by one, starting from the cervical to caudal end. Spinal cord should remain attached to the ventral side of the spinal column. Gently remove the spinal cord by cutting the meninges between the cord and the column.
- 6 Place dissected brain and spinal cords in cryoprotection solution, overnight at 4°C

Sectioning

- 7 Add absolute ethanol and dry ice to the stage of the freezing microtome.
- 8 Make a small platform with 30% sucrose to mount the tissue. Remove tissue from cryoprotection solution and mount on base in the desired orientation (horizontally, coronally or sagittally. Tissue will take approximately 10 minutes to become completely frozen.
- 9 Cervical and lumbar regions of the spinal cord are cut to 1 cm and placed side by side to be sectioned horizontally at 30–50 µm.
- 10 Section at desired thickness, we recommend 30–50µm. Store sections in cryostorage solution at –20°C in 24-well plates

Basic *PROTOCOL 4. IMMUNOCYTOCHEMISTRY IN FREE FLOATING TISSUE SECTIONS*

Free floating sections constitute a good resource for investigating the distribution of cells populations in tissues of interest. The thickness of CNS sections, which can range from 30 to 100µm allows for the screening of microglia behavior and function throughout the entire brain conveniently with relatively few sections. The thickness of the sections allows for the visualization of complete cells with their corresponding network of interconnected processes in the same field. Additionally, the in situ visualization of microglia cells provides a more comprehensive study of microglia behavior in specific CNS regions. Immunofluorescence detection of microglial cells can be combined conveniently with other CNS markers to thoroughly investigate the relationship and interaction of these cells with other cell types.

Materials

50µm sections from well perfused mice
 1x DPBS/Modified (HyClone, cat. No. SH30264.01)
 9-well clear glass spot plate
 Glass reservoir
 Paint brush
 Antigen retrieval solution (Dako, cat. No. S1699)

Blocking solution

DPBS/0.1% Triton-100X

Multi-platform shaker

Diluted primary antibody rabbit anti-Iba-1 1:4000 (WAKO, 01919741)

Diluted secondary antibody goat anti-mouse Cy2 1:1000 (Jackson ImmunoResearch, cat. No. 115-225-146)

Diluted biotin conjugated secondary antibody goat anti-rabbit 1:1000 (Vector Labs, cat. No. BA-1000)

Diluted Avidin/Biotin complex ABC 1:400 (Vector Labs, cat. No. PK-6100)

DAB (Invitrogen, cat. No. 750118) Aliquot and store at -20°C

Microscope slides Superfrost

Oven

Coverslips

Xylenes

Mounting medium (permount or prolong)

Prepare tissue sections

- 1 Select tissues and transfer them gently (using a paint brush) to a container filled with 1X DPBS to wash away cryostorage solution. Place tissues on 9-well glass spot plate

Alternatively, staining can be performed in a 24 well-plate. If tissues have been overfixed, antigen retrieval may be implemented to change protein conformation allowing surface antigens to be accessible.

- 2 Add 200 μl of blocking solution and incubate 1–3 hour at 4°C in constant agitation.

If the thickness of the tissues is less than 50 μm , blocking step can be done in 1 hour at room temperature

Stain tissues with antibodies

- 3 Remove blocking solution and immediately add primary antibody diluted in DPBS/0.1% Triton X-100. Incubate overnight at 4°C , under constant agitation.
- 4 Wash tissues 5 times with 500 μl of DPBS/0.1% Triton X-100 at room temperature while gently rocking.
- 5 Incubate tissues with 200 μl of secondary antibody diluted in DPBS/0.1% Triton X-100

Fluorescently labeled secondary antibodies can be used for the colocalization with other microglial marker (GFP in the case of transgenic mice expressing green fluorescent protein in microglia cells) or any other marker of interest

- 6 Wash 5 times with 500 μl of DPBS/0.1% Triton X-100, room temperature under constant agitation. Prepare ABC complex as it needs to incubate for 20–30 min

prior adding to the tissues if immunohistochemistry is the method to visualize microglia

- 7 Add Avidin/Biotin complex diluted 1:400 in DPBS/0.1% Triton X-100. Incubate 1 hour at room temperature
- 8 Wash 5 times with 500µl of 1X DPBS, room temperature under constant agitation
- 9 Develop with DAB (pre-filtered) for 1–3 min (monitor color intensity until desire contrast)

Alternatively, if fluorescence staining is desired skip this step and continue with immunofluorescence protocol of choice.
- 10 Wash 5 times with 500µl of 1X DPBS, room temperature under constant agitation

Mount tissues onto slide

- 11 Mount tissues on slides in DPBS. Under stereomicroscope, flatten tissue against slide removing all folds and creases using a fine tipped paint brush.
- 12 Bake tissues for 30 min at 65°C, to achieve complete attachment and dehydration of the tissues

Only bake tissues onto slide for immunohistochemistry. If immunofluorescence is used, sections must be coverslipped immediately using Prolong mounting medium.
- 13 Soak slides in Xylenes for 3 min.
- 14 Add coverslip using permount.

REAGENTS AND SOLUTIONS

100% Isotonic Percoll

Add 5mL of 10x HBSS to 45mL Percoll. You will need 3mL per sample, scale up or down accordingly.

70% Percoll

Add 21mL of 100% Isotonic Percoll (made in previous step) to 9mL 1x HBSS/ 10mM HEPES. 2mL of 70% percoll is required for each sample.

Myelin Removal Buffer

Supplement PBS (phosphate buffered saline) pH 7.2, with 0.5% bovine serum albumin (BSA). Keep solution cold at 4°C and degas before using with magnetic columns.

CD11b Enrichment Buffer

Supplement PBS (phosphate buffered saline) pH 7.2, with 0.5% bovine serum albumin (BSA) and 2mM EDTA. Keep solution cold at 4°C and degas before using with magnetic columns. Buffers containing Ca⁺ and Mg⁺ are not recommended.

1xHBSS/10mM HEPES

Add 5mL of 1M HEPES to 500mL of 1x HBSS. Mix by inversion and store at room temperature.

Sorenson's Buffer

Prepare Sorenson's Buffer by adding 3.59g sodium phosphate monobasic and 24.7g sodium phosphate dibasic heptahydrate to 400mL ddH₂O. Ensure that pH is between 7.0–7.3. Adjust final volume to 500 mL with ddH₂O and store at room temperature.

4% Paraformaldehyde

4% Paraformaldehyde in phosphate buffer (Sigma): First prepare 8% PFA by dissolving 40g paraformaldehyde to 400mL ddH₂O and stir for 15 minutes at 55–65°C. Add 100 µL 5M NaOH for 100mL of 8% PFA solution. Stir at 30°C and add ddH₂O for a final volume of 500mL. Add 200 mL of Sorenson's Buffer to 500 mL of 8% PFA and 300 mL ddH₂O. Filter sterilize, aliquot in 100mL bottles and store at –20°C.

Blocking solution

Supplement 10% Goat serum with 0.3% Triton-X100

Cryoprotection solution

Mix 200mL glycerol with 200mL Sorenson's buffer and finish with 600mL of double distilled water. Mix with stir bar until confluent and store at 4°C.

Cryostorage solution

Make 0.2M PO₄ solution by mixing part A into part B until the pH reaches 7.4.

Part A: 5.51g sodium phosphate monobasic in 200mL ddH₂O

Part B: 19.87g sodium phosphate dibasic in 700mL ddH₂O

Combine the following ingredients in order:

500mL 0.2M PO₄ solution as made above

10g polyvinylpyrrolidone (PVP-40)

300g sucrose

300mL ethylene glycol

Solution may take about 45 minutes to become confluent. Adjust to 1L with 0.2M PO₄ solution if needed. Store at 4°C.

COMMENTARY

Background Information

Analyses of cell surface markers on microglial cells to assess activation state and phenotypic features in vivo from normal and inflamed CNS tissues has been utilized for several decades (Sedgwick et al., 1991; Ponomarev et al., 2005; Ford et al., 1995). Most isolation protocols are based on density gradient centrifugation, although cell sorting is also highly implemented to analyze pure cell population for transcriptome or proteomic analysis. Furthermore to study the contribution of myeloid cells during CNS pathologies the combination of flow cytometry staining and the usage of bone marrow chimeric mice has provided valuable data into the function of distinct myeloid cell populations during CNS inflammatory conditions (Djukic et al., 2006; Prinz & Priller, 2010; Ponomarev, Shriver, Maresz & Dittel, 2005; Mizutani et al., 2012). Notably, several knock-in strains of mice available commercially have facilitated the analysis of microglial cells in vivo. Particularly, studies using the CX3CR1-green fluorescent protein knock in mice (Cardona et al., 2006; Jung et al., 2000) containing the expression GFP exclusively on microglial cells in naïve

CNS tissues has immensely contributed to the understanding of microglial and myeloid cell biology in different pathologies (Auffray et al., 2009; Auffray et al., 2007; Landsman et al., 2009; Davalos et al., 2012). Protocols to isolate microglia combined with in situ immunocytochemical approaches provide powerful tools to address multiple avenues in the investigation of microglial cells during CNS inflammation.

Critical Parameters

Microglial cells, similar to macrophages are responsive to endotoxin; therefore, all reagents must be endotoxin-free. During the isolation of CNS mononuclear cells, including microglia, the setup of the gradient is the most critical. The cell suspension in 30% percoll must be layered over the 70% solution avoiding mixing of the 30 and 70% layers in order to generate a defined step gradient. Also, after collecting the interphase be sure to check for sufficient cell yield and viability by counting cells on a hemocytometer in trypan blue. This will eliminate efforts in flow cytometry staining in the event that not enough cells were obtained. In addition, some markers are particularly prone to receptor mediated endocytosis such as CD115, so it is recommended to keep cells cold at all times. Regarding enzymatic digestion, when preparing Solution 2 for enzyme mix 1 for the neural tissue dissociation protocol, it is recommended to add beta-mercaptoethanol to a final concentration of 0.067mM to stabilize the enzymes. For example, add 13.5 μ L of 50mM beta-mercaptoethanol to 10mL Solution 2. The enzyme solution will be stable for 1 month at 4°C. Manual dissociation by pipetting can replace the use of the gentleMACS dissociator. If this method is followed, be sure to dissociate tissues completely so that the enzyme can be exposed to all cells. The gentleMACS dissociator is a tool used to process many samples in a short amount of time. When applying histochemistry and/or immunofluorescent protocols, preparation and proper treatment of mice prior isolation of tissues is as imperative as the isolation itself. These procedures require the combination of a set of skills and techniques that also become easier and faster with practice. The protocol described will allow the initiation of an extensive investigation of the CNS that includes assessment of cellular interactions, compartmentalization and functions in great detail. Perfusion with HBSS and 4% PFA are essential for removing cells of the periphery making it possible to only consider CNS bound cells and respective markers. In addition, PFA fixes the cells within the tissue as they are so that we can observe them in situ. On an additional note, the fixation of mice for the purpose of tissue harvesting is critical in preserving the GFP expressed on microglia signaling the replacement of CX3CR1. PFA fixation may mask antigens therefore antigen retrieval protocols may be necessary. Fluorescent immunostaining procedures should always be performed in a dark environment if allowed, or protect specimens from any direct light source by covering them with aluminum foil. Once an immunofluorescent procedure is completed, samples should be stored at 4°C to slow down tissue/cell structure degradation and photo bleaching.

Troubleshooting

In the scenario that no interphase is observed after gradient centrifugation, the number of cells is likely very low which will make further analysis very difficult. Therefore, fresh cellular suspensions may be required. Before the experiment, make sure that all of the antibodies used in flow cytometry work by doing a titration prior staining of the CNS cells. This is especially important if the enzymatic dissociation was used, as it is possible for the enzymes to cleave some cell markers of interest. In the process of tissue collection for histology, although fixation is crucial, certain fixatives or over fixation can result in protein cross-linking causing alterations in the conformation of cellular proteins that in turn mask the epitopes of interest for antibody binding. In this case, antigen retrieval should be considered to unmask antigens hidden by the fixation process.

Anticipated Results

Cell suspensions obtained either by using the down homogenizer of enzymatic dissociation should appear as smooth white solutions without visible pieces of tissues. After the centrifugation of the 30%/70% gradients the interphase should have a visible defined white halo. If you do not see the interphase in a particular sample, try to collect the suspension located around the 2mL graduation which is where the cells should be layered. When counting cells, pay attention to cell viability. If you see many dead cells check the pH and temperature of solutions and make sure that you are washing the interphase cells removing any access percoll using 1x HBSS/ 10mM HEPES. You can expect to recover approximately $4-9 \times 10^5$ cells from 2 pooled naïve brains, and $2-3 \times 10^5$ cells from 2 naïve spinal cords following the protocol for isolation of mononuclear cells, and inflamed tissues typically yield $> 1-3 \times 10^6$ cells depending on the neuroinflammatory insult. If the CD11b enrichment protocol is utilized in conjunction with the percoll gradients, you can anticipate roughly 25% of your original cell count to be CD11b+. When analyzing by flow cytometry, the CD45lo population will be made up of mostly CD11b+ microglia whereas the CD45hi population contains myeloid cells including infiltrating monocytes and monocyte derived dendritic cells. MHC II expression is observed in higher levels from the CD45hi populations.

Time Considerations

The harvesting of brain leukocytes should take less than 4 hrs. It will take approximately 8 minutes per mouse to sedate, perfuse and remove tissues. Homogenization, layering of cells on percoll, and collecting the interphase can take 1–2 hours depending on how many samples are processed. CD11b enrichment, including myelin removal, should take approximately 1 ½ hours. Staining of cells for flow cytometry takes between 1–1 ½ hours depending on the number of samples. This procedure becomes much faster with experience, but in general the entire procedure will take approximately 5 hours to process 5–10 samples.

The collection of CNS tissues for histochemistry might take between 15 to 30 minutes. If immunohistochemistry or immunofluorescence are required, tissues need to be harvested, postfixed and cryoprotected to prevent damage due to ice formation during the sectioning process, processes that are recommended to be performed overnight. Once the tissues are sectioned they can be stored in cryostorage solution at -20°C for years. Once the staining is initiated it requires two consecutive days for the completion of the protocol. Observation and analysis of staining is recommended to be done shortly (within 2–3 days after finishing staining) mainly if immunofluorescence was used, to preserve the integrity of the fluorochromes and prevent photo bleaching.

Acknowledgments

This work was supported in part by the US National Multiple Sclerosis Society (grant TA3021A1/T to AEC), The San Antonio Area Foundation (grant 201135345 to AEC), and National Institutes of Health (grant SC1GM095426 to AEC).

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Figure 14.35.1.

The total leukocyte population is gated from the raw data to exclude any debris that typically lies along the Y axis. It is imperative to only select the true leukocyte population in order to obtain clean plots. CD45 is sub gated from the all population into 3 different groups. CD45Hi population is made up of hematogenous derived cells that infiltrated the CNS, whereas the CD45lo population represents the CD11b+ microglial population. The CD45 negative population will be omitted from this analysis and likely contains astrocytes, or other glial and neuronal populations. Within the CD45Hi population, there are 2 populations of interest: infiltrating monocytes (CD11b+ CD11c-) and monocyte derived dendritic cells (CD11b+ CD11c+). From the CD45lo population graph CD11b by CD11c to determine the percentage of true resident microglia (CD11b+ CD11c-) and CD11b+ CD11c+ populations.

Table 14.35.1

Common antibodies used for analysis of microglial cells by flow cytometry

Antibody Markers	Clone
CD45	30-F11
CD11b (microglia)	M1/70
CD11c (dendritic cells)	N418
CD80	16-10A1
CD86	GL-1
I-A/I-E (MHC II)	M5/114.15.2
CD274 (PD-L1)	10F.9G2
F4/80 (macrophage / blood monocytes)	BM8
Ly6C (macrophage)	ER-MP20
CD115 (monocyte, macrophage)	AFS98
Ki-67 (cell proliferation) ** intracellular	B56