Video Article

Isolation of Brain and Spinal Cord Mononuclear Cells Using Percoll Gradients

Paula A. Pino[1], Astrid E. Cardona[1]

[1]Department of Biology and South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio - UTSA

Correspondence to: Astrid E. Cardona at astrid.cardona@utsa.edu

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Abstract

Isolation of immune cells that infiltrate the central nervous system (CNS) during infection, trauma, autoimmunity or neurodegeneration, is often required to define their phenotype and effector functions. Histochemical approaches are instrumental to determine the location of the infiltrating cells and to analyze the associated CNS pathology. However, in-situ histochemistry and immunofluorescent staining techniques are limited by the number of antibodies that can be used at a single time to characterize immune cell subtypes in a particular tissue. Therefore, histological approaches in conjunction with immune-phenotyping by flow cytometry are critical to fully characterize the composition of local CNS infiltration. This protocol is based on the separation of CNS cellular suspensions over discontinous percoll gradients. The current article describes a rapid protocol to efficiently isolate mononuclear cells from brain and spinal cord tissues that can be effectively utilized for identification of various immune cell populations in a single sample by flow cytometry.

Video Link

The video component of this article can be found at https://www.jove.com/video/2348/

Protocol

Preparation of reagents

Prepare stock isotonic percoll (SIP), 4 ml per brain, by mixing 9 parts of percoll with one part of 10X HBSS without Ca++ and Mg++.

Prepare SIP at 70% in 1X HBSS without Ca++ and Mg++, 2 ml per brain dispensed into a 15 ml polypropylene conical tube.

Note: It is recommended that percoll should be used at room temperature, if used cold, cells tend to clump and cell separation is less efficient.

Tissue collection and homogenization

Anesthetize mice and perfuse through the left ventricle with ice-cold 1X HBSS. Dissect brain and spinal cord and maintain in RPMI without phenol red until all mice are sacrificed.

Place tissues in 7 or 15 ml dounce homogenizer containing 3 ml of RPMI, gently make a cell suspension with a loose-fitting pestle (A size) followed by a tigh-fitting pestle (B size) to further dissociate the tissues. Complete the volume of the cell suspensions to 7 ml with RPMI.

Gradient set up

Add 3 ml of SIP to the cell suspension to make a final 30% SIP

Slowly layer the 10 mL cell suspension on top of the 70% SIP. This is the most critical step of the procedure, use a pipette-aid set in the gravity mode avoiding mixing of the 70% and 30% solutions. A very clear flat line should be visible at the 70%-30% junction.

Centrifuge 30 min at 500G 18°C. Make sure centrifuge will stop with minimal or no brake so that the interphase is not disturbed.

Using a transfer pipet, gently remove the layer of debris from the top of the tube and collect 2.0-3.0 ml of the 70%-30% interphase into a clean conical tube containing 8 ml of 1X HBSS. Ensure that the percoll containing the interphase is diluted about three fold, mix a few times by inversion and centrifugre 7 min at 500G at 18°C.

Resuspend pellet in 1 ml of cell staining buffer and transfer it to a 1.5 ml tube and wash one more time using a micro-centrifuge at 10,000G 1 min at 4°C.

Antibody staining

Resuspend pellets in 50 µl of purified antimouse CD16/CD32 diluted 1:200 in cell staining buffer to block the Fc binding sites. Incubate over ice for 10 min. Count cells using a hemocytometer.

Add 50 µl of antibody cocktail mix and incubate on ice for 30 min. Each antibody must be first titrated to assess optimal dilution. Use appropriate fluorochrome combinations chosen according the capabilities of laser and filter settings of your particular flow cytometer.

Wash cells in cell staining buffer, resuspend pellets in 100-200 µl of cell staining buffer and analyze immediately in the cytometer, or alternatively cells can be resuspended in 2% paraformaldehyde (PFA) prepared in PBS and stored over-night at 4°C.

Representative Results:

After spinning the gradients, the 70%-30% interphase should have a defined white halo, and quantitation of the cells recovered from a naíve mouse should yield 3-5 x10^5 cells per mouse (brain plus spinal cord). Inflamed brain might have ranging numbers of inflammatory cells depending of the CNS insult or disease model (Figure 1).

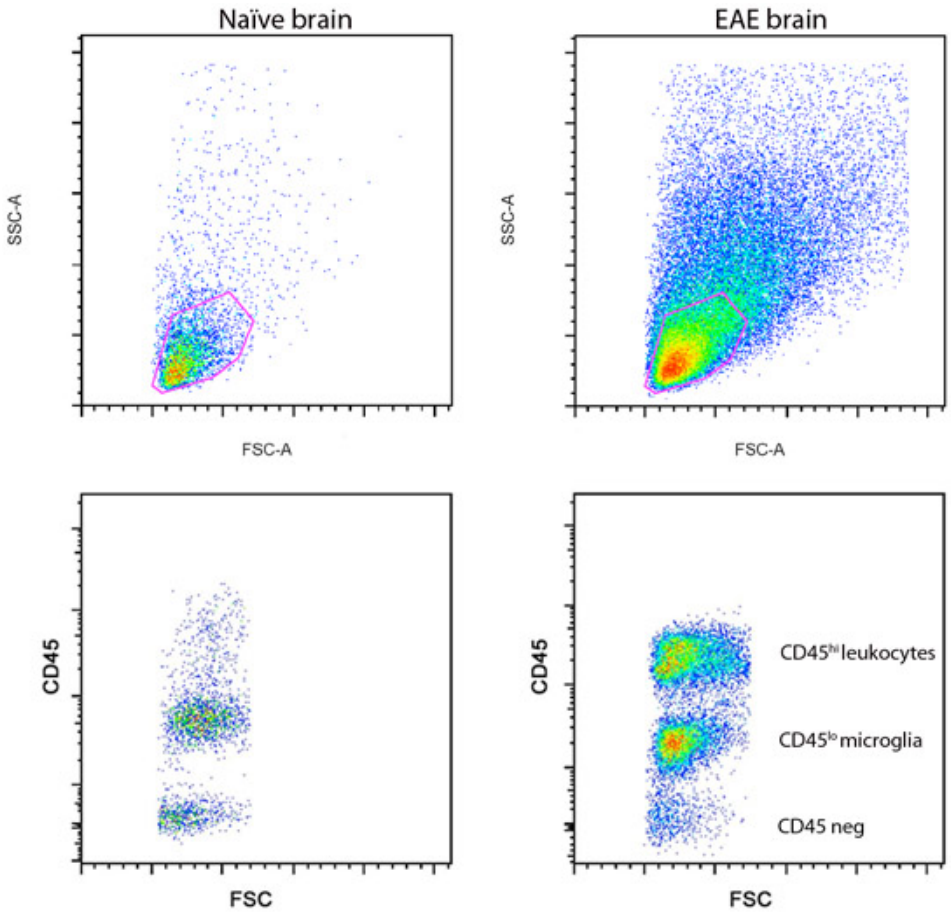

Figure 1. Isolation of mononuclear cells, from naíve and EAE-brain at peak disease. Brain cells suspensions were separated over discontinuous 70%/30% percoll gradients. Cells stained were incubated with Fc-block followed by anti-CD45 antibodies for 30 min on ice. Cells were rinsed in cell staining buffer, and fixed in 2% PFA prior acquisition in an LSR-II. CD45 intensity is measured in the Y-axis, where three distinct populations are visualized. Further characterization of the immune phenotype of $CD45^{hi}$ infiltrating cells is implemented using additional antibodies and subsequently analyzed by flow cytometry.

Discussion

Analyses of cell surface markers in CNS leukocytes from normal and inflamed mouse tissues has been utilized for several decades[1-3]. Isolation protocols are based in the separation of microglia and leukocytes by density centrifugation[4]. Here we describe a fast and effective method of isolating CNS leukocytes using discontinuous percoll gradients. After isolation of cells, staining with various antibodies such as -but not limited to- CD45, CD4, CD8, CD11b, CD19, etc. allows the identification of immune populations that infiltrate the CNS upon induction of a particular pathology. Staining with anti-CD45 antibodies reveals three distinct populations by flow cytometry; (1) CD45 negative population consisting of neuronal cells, astrocytes and oligodendrocytes among others nerve cells, (2) $CD45^{lo}$ or intermediate which contains mostly surveillant microglial cells, (3) $CD45^{hi}$ population consisting of infiltrating hematogenous leukocytes. Studying the contribution of myeloid cells during CNS pathologies

has been challenging. However, the combination of various cells surface markers[5, 6] and the usage of bone marrow chimeric mice have shown insights into the functional distinctions of myeloid cells during CNS inflammation[2, 7, 8]. Upon immunization with myelin peptides, an autoimmune reaction is induced and a massive influx of blood cells is recruited to the brain. After this point, there is a wide repertoire of antibodies available to study the phenotype of these cells either by the presence of various cell surface molecules, as well as expression of intracellular proteins such as cytokines or transcription factors.

When homogenizing the tissues, use extreme caution handling the homogenizer with ease. Mononuclear phagocytes are highly susceptible to autolysis. If experiencing clumping of the cells, practice a gentler homogenization protocol and add DNase to the buffer during the homogenization step. Therefore, assess percentage of cell dead in your isolated population. This protocol can be easily modified to add various digesting enzymes, such as collagenase, dispase among others. The yield of cells is usually higher. However some cell surface markers are susceptible to this treatment and therefore will limit their detection by flow cytometry due to their cleavage by proteases. In our protocol, we routinely avoid the usage of digesting enzymes, dissect tissues as quick as possible without compromising the perfusion, and avoid leaving the tissues for long periods of time on ice (> 1hr) prior homogenization. The protocol described has the potential to be applied to gene expression, protein and cell culture of the isolated population.

Disclosures

No conflicts of interest declared.

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