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Meningeal whole mount preparation and characterization of neural cells by flow cytometry

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Abstract

Neuroimmunologists aim to understand the interactions between the central nervous system and the immune system under both homeostatic and pathological conditions. The meninges, contrary to the brain parenchyma, are populated by numerous immune cells. Soluble factors produced by these cells are capable to diffuse into the brain parenchyma and influence the brain cells within the parenchyma, including neurons. In this unit, we will describe two protocols: analysis the meningeal compartment of rodents and the use flow cytometry to study the cells of the brain parenchyma (particularly neurons).

Keywords

Neuroimmunology; meninges; immune cells; neurons

Introduction

While coronal sections of the brain with the attached meninges can provide some information about the meningeal compartment, the processing of the tissue usually results in a poor spatial representation of this compartment and is usually missing parts of the meninges (dura + arachnoid) that remains on the skull in most brain dissection protocols. In the first part of this protocol, we provide a detailed description of the dissection and whole-mount preparation of the meninges for immunohistochemistry analysis.

Immune cells of the meningeal compartment have been shown to be able to directly impact brain function, through the secretion of cytokines. In the second part of this protocol, we describe a flow cytometry-based approach to analyze protein expression on the cells of the brain parenchyma, notably neurons.

Basic Protocol 1: Preparation and analysis of meningeal whole mount

In this section we will describe steps to analyze the meningeal compartment in mice using immunohistochemistry. First we will describe the steps to dissect and isolate the meninges (Movie 1) followed by the immunostaining protocol.

Materials

Large Scissor
Dumont #5 forceps
Dumont curved forceps
Angled scissors
10ml syringe with 30G needle
Dissecting microscope
Shaker
24-well plate
10mm petri dish
Glass slides
Coverslips
Perfusion Buffer (see reagents and solutions)
Fixation solutions (see reagents and solutions)
Block-Permeabilization solution (see reagents and solutions)
Antibodies solution (see reagents and solutions)
Aqua-mount (Thermo Scientific, Cat No 14-390-5)
ProLong Gold antifade Reagent (Invitrogen, Cat No P36930)
Small paintbrush
Anti-mouse CD16/CD32 (eBiosciences, Cat No 14-0161-85)

Harvest the skullcap

- 1 After appropriate euthanasia (UNIT 1.8), transcardially perfuse (UNIT 15.1) the mouse with 10ml of ice-cold PBS containing 5 units/ml of heparin (Perfusion buffer) using a 30G syringe until the mouse is exsanguinated.

It is important for the perfusion to be efficient since the presence of blood within the meningeal vasculature will increase the auto-fluorescence of the tissue (because of the remaining red blood cells) and will alter the analysis of the meningeal resident immune cells.
- 2 Decapitate the mouse above the shoulders, remove the skin from the skull, and sever optic nerves to remove the eyes.
- 3 Turn the head over to face the ventral side of the jaw. Slide angled dissection scissors through the oral cavity until the resistance of the mandibular junction is felt. Cut the large muscles connecting the lower jaw to the skull and remove.

- 4 Using angled scissors, remove the lower orbits of each side of the skull and clean all flesh from the skull.

Efficient removal of the flesh will facilitate removal of the meninges from the skullcap.
- 5 With curved scissors angled towards the outside (to prevent major damages of the brain parenchyma) carefully cut clockwise around the skull inferior to the posttympanic hook and remove the lower portion of the skull.
- 6 Sever the nasal bone, anterior to the olfactory bulbs.
- 7 Scoop the brain out of the upper half of the skull and place the skullcap in a 24-well plate with 1ml of the appropriate Fixation solution placed on ice.

Fixation of the meninges

- 8 Depending on the antibodies that will be used later on, fix the meninges, left in the upper half of the skullcap either overnight at 4°C with PFA2% in PBS or 20min at -20°C with a 1:1 solution of ethanol:acetone. After fixation, transfer the skullcap into PBS.

For choice of fixation, please refer to the manufacturer's datasheet of your antibody.

If you want to preserve the skullcap for a longer period of time prior to dissection and analysis, it is advised to add azide (0.02%) into the PBS solution and store the skullcap at 4°C.

Dissection of the meninges

- 9 Place the skullcap (Figure 1), with PBS, in a 10mm petri dish under a dissecting microscope.
- 10 With fine Dumont #5 forceps, secure the skullcap, at the section that covers the olfactory bulbs, and grab the thick piece of meninges and gently pull it towards the superior sagittal sinus. This will lead to the initial detachment of the meninges from the skullcap and will facilitate the rest of the dissection.

To maintain the skullcap at the bottom of the petri dish, curved forceps are used.

- 11 Moving along the edge of the interior of the skullcap, gently detach (scrape) the meninges from the skull using fine forceps until you reach 360°.

The meninges, at the tip of the transverse sinuses and above the cisterna magna are thicker and will require to be pulled more firmly to be detached from the skull.

- 12 Grab the meninges at the tip of the transverse sinuses and pull them towards the pineal gland (at the junction of the superior sagittal sinus and the transverse sinuses) to detach them from the bone junction. Grab the meninges from the

pineal gland region and carefully pull it out of the skullcap. If done correctly, the whole mount should consist of one piece without tears.

- 13 If small pieces of skull bone remain attached to the dissected meninges (particularly at the end of the transverse sinuses), remove them using fine forceps. Leaving bone pieces would negatively impact the immunostaining (Video).
- 14 Place the dissected meninges in a 24-well plate with 500µl of PBS (supplemented with azide 0.02% if long term storage is expected prior to immunostaining).

Immunostaining

- 15 If the meninges were stored with azide, wash the meninges for 5 min with PBS (Incubate on a shaker).
- 16 Incubate the meninges on a shaker for 1h at room temperature in 300µl of PBS with 2% serum, 1% BSA, 0.001% anti-mouse CD16/CD32 (for blockade of endogenous Fc receptors), 0.1% Triton-X-100 and 0.05% of Tween-20.
- 17 Incubate the meninges on a shaker overnight at 4°C in primary antibodies diluted in PBS with 1% BSA and 0.5% Triton-X-100.
- 18 Wash the meninges 3×5min with PBS on a shaker.
- 19 Incubate the meninges on a shaker for 1h at room temperature in 300µl of secondary antibodies diluted in PBS with 1%BSA and 0,05% Triton-X-100.
- 20 If desired, incubate the meninges for 5min at room temperate in 300µl of DAPI.
- 21 Wash the meninges 3×5min on shaker with PBS.
- 22 Using a fine paintbrush, flatten the meninges on a glass slide and let them dry, in the dark for 10–15 minutes.
- 23 Cover the meninges with a coverslip and the appropriate mounting media (e.g. Aquamount) or subsequent microscopy. Samples can be stored for a week at 4°C prior to imaging. For longer preservation, one might consider another mounting media (e.g. ProLong antifade Reagent)

Basic Protocol 2: Characterization of neural cells by flow cytometry

In this section, we will describe how to process brain tissue into single cell suspension allowing analysis of neurons by flow cytometry.

Materials

Neurobasal Media (Invitrogen; Cat No 12348-017)
Fetal Bovine Serum
DNase I (Sigma Aldrich; Cat No DN25-1G)

Dumont #5 forceps
Dissecting microscope
2ml dounce homogenizer
V-shaped 96 well plate
70µm nylon filter (Component Supply; Cat No U-CMN-70-C)
FACS Buffer (see reagents and solutions)
Hoeschst 33342 (Molecular Probes; Cat No H3570)
Cytofix/Cytoperm (BD Biosciences; Cat No 554714)
Permeabilization wash buffer (see reagents and solutions)
Live Dead Dye (Zombie Aqua Fixable viability, Biolegend, Cat No 423102)

Harvesting of the brain tissue

- 1 Euthanize and perfuse the mouse as described above and place the brain in Neurobasal media containing 10% of fetal bovine serum kept on ice.
- 2 Using forceps, under a dissecting microscope, peel the meninges from the surface of the brain.
- 3 Micro-dissect the region of interest (for example prefrontal cortex, hippocampus or cerebellum).

Single cell solution preparation

- 4 Place the brain tissue into a 2ml dounce homogenizer containing Neurobasal media supplemented with 50U/ml of DNase I. On ice, gently homogenize the brain tissue until no visible pieces of brain can be visualized.
- 5 Filter the brain homogenate through a 70µm nylon filter and wash with ice-cold FACS buffer.

Immunostaining

- 6 Centrifuge the brain homogenate for 10min at 320g/4°C.
- 7 Resuspend the brain pellet in FACS buffer and transfer into V-shaped 96 well-plate.
- 8 Centrifuge the plate for 5min at 320g/4°C.
- 9 Wash the cells twice with FACS buffer.
- 10 Incubate the cells with 100µl solution of extracellular antibodies with Live Dead dye and Hoechst (to allow gating of cells during the analysis) diluted in FACS buffer with 0.001% of anti-mouse CD16/CD32 antibodies. Incubate for 30min at 4°C.
- 11 Add 100µl of FACS buffer.

- 12 Centrifuge the plate for 5min at 320g/4°C.
- 13 Permeabilize the cells with 100µl of Cytofix/Cytoperm solution and incubate for 40min at room temperature in the dark.
- 14 Add 100µl of Permeabilization wash buffer.
- 15 Centrifuge the plate for 5min at 320g/4°C.
- 16 Incubate the cells with 100µl of intracellular antibodies (e.g. anti-NeuN) diluted in permeabilization wash buffer overnight at 4°C.
- 17 Add 100µl of Permeabilization wash buffer.
- 18 Centrifuge the plate for 5min at 320g/4°C.
- 19 Resuspend the cells in FACS buffer. Pass the cells through a 70µm nylon filter (to remove any aggregates that could impact reading to the samples).
- 20 Analyze the samples using a flow cytometer (see Chapter 5). The samples can be stored in FACS buffer up to 48h at 4°C prior to run on the flow cytometer.

Reagents and Solutions

Perfusion buffer:

PBS (0.1M; pH 7.4)

5U/ml of heparin

Fixation solutions:

PBS with 2% of paraformaldehyde (keep at 4°C)

1:1 solution of Acetone:Ethanol

Block-Permeabilization solution:

PBS (0.1M; pH7.4)

2% Serum

1% Bovine Serum Albumin

0.001% anti-mouse CD16/CD32 (eBiosciences; Cat No 14-0161-85)

0.1% Triton-X-100

0.05% Tween 20

Antibodies solution

PBS (0.1M; pH7.4)

1% Bovine Serum Albumin

0.5% Triton-X-100

FACS Buffer

PBS (0.1M; pH7.4)
1mM EDTA
1% Bovine Serum Albumin
Permeabilization wash buffer
PBS (0.1M; pH 7.4)
10% Fetal Bovine Serum
1% Sodium Azide
1% Saponin

COMMENTARY

Background information

Understanding the interactions between the central nervous system and the immune system has been fascinating scientist for decades. Recent studies have demonstrated that the immune system is necessary for proper brain function under physiological conditions (Baruch et al., 2013; Brombacher et al., 2017; Derecki et al., 2010; Filiano et al., 2016; Ziv et al., 2006), and that under pathological conditions, the immune system can be both beneficial and detrimental for brain function (Kipnis, 2016; Kokaia, Martino, Schwartz, & Lindvall, 2012; McKee & Lukens, 2016; Möhle et al., 2016; Steinman, 2008; Walsh et al., 2014).

While the brain parenchyma is naturally devoid of immune cells, to the exception of microglia (and mast cells), the surrounding of the brain i.e. the meninges are populated by numerous immune cells. Meningeal immunity ensures brain-immune surveillance (Kipnis, Gadani, & Derecki, 2012; Ransohoff & Engelhardt, 2012), contributes to proper brain function (Brombacher et al., 2017; Derecki et al., 2010; Filiano et al., 2016), and can be manipulated to affect development of neurological disorders (Baruch et al., 2016; Schläger et al., 2016). Cytokines and other molecules, secreted by the immune cells of the meningeal immune compartment, likely can diffusion into the parenchyma and act directly on the cells within the parenchyma to impact neuronal function. The meningeal compartment, by its location, and its direct communication with the brain (through the cerebrospinal fluid) and with the periphery (via the meningeal lymphatic system (Aspelund et al., 2015; Louveau et al., 2015), therefore, represents a key player in understanding the interactions of the central nervous and the immune systems under both homeostatic and pathological conditions.

Critical parameters/troubleshooting

For the whole-mount preparation, the critical parameter is with the dissection of the meninges. One needs to consider practicing the dissection prior to performing real experiment as initial dissections usually result in tears of the tissue. The cleaning of the skullcap from flesh will be important to obtain a good whole-mount dissection.

For the flow cytometry, the critical parameter is the homogenization of the brain to obtain good cell viability. It is important to work on ice during the dissection and homogenization of the brain to avoid cell death. If starting from a large portion of the brain, Hoechst staining (to identify nucleated cells versus debris) will be critical to avoid autofluorescent debris interfering with the analysis and complicating the gating.

Anticipated Results

For the whole-mount meninges, it is expected to obtain a complete meningeal covering of the mouse brain with identifiable structures: superior sagittal sinus and transverse sinuses, along with meninges covering both cortexes (including the middle meningeal artery) but also the cerebellum and cisterna magna (Louveau et al., 2015).

For the flow cytometry on the brain, it expected the cell viability to be ~40% (Filiano et al., 2016)

Time considerations

The whole mount dissection/immunostaining protocol will span a 3 day-period, with fixation on the first day, dissection and first part of immunostaining on the second day, and final immunostaining on the third day. The dissection itself will be of about 3 minutes per meninges once efficiency is achieved.

The entirety of the flow cytometry protocol will be performed over the course of 2 day, with the tissue preparation and staining in a matter of a couple of hours (3–4h) on the first day and staining the next day.

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Figure 1. Skullcap Meninges

Representative images of the skullcap of adult mouse prior to meninges dissection. Dorsal (A), ventral (B) and side (C) view of the skullcap. Scale bar = 1 cm.