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Isolating Central Nervous System Tissues and Associated Meninges for the Downstream Analysis of Immune Cells

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Corresponding Author:	Krista DiSano Dartmouth-Hitchcock Medical Center Lebanon, NH UNITED STATES
Corresponding Author's Institution:	Dartmouth-Hitchcock Medical Center
Corresponding Author E-Mail:	krista.d.disano@hitchcock.org
Order of Authors:	Krista DiSano
	Michael R. Linzey
	Nora C. Welsh
	Joshua S. Meier
	Andrew R. Pachner
	Francesca Gilli
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Krista DiSano
Department of Neurology 558W
Dartmouth Hitchcock Medical Center
1 Medical Center Drive
Lebanon, NH 03756
Krista.d.disano@hitchcock.org

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Editorial Office

Attached is a manuscript entitled "Isolating Central Nervous System Tissues and Associated Meninges for Downstream Analysis of Immune cells Using Histology and Single-cell Techniques" by Krista D. DiSano, Michael R. Linzey, Nora C. Welsh, Joshua S. Meier, Andrew R. Pachner, and Francesca Gilli for consideration for publication as an original article in the *Journal of Visualized Experiments*. The manuscript has 6 figures.

Cells residing in meninges are widely recognized as being important contributors to homeostasis in the Central Nervous System (CNS) compartment. Moreover, during inflammatory conditions resulting from stroke, infection, injury, autoimmunity, and inflammatory demyelinating diseases such as Multiple Sclerosis (MS), the meninges support the accumulation of diverse immune cells which may either play a beneficial or detrimental role in CNS disease pathogenesis. However, conventional methods for extracting CNS tissues for analysis often omit the meninges, either due to the technically difficulty or time required for extraction. Omission of the meninges results in an incomplete analysis of cells in the CNS compartment during steady-state conditions and during disease pathogenesis.

In this article, we propose two distinct methods for the rapid extraction of CNS tissues, including the spinal cord, brain, and meninges. The first methodology focuses on the rapid extraction of brains in the intact cranium and spinal cords within the vertebral column and a subsequent decalcification and tissue embedding protocol. This method allows all meningeal layers to remain intact and is suitable for downstream histology applications, including immunohistochemistry or *in situ* hybridization, allowing the simultaneous analysis of the CNS parenchyma and meningeal compartment. The second methodology focuses on the rapid extraction of the brain, spinal cord, and the dural/arachnoid meninges from both tissues. The processing protocol describes the isolation of single-cell suspensions from the brain, spinal cord, and meninges for downstream single-cell techniques, including flow

cytometry, *in vitro* culture assays, and bulk and single-cell transcriptomics. Altogether, the described protocols allow for the quick extraction of CNS tissues for analysis by histology or single-cell techniques, creating a comprehensive analysis of cells residing in the CNS compartment during homeostatic and pathogenic conditions.

All authors concur with the submission and the work has not been published elsewhere in any form. Furthermore, there are no conflicts of interest.

Sincerely,

Krista DiSano

TITLE:

Isolating Central Nervous System Tissues and Associated Meninges for the Downstream Analysis of Immune cells

AUTHORS AND AFFILIATIONS:

Krista D. DiSano[1], Michael R. Linzey[1,2], Nora C. Welsh[1,2], Joshua S. Meier[1], Andrew R. Pachner[1], Francesca Gilli[1]

[1]Department of Neurology, Geisel School of Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA

[2]Program in Experimental and Molecular Medicine, Dartmouth College, Hanover, NH, USA

Corresponding Author:

Krista D. DiSano (Krista.D.DiSano@dartmouth.edu)

Email Addresses of Co-authors:

Michael R. Linzey (Michael.Linzey.GR@dartmouth.edu)
Nora C. Welsh (Nora.C.Welsh.GR@dartmouth.edu)
Joshua S. Meier (Joshua.S.Meier@hitchcock.org)
Andrew R. Pachner (Andrew.R.Pachner@dartmouth.edu)
Francesca Gilli (Francesca.Gilli@dartmouth.edu)

KEYWORDS:

meninges, central nervous system, decalcification, immunohistochemistry, single-cell suspension, immune cells, mice

SUMMARY:

This paper presents two optimized protocols for examining resident and peripherally derived immune cells within the central nervous system, including the brain, spinal cord, and meninges. Each of these protocols helps to ascertain the function and composition of the cells occupying these compartments under steady state and inflammatory conditions.

ABSTRACT:

The central nervous system (CNS) is comprised of the brain and spinal cord and is enveloped by the meninges, membranous layers serving as a barrier between the periphery and the CNS. The CNS is an immunologically specialized site, and in steady state conditions, immune privilege is most evident in the CNS parenchyma. In contrast, the meninges harbor a diverse array of resident cells, including innate and adaptive immune cells. During inflammatory conditions triggered by CNS injury, autoimmunity, infection, or even neurodegeneration, peripherally derived immune cells may enter the parenchyma and take up residence within the meninges. These cells are thought to perform both beneficial and detrimental actions during CNS disease pathogenesis. Despite this knowledge, the meninges are often overlooked when analyzing the CNS compartment, because conventional CNS tissue extraction methods omit the meningeal layers. This protocol presents two distinct methods for the rapid isolation of murine CNS tissues (i.e.,

brain, spinal cord, and meninges) that are suitable for downstream analysis via single-cell techniques, immunohistochemistry, and in situ hybridization methods. The described methods provide a comprehensive analysis of CNS tissues, ideal for assessing the phenotype, function, and localization of cells occupying the CNS compartment under homeostatic conditions and during disease pathogenesis.

INTRODUCTION:

The central nervous system (CNS) is an immunologically specialized site. The CNS parenchyma, excluding the CSF space, the meninges, and the vasculature, is classically viewed as an immune-privileged site[1-5] and is relatively devoid of immune cells during homeostatic conditions[2,6,7]. In contrast, the meninges, comprised of the dura, arachnoid, and pia layers, are crucial components of the CNS compartment, actively participating in homeostatic immune surveillance and inflammatory processes during disease pathogenesis[3,6-8]. During steady state conditions, the meninges support numerous immune sentinel cells, including innate lymphoid cells (ILC), macrophages, dendritic cells (DC), mast cells, T cells, and to a lesser extent, B cells[9-11].

The meninges are highly vascularized structures and contain lymphatic vessels that provide a lymphatic connection between the CNS and its periphery[8,12-14]. In inflammatory conditions induced by CNS injury, infections, autoimmunity, or even neurodegeneration, peripherally derived immune cells infiltrate the parenchyma and alter the immune landscape within the meninges. Following cell infiltration, the meninges may represent a functional niche for peripherally derived immune cells, promoting immune cell aggregation, local immune cell activation, and long-term survival in the CNS compartment. Prominent meningeal inflammation is observed in multiple diseases affecting the CNS, including multiple sclerosis (MS)[15-19], stroke[20,21], sterile injury[22,23] (i.e., spinal cord injury and traumatic brain injury), migraines[24], and microbial infection[25-29]. Thus, the characterization of resident cells and peripherally derived immune cells in the meningeal compartment is essential for understanding the role of these cells during steady state conditions and disease pathogenesis.

The extraction of the brain, spinal cord, and meninges from the cranium and vertebral bodies is technically challenging and time-consuming. There are currently no techniques available for the rapid extraction of the brain with all three meningeal layers intact. While laminectomy yields excellent spinal cord tissue morphology and preserves the meningeal layers, it is both extremely time-consuming and complicated[30,31]. Conversely, more conventional extraction methods such as the removal of the brain from the cranium and the hydraulic extrusion of the spinal cord facilitate the quick extraction of the CNS tissue, but both the arachnoid and dural meninges are lost with these techniques[30,31]. The omission of dura and arachnoid layers during conventional isolation of brain and spinal cord tissues results in an incomplete analysis of the cells within the CNS compartment. Thus, the identification of new techniques focused on the quick extraction of CNS tissues with intact meninges is crucial for the optimal analysis of the CNS compartment.

This manuscript presents two methods for the rapid extraction of the brain, spinal cord, and meninges from mice, facilitating the downstream analysis of resident cells and peripherally derived immune cells in the CNS parenchyma and meninges. These optimized protocols focus on

1) isolating single-cell suspensions for downstream analysis and 2) preparing tissue for histological processing. Obtaining single-cell suspensions from the brain, spinal cord tissue, and dural and arachnoid meninges[32] allows for the simultaneous analysis of cells residing in both the parenchymal and meningeal compartments. Single-cell suspensions can be used in different applications, including cell culture assays to perform in vitro stimulation[33], enzyme-linked immunospot (ELISpot)[28,34,35], flow cytometry[36,33], and single-cell[37] or bulk transcriptomics. Additionally, the optimized protocol for decalcification of whole brains and spinal cords with intact skulls or vertebral columns, respectively, allows for the gentle decalcification of the surrounding bone, leaving the meninges intact and preserving the tissue morphology. This method allows for the selective identification of proteins or RNA using immunohistochemistry (IHC) or in situ hybridization (ISH) techniques within both the parenchymal and meningeal spaces. The characterization of the phenotype, activation state, and localization of resident cells and peripherally derived immune cells within the CNS may provide information essential to understanding how individual cell types in the CNS compartment contribute to homeostasis and disease pathogenesis.

PROTOCOL:

All animal work utilizes protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Geisel School of Medicine at Dartmouth.

1. Processing brain and spinal cord samples for decalcification

1.1. Isolating brain and spinal cord samples

1.1.1. Euthanize the mouse via CO_2 inhalation. Ensure that the CO_2 flow rate displaces 10%–30% of the cage volume per minute.

1.1.2. Using forceps, lift the xiphoid process, and cut the abdominal wall laterally just below the rib cage with scissors, pulling up to avoid cutting underlying blood vessels or organs. Cut through the diaphragm laterally.

1.1.3. Cut the rib cage along the lateral edges parallel to the lungs up to the collarbone. Using forceps, lift the sternum and clamp the sternum with a hemostat. Place the hemostat over the head to lift the rib cage away and expose the heart.

1.1.4. Using forceps, grasp the heart near its apex and make an incision in the right atrium of the heart to provide an outlet. Insert a 25 G needle with a 10 mL syringe attached to slowly administer 10 mL of ice-cold 1x phosphate buffered saline (PBS) into the left ventricle to transcardially perfuse the mouse.

NOTE: Perfusion should occur over 4–5 min until the liver is cleared of blood. A total of 10 mL of 1x PBS is usually enough for perfusion, but more may be utilized if necessary. A clear liver usually indicates adequate perfusion.

1.1.5. Using sharp scissors, remove the head by decapitation (**Figure 1;** 1). Make a midline incision in the skin (**Figure 1;** 2) and flip the skin over the eyes to free the skull.

1.1.6. Cut at the nasal bone to release the mandible from the skull (**Figure 1;** 3). Remove the mandible, tongue, and eyes. Cut along the lateral aspects of the skull to release the tissue along the external auditory meatus (**Figure 1;** 4). Trim to remove all excess skin, muscle, and tissue overlaying the skull.

1.1.7. Separate the rib cage from the spinal column by cutting parallel to the spine with sharp scissors (**Figure 1;** 5 and 6). Make a small cut at the lower lumbar region to isolate the spinal column (**Figure 1;** 7). Trim and remove any remaining muscle along the spine to expose the vertebrae (**Figure 1;** 8).

NOTE: Removing excess tissue from the spinal column and skull is necessary to obtain adequate penetration of fixative paraformaldehyde (PFA) and ethylenediaminetetraacetic acid (EDTA) decalcification buffer.

1.2. Post-fixation, decalcification, and cryopreservation

1.2.1. Using forceps, place the brain with the intact skull or spinal column in a 15 mL conical tube containing 10 mL of 4% PFA. Place the tubes at 4 ˚C for at least 48 h for adequate fixation.

NOTE: To avoid overfixation of the tissue, do not exceed 72 h of fixation. Fixation times are extended for bone specimens before decalcification. Adequate fixation will protect tissue from the effects of the decalcification and ensure better tissue morphology.

1.2. Rinse the brain or spinal cord by removing the tissue from the 4% PFA with forceps and placing the tissue in a disposable 14 mL tube with 10 mL of 1x PBS for 5 min. Transfer the brain or spinal cord into a 50 mL conical tube with 10 mL of 10% EDTA (pH = 7.2–7.4).

NOTE: Using a larger tube with 10 mL of EDTA gives the EDTA a greater contact area with the tissue and accelerates the decalcification process.

1.2.3. Check daily if the bone is soft and pliable: remove the tissue from the EDTA solution with forceps, place it on a Petri dish, and gently test the bone softness with a 25 G needle. If the needle easily penetrates the bone, the decalcification process is complete.

1.2.4. Remove the tissue from the EDTA solution and transfer the brain or spinal cord to a 14 mL disposable tube containing 10 mL of 1x PBS and wash for 10 min. Repeat the wash.

NOTE: Decalcification usually takes 2–3 days. The solution should be changed every 2–3 days if the bone is not yet adequately decalcified. However, prolonged incubation in EDTA after the bone has been decalcified can damage the tissue morphology.

1.2.5. Prepare 10%, 20%, and 30% sucrose solutions by adding sucrose to 1x PBS. For example, for 10% sucrose, add 10 g of sucrose and bring the volume to 100 mL using sterile 1x PBS. Store the solution at 4 ˚C for up to 1 month.

NOTE: Sucrose solutions are prone to microorganism growth, so samples should not be stored for prolonged periods of time in these solutions.

1.2.6. Remove the tissue from the 1x PBS, place it in 10 mL of 10% sucrose solution and store it at 4 ˚C. Let the tissue sit for 24 h or until it sinks to the bottom of the tube.

1.2.7. Repeat this process, moving the tissue to a 20% sucrose solution first and finally to a 30% sucrose solution. Allow the tissue to sink in 30% sucrose (at least 24 h) and proceed to tissue embedding.

1.3. Tissue embedding and freezing

1.3.1. Using forceps, remove the tissue from the 30% sucrose, place it on a Petri dish, and tilt the dish to get rid of any excess sucrose solution on the tissue. Using a scalpel, cut the tissue into desired segments.

1.3.2. Create a thin layer of optimal cutting temperature (OCT) compound at the bottom of the cryomold and place the tissue piece(s) in the mold. Cover the tissue completely with the OCT compound, ensuring no bubbles are present.

1.3.3. Flash freeze the blocks by hovering over liquid nitrogen[38] or setting the blocks on a 100% isopropanol/dry ice slurry[39] until the block is opaque. Wrap the cryomolds in aluminum foil and store the blocks at -80 ˚C for long-term storage. Move blocks to -20 ˚C before sectioning.

NOTE: Care must be taken when sectioning and performing histology protocols on decalcified brains as the skull and meningeal layers may be lost if the sections are handled roughly.

2. Preparation of the meninges and CNS tissues for flow cytometry staining

2.1. Extracting the skull cap and brain

2.1.1. Using sharp scissors, remove the head by decapitation (**Figure 2A;** 1). Using scissors, make a midline incision in the skin (**Figure 2A;** 2) and flip the skin over the eyes to free the skull.

2.1.2. Place the scissors within the foramen magna and begin cutting the skull laterally along the cortices towards the olfactory bulb, keeping the incisions above the external auditory meatus and mandible (**Figure 2A;** 3). Perform the same cuts on the opposite side, with cuts meeting at the olfactory bulb to free the skull cap from the brain (**Figure 2A;** 3).

2.1.3. Using forceps, peel back the skull cap and place the skull cap into a 15 mL conical tube containing 5 mL of cold RPMI medium supplemented with 25 mM HEPES. Keep the tube on ice.

2.1.4. Using curved forceps, place the forceps below the base of the brain, and lift to free the brain from the skull cap. Place the brain into a 15 mL conical tube containing 5 mL of cold RPMI supplemented with 25 mM HEPES. Keep the tube on ice until processing.

2.2. Extracting the vertebral column and spinal cord tissue

2.2.1. Using forceps and sharp scissors, separate the rib cage from the spinal column by cutting parallel to the spine (**Figure 2A;** 4 and 5). Make a small cut at the lower lumbar region to isolate the vertebral column (**Figure 2A;** 6). Trim and remove any remaining muscle along the spine to expose the vertebrae (**Figure 2A;** 7).

2.2.2. Place the extra fine surgical scissors within the vertebral column and cut along the lateral edge of the column (**Figure 2C**). Cut the opposite lateral edge completely to divide the vertebral column into an anterior and posterior portion.

NOTE: The spinal cord will remain attached to the vertebral column.

2.2.3. Using forceps, slowly and carefully peel away the spinal cord from the vertebral column and place the tissue in a 15 mL conical tube containing 5 mL of cold RPMI with 25 mM HEPES. Transfer the anterior and posterior portions of the spinal column to a 15 mL conical tube containing 5 mL of cold RPMI with 25 mM HEPES.

2.3. Removing the meninges to prepare single-cell suspensions

2.3.1. Using forceps, remove the skull cap from the RPMI media. Using sharp forceps (#7 forceps; **Table of Materials**), score around the outer edge of the skull cap (**Figure 2B**) and peel the meninges away from the edge of the skull cap, scraping to remove the dural and arachnoid meninges. Place the meninges on a Petri dish.

NOTE: Removal of the meninges from both the brain and spinal cord requires practice. If the user experiences difficulty extracting the meninges, use a dissecting microscope to aid in the removal.

2.3.2. Remove the vertebral column from the tube. Using sharp forceps, score around the edges of the vertebral column to free the meninges and peel away the meninges from the edge of the vertebra using curved forceps. Place the meninges on a Petri dish.

2.3.3. Place a nylon mesh strainer in a 50 mL conical tube. Move the meninges into the strainer and add 3 mL of RPMI supplemented with 25 mM HEPES. Using the plunger from a 5 mL syringe, grind the tissue and media through the strainer.

2.3.4. Using a 5 mL serological pipette, wash the strainer with an additional 2 to 3 mL of

RPMI/HEPES media until all visible tissue has passed through the strainer.

NOTE: To obtain adequate cell numbers for flow cytometric analysis, meninges from multiple animals may need to be pooled together. In this experiment (**Figure 3** and **Figure 4**), the brain and spinal cord meninges from 4–5 mice were pooled together. If the samples are pooled, additional media will be required to grind the tissue through the nylon mesh strainer to prevent overheating of the tissue.

2.3.5. Using a 10 mL serological pipette, transfer the cells and media to a fresh 15 mL conical tube. Using a 10 mL serological pipette, wash the 50 mL conical tube with 5 mL of media to collect any remaining cells. Centrifuge at 450 x *g* for 5 min at 4 °C to pellet the cells.

2.3.6. Using a Pasteur pipette with vacuum, aspirate the supernatant being careful to avoid the cell pellet and resuspend the cells in an appropriate volume and buffer.

2.3.7. To count the single-cell suspension, dilute a small volume of the cells (i.e., 5–10 µL) using trypan blue exclusion dye (1:10 dilution) and RPMI. Add 10 µL of the dilution to the hemocytometer.

2.3.8. Count the cells as previously described[40,41], averaging at least two 16 square grids for accuracy.

NOTE: In **Figure 3**, for example, pooled, pelleted cells from the meninges were resuspended in 250 µL of fluorescence-activated cell sorting (FACS) buffer (1x PBS with 1% FBS) for downstream surface staining. The cells were diluted 1:10 for counting (5 µL cells, 5 µL trypan blue, 40 µL RPMI). This dilution yielded between 50–100 cells per 16 square grids, ensuring more accurate cell counting because the cells are neither too dense and overlapping nor too sparse. Nucleated cell counts from pooled brain and spinal cord meninges per mouse were as follows: For meninges from sham-treatment mice = 100,000–150,000 cells and for meninges from Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) mice = 300,000–350,000 cells. Cell counts will vary depending on the precision of collection, processing, and if meningeal inflammation is present.

2.3.9. Proceed to the desired single-cell technique such as a FACS surface (**Figure 3**)[14,36,42-44], intracellular staining protocols[33,45], in vitro stimulation, cell culture assays[33,46,47], ELISPOT assay[28,34,35], and bulk or single-cell transcriptomics[37,48].

NOTE: Keep all tubes on ice in between processing steps.

2.4. Preparing single-cell suspensions of brain and spinal cord tissue

2.4.1. Transfer the brain or spinal cord tissue with media to the top of the 100 mm Petri dish by pouring the tissue and media from the tube. Using forceps, move the tissue to the bottom of the Petri dish. Finely mince the brain or spinal cord with a sterile razor blade. Using the razor blade,

move the minced tissue to the bottom of the plate by scraping to gather the tissue.

NOTE: For the enzymatic digestion protocol below, up to two spinal cords may be pooled together for processing. Brains should be processed individually.

2.4.2. Using a 5 mL serological pipette, add 3 mL of RPMI supplemented with 10% fetal calf serum (FCS) to the Petri dish. Using a 10 mL serological pipette, pipette up and down to resuspend the tissue in the media and transfer to a 15 mL conical tube.

NOTE: If the downstream application is single-cell or bulk RNA sequencing analysis or cell culture, FCS lots should be tested to ensure cells are not activated prior to analysis. Alternatively, cells can be processed using 1x PBS with 0.04% BSA instead of RPMI with 10% FCS.

2.4.3. Using a 10 mL serological pipette, wash the Petri dish with an additional 2 mL of media to collect any residual tissue and transfer to a conical tube for a 5 mL total volume. Keep the tubes on ice between processing steps.

2.4.4. Using a pipette, resuspend the collagenase I powder in Hank's Balanced Salt Solution (HBSS) media to obtain the desired concentration (i.e., 100 mg/mL). Add collagenase type I to the conical tube containing the minced tissue sample to obtain the desired final concentration (i.e., 50 µL for 1 mg/mL).

NOTE: Higher concentrations of collagenase will increase cell yields but can cleave cell surface markers. Therefore, collagenase I lots should be titrated to determine the optimal concentration needed to obtain the highest number of viable cells with all required cell surface markers intact. For example, collagenase I was tested at final concentrations of 0.5 mg/mL, 1 mg/mL, and 2 mg/mL on single brain or spinal cord samples. Cell viability was determined using the trypan blue exclusion method and cell surface markers CD45, CD19, and CD4 were assessed by flow cytometry. A 1 mg/mL concentration of collagenase I yielded the highest live cell count while retaining all cell surface markers of interest. Thus, this concentration was used for further experiments examining these cell types.

2.4.5. Gently resuspend DNase I powder using 0.15 M sodium chloride to the desired stock concentration. Add the resuspended DNase I to the conical tube containing the minced tissue sample to obtain a final concentration of 20 U/mL.

NOTE: DNase I lots vary by units of activity per mL. The concentration to be added to the tissue sample will change based on the stock vial's units of activity per milliliter. The final desired concentration per sample is 20 U/mL.

2.4.6. Place the tubes in a tube rack in a 37 °C water bath and incubate for 40 min. Invert the tubes every 15 min to thoroughly mix the tissue with the enzymes. After incubation, add 500 µL of 0.1 M EDTA (pH = 7.2) to each tube for a final concentration of 0.01 M EDTA and incubate for an additional 5 min to inactivate the collagenase.

2.4.7. Using a 10 mL serological pipette, add 9 mL of RPMI supplemented with 10% FCS to each tube to bring the volume of each tube to ~14.5 mL. Centrifuge at 450 x *g* for 5 min at 4 ˚C. Using a Pasteur pipette with vacuum, aspirate the supernatant being careful not to touch the cell pellet.

2.4.8. Using a 5 mL serological pipette, add 3 mL of 100% stock isotonic density gradient solution to the tube containing the cell pellet. Using a 10 mL serological pipette, add additional RPMI 10% FCS media to bring the final volume to 10 mL and resuspend the cell pellet to create a 30% stock isotonic density gradient solution layer.

NOTE: Prepare the 100% stock isotonic density gradient medium in advance, aliquot, and store at 4 °C for up to 3 months. To prepare the 100% stock isotonic density gradient solution, dilute the density gradient media (**Table of Materials**) with density gradient media dilution buffer. Prepare the density gradient media dilution buffer (80.0 g/L NaCl, 3.0 g/L KCl; 0.73 g/L Na_2HP0_4, 0.20 g/L KH_2HP0_4; 20.0 g/L glucose) and filter sterilize using a vacuum filter system. Make the 100% stock isotonic density gradient solution by mixing 1 part of density gradient dilution buffer and 9 parts density gradient media. Mix well.

2.4.9. Invert and mix each tube well prior to adding the 70% stock isotonic density gradient solution underlay. Insert a 1 mL serological pipette containing 1 mL of 70% stock isotonic density gradient solution into the bottom of the tube. Slowly underlay 1 mL of the solution, being careful not to make bubbles. Slowly remove the serological pipet from the tube, being careful not to disturb the gradient.

NOTE: For the 70% underlay, the 100% stock isotonic density gradient solution should be diluted to 70% using RPMI media (i.e., 7 mL of 100% stock isotonic density gradient solution and 3 mL of RPMI media mixed well). Additionally, creating a clean, undisturbed 70% underlay is essential for the removal of myelin debris and for obtaining pure single-cell suspensions at the gradient interface following centrifugation.

2.4.10. Centrifuge at 800 x *g* for 30 min at 4 °C with no brake. Aspirate the supernatant, including the myelin debris layer until 2–3 mL remains in the tube, being careful not to disturb the cell layer. Harvest the cell layer between the 30/70% density gradient using a 1 mL pipette and transfer to a new 15 mL conical tube.

2.4.11. Using a 10 mL serological pipette, add RPMI 10% FCS media to bring the final volume to 15 mL. Centrifuge 450 x *g* for 5 min at 4 °C.

NOTE: During this step, cell layers from two tubes can be pooled if needed. Do not pool more than two tubes or the cells will not pellet due to a high-density gradient media concentration.

2.4.12. Aspirate the supernatant carefully to not disturb the cell pellet. Resuspend the cells in an appropriate volume/buffer to count the cells using a hemocytometer (e.g., resuspend a single spinal cord in 250 µL of FACS buffer for downstream surface staining) (**Figure 3**).

2.4.13. Using a 1 mL pipette, transfer the cell suspensions to the top of a filter top tube (**Table of Materials**) and allow the cells to filter to the bottom of the tube to remove any remaining myelin debris.

2.4.14. Using trypan blue exclusion dye, dilute, and count the cells on a hemocytometer by averaging at least two 16 square grids for accuracy[40,41].

2.4.15. Proceed with the desired single-cell analysis technique.

NOTE: Using various forms of collagenase (i.e., D, type I, type II, type IV), immune cells, microglia (**Figure 3**), astrocytes, pericytes, endothelial cells[49], and neurons[50] can all be efficiently isolated. Nucleated cell counts obtained for the results were as follows using the titrated collagenase I enzyme: Whole sham-treated brain = 500,000–600,000 cells; Whole TMEV-IDD brain = 800,000–1,000,000 cells; Whole sham-treated spinal cord = 150,000–200,000 cells; Whole TMEV-IDD spinal cord = 300,000–400,000. Cell counts will vary depending on the precision of collection, processing, and whether CNS inflammation is present.

REPRESENTATIVE RESULTS:

This representative experiment was aimed at quantifying B and T cells and describing B and T cell localization in the meningeal and parenchymal CNS compartments in homeostatic conditions as well as in a murine progressive MS model (i.e., TMEV-IDD). TMEV-IDD was induced in 5-week-old female SJL mice by intracranial infection with 5 x 10^6 plaque forming units (PFU) of TMEV BeAn as previously described[29].

The present study assessed B and T cells in the meninges, brain, and spinal cord during chronic TMEV-IDD at day 120 postinfection. Age-matched sham-treated mice were used as controls. The study was comprised of two experiments. The first focused on obtaining single-cell suspensions for flow cytometric assessment, a well-established technique to analyze and quantify cell composition by evaluating cell surface and/or intracellular antigens (n = 4 sham-treated; n = 5 TMEV-IDD). The second experiment focused on describing B and T cell localization in the CNS compartment by utilizing immunohistochemistry on decalcified brain and spinal cord tissues (n = 3 sham-treated; n = 8 TMEV-IDD).

Following the isolation of single-cell suspensions from the brain, spinal cord, and pooled meninges (brain and spinal cord) from TMEV-IDD and sham-treated mice, a surface staining protocol was applied to all samples. Briefly, single-cell suspensions were incubated with a fixable viability exclusion stain (780) for 15 min, washed, blocked with Fc block in the presence of mouse serum for 15 min, and stained with conjugated antibodies for cell surface markers, including CD45 (30-F11; PerCP-Cy5.5), CD19 (1D3; PE-CF594), and CD4 (GK1.5; PE) for 30 min as previously described[28,29]. Cells were then washed and analyzed using a flow cytometer[28,29]. Viability gating was conducted as previously described[28]. CD45 expression was assessed to distinguish $CD45^{hi}$ peripherally derived infiltrating immune cells (P1) from $CD45^{lo}$ microglia (P2) and $CD45^{-}$ neurons, astrocytes, and oligodendrocytes in the brain and spinal cord (P3; **Figure 3A**). In sham-treated

mice, few CD45^{hi} cells were present in the spinal cord and brain tissue. In the meninges, the same gating cut-off for CD45^{hi} expression used for the brain and spinal cord data was applied to identify CD45^{hi} immune cells (P1; **Figure 3C**) and exclude nonimmune cells present in the meninges (i.e., fibroblasts, endothelial cells). In sham-treated mice, few CD45^{hi} cells (<0.1%) were present in the meninges. Among CD45^{hi} immune cells in TMEV-IDD CNS tissues, B cells and CD4 T cells were identified by surface expression of CD19 and CD4, respectively (**Figure 3B–D**). In all TMEV-IDD CNS tissues, increased percentages of CD45^{hi} immune cells were observed compared to sham-treated mice (**Figure 4A**). During chronic TMEV-IDD, the percentage of B cells among CD45^{hi} immune cells in the brain and spinal cord was higher compared to the meningeal compartment (**Figure 4B**).

To identify the localization of B cells and T cells within the CNS compartment during chronic TMEV-IDD, decalcified brains and spinal cords were evaluated using immunohistochemistry using the staining protocol previously described[29]. The meninges and vasculature were demarcated using laminin, a basement membrane component[29,51] and ER-TR7, a fibroblast reticular cell marker[52,53]. During conventional extraction of brain from the skull cap, the pia layer was intact, but the remaining meningeal layers were excluded (**Figure 5A**). In the spinal cord, hydraulic extrusion resulted in the absence of all meningeal layers (data not shown). In both decalcified brains and spinal cords, all meningeal layers were intact (**Figure 5B**). To examine B and T cell localization in chronic TMEV-IDD, IgG expression was used to determine the localization of isotype-switched B cells[29], and CD3 was used to visualize all T cells[29]. Costaining IgG with ER-TR7 revealed that isotype-switched IgG$^+$ B cells were present in the CNS parenchyma and the meninges (**Figure 6A**). Cellular aggregates within ER-TR7$^+$ meninges contained multiple IgG$^+$ B cells and CD3$^+$ T cells (**Figure 6B**).

The representative results show high percentages of both B cells and T cells in the parenchymal and meningeal compartments in chronic TMEV-IDD mice in contrast to age-matched sham-treated mice. IHC analysis further demonstrated that in TMEV-IDD tissue, B cells and T cells were dispersed in the parenchyma, but were closely associated in the meninges, forming inflammatory aggregates. In progressive MS patients, meningeal inflammatory aggregates are associated with adjacent tissue injury and worse disease outcomes. In chronic TMEV-IDD, persistent B cell and T cell presence in the CNS compartment and aggregation in the meninges may be associated with tissue injury and disease progression. Further studies are needed to understand how meningeal versus parenchymal inflammation affect demyelination, neurodegeneration, and clinical disability.

FIGURE LEGENDS:

FIGURE 1: Isolating brains and spinal cords for decalcification. Blue dotted lines indicate cuts made to isolate the brain with intact skull (cuts 1-4) and vertebral column (cuts 5-8).

FIGURE 2: Isolating brains, spinal cords, and meninges for single-cell techniques. (**A**) Blue dotted lines indicate cuts made to isolate the skull cap with intact meninges and brain (cuts 1-3) and vertebral column (cuts 5-7). Lateral cut 3 is made on both sides of the skull. (**B**) Blue line indicates incision made to score and remove the meninges in the skull cap and the cuts made to the

vertebral column (both lateral sides) to isolate the spinal cord and meninges.

FIGURE 3: **Identification of CD45^{hi} infiltrating immune cells in CNS tissues.** (**A**) Gating strategy for the identification of CD45^{hi} infiltrating immune cells (P1), CD45^{lo} microglia (P2), and CD45^{-} oligodendrocytes, astrocytes, and neurons among total live cells in spinal cords from sham-treated (red) or TMEV-IDD (blue) mice. Minimal CD45^{hi} cells were detected in sham-treated brains and spinal cords. (**B**) Gating strategy for identifying CD19^{+} B cells and CD4^{+} T cells among CD45^{hi} infiltrating immune cells (P1) in TMEV-IDD mice. Gating strategies were similar for brain and spinal cord tissue. (**C**) Gating strategy for identifying CD45^{hi} cells (P1) in the meninges of sham-treated (red) and chronic TMEV-IDD mice (blue). (**D**) Gating strategy for identifying CD19^{+} B cells and CD4^{+} T cells among CD45^{hi} infiltrating immune cells (P1) in the meninges of chronic TMEV-IDD mice.

FIGURE 4: CD45^{hi} immune cells and CD19^{+} B cells increased in CNS tissues in chronic TMEV-IDD. Bar graphs summaries of flow cytometry data obtained from sham-treated or TMEV-IDD meninges, brain, and spinal cords show percentages of CD45^{hi} infiltrating immune cells (**A**) or percentages of CD19^{+} B cells (**B**). For TMEV-IDD tissues, flow cytometry data were obtained from five mice, with spinal cords and brains processed individually, while meninges were pooled for analysis. For sham-treated mice, flow cytometry data were obtained from four mice, with spinal cords and brains processed individually, while meninges were pooled for analysis. Data for spinal cords and brains are shown as mean ± SEM.

FIGURE 5: Intact meningeal layers in decalcified brains and spinal cords. (**A**) Brains extracted from the skull cap or (**B**) decalcified brains with intact skulls and vertebral columns from chronic-TMEV-IDD mice were assessed for DAPI (blue), meningeal markers laminin (green), and ER-TR7 (red). Scale bar = 50 μm.

FIGURE 6: Immune cell aggregation was evident in the meninges during chronic TMEV-IDD. (**A**) Decalcified vertebral columns from chronic-TMEV-IDD mice were examined for the presence of IgG (green) to identify isotype-switched immune cells in the parenchyma and in the ER-TR7+ meninges (red). (**B**) CD3^{+} T cells (green) and IgG^{+} isotype-switched B cells (red) were costained with ER-TR7^{+} meninges to assess localization within the spinal cord tissue. White arrows highlight B and T cells within the meninges. Scale bar = 50 μm. White boxes delineate areas selected for cropped images (right; scale bar= 10 μm).

DISCUSSION:

Methods for evaluating the cellular composition in the CNS compartment during homeostasis and disease are essential for understanding the physiological and pathological states of the CNS. However, despite serving as an important barrier in the CNS and housing a diverse array of immune cells, the meninges are often omitted from analysis because many conventional tissue extraction methods for the brain and spinal cord do not allow for the collection of these membranes. This omission is a critical limitation in the advancement of our understanding of the cellular composition and function of the meninges and its role in steady state and inflammatory conditions. Recent studies revealed that both resident and peripherally derived immune cells

residing in the meningeal compartment play an essential role in maintaining homeostasis in the CNS, as well as in driving CNS disease pathogenesis. These studies emphasize the need to analyze not only the parenchymal compartment but also the surrounding meningeal layers. The protocols described here allow for the rapid isolation of brain and spinal cords while preserving the meninges, ultimately enabling a comprehensive downstream analysis of the CNS compartment utilizing both histologic and single-cell studies. The representative results focus on assessing immune cells in the CNS compartment; however, the protocols may be adapted to analyze microglia, astrocytes, neurons, pericytes, endothelial cells, or other CNS resident cells.

A critical step in the described methods is the careful extraction of the tissue, essential for both isolating pure single-cell suspensions from brain, spinal cord, and meninges and for obtaining CNS tissues with either intact skulls or vertebral columns, allowing high-quality tissue morphology. Practicing the technique for obtaining single-cell suspensions is the key to a successful tissue extraction because it will not only enhance the purity of the sample but also improve cell yields for downstream applications. Similarly, practice in the isolation of CNS tissues with intact skulls or vertebral columns to adequately remove excess tissue surrounding the bone is an essential step that ensures better fixation, decalcification, and cryopreservation of the tissue, all crucial components for obtaining tissue sections with high-quality morphology and intact antigen targets.

One limitation of our protocol for isolating single-cell suspensions from the meninges is that it focuses on obtaining dural and arachnoid meninges[32,54] while omitting the isolation of the pia, which remains attached to the brain or spinal cord via astrocyte processes. Although the cells residing in the pia mater are an essential component of the meningeal compartment, it was decided to exclude the pia mater during the single-cell suspension preparation due to the extensive amount of time required to adequately isolate it[55]. Similarly, the protocol only focuses on obtaining meninges in the superior portion of the cranium and excludes the isolation of the invaginating meninges in the brain and choroid plexus. Collection of the invaginating meninges and choroid plexus can be conducted according to previously published protocols[55], although it is a time-consuming procedure. In both cases the choice to omit part of the meningeal layers was due to the amount of time it requires to isolate them. The timing of the protocol used to prepare single-cell suspensions required for downstream applications such as flow cytometry, cell culture assays, and RNA sequencing (RNAseq) is critical to improve cell viability and data quality. Therefore, depending on the specific research question, a balance should be struck between sample isolation time and the thoroughness in extracting the meninges. In the current protocol, only single-cell suspension preparations from the dura and arachnoid meninges are obtained, which limits the ability to extrapolate results to the entirety of the CNS meninges. However, if these methods are used in combination with IHC or ISH analysis of whole tissue sections with the three layers of meninges, a more thorough understanding of the cellular and molecular dynamics in the parenchymal and meningeal compartments can be gained.

Another limitation of the protocol is the low cell numbers obtained from the dural and arachnoid meninges, especially during homeostatic conditions. However, if cell numbers in the spinal cord, brain, or the meninges are deemed to be too low for analysis of a particular target cell population,

the minimal cell number required for that analysis can be determined by estimating the total number of events required for a given precision (e.g., 5% coefficient of variation) as described in previous literature specializing in rare event analysis[56]. These calculations can then be used to determine if samples from multiple animals must be pooled in order to acquire sufficient cell numbers to analyze the cell population of interest.

The methods described provide an essential advancement in the investigation of the cellular dynamics within the CNS compartment by extending analyses to include the commonly neglected meningeal compartment. These protocols allow for the individual extraction of the brain, spinal cord, and the dural and arachnoid meninges to generate single-cell suspensions. Additionally, the decalcification protocol allows histological analyses of tissue sections comprising brain or spinal cord tissue inclusive of the three meningeal layers. Utilizing EDTA provides a slow but gentle decalcification process, which is most appropriate for specimens where high-quality tissue morphology is required (e.g., IHC and ISH). The protocol uses a pH of 7.2–7.4 to slow the rate of decalcification and ensure the maintenance of intact tissue morphology, a clear advantage over strong and weak acids (e.g., hydrochloric acid and trichloroacetic acid), which have a shorter decalcification time, but impact the quality of the tissue morphology.

Future methods seeking to isolate single-cell suspensions from the meninges should focus on developing protocols to shorten the time required to thoroughly isolate the pial layer from CNS tissues and the invaginating meninges in the brain. Obtaining the complete complex of the three meningeal layers will not only increase the cell numbers for any analysis but will also provide a more comprehensive assessment of the resident and infiltrating immune cells occupying the meninges, which enclose the CNS. Concurrently, future protocols focused on preparing decalcified brains and spinal cords should seek to identify novel agents aimed at expediting tissue decalcification while preserving the quality of tissue morphology. However, altogether, downstream applications analyzing CNS single-cell suspensions inclusive of dual and arachnoid meninges, performed in combination with comprehensive tissue section analysis, can provide a detailed understanding of cell phenotype, function, and localization within the CNS compartment.

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The authors have nothing to disclose.

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Figure 1

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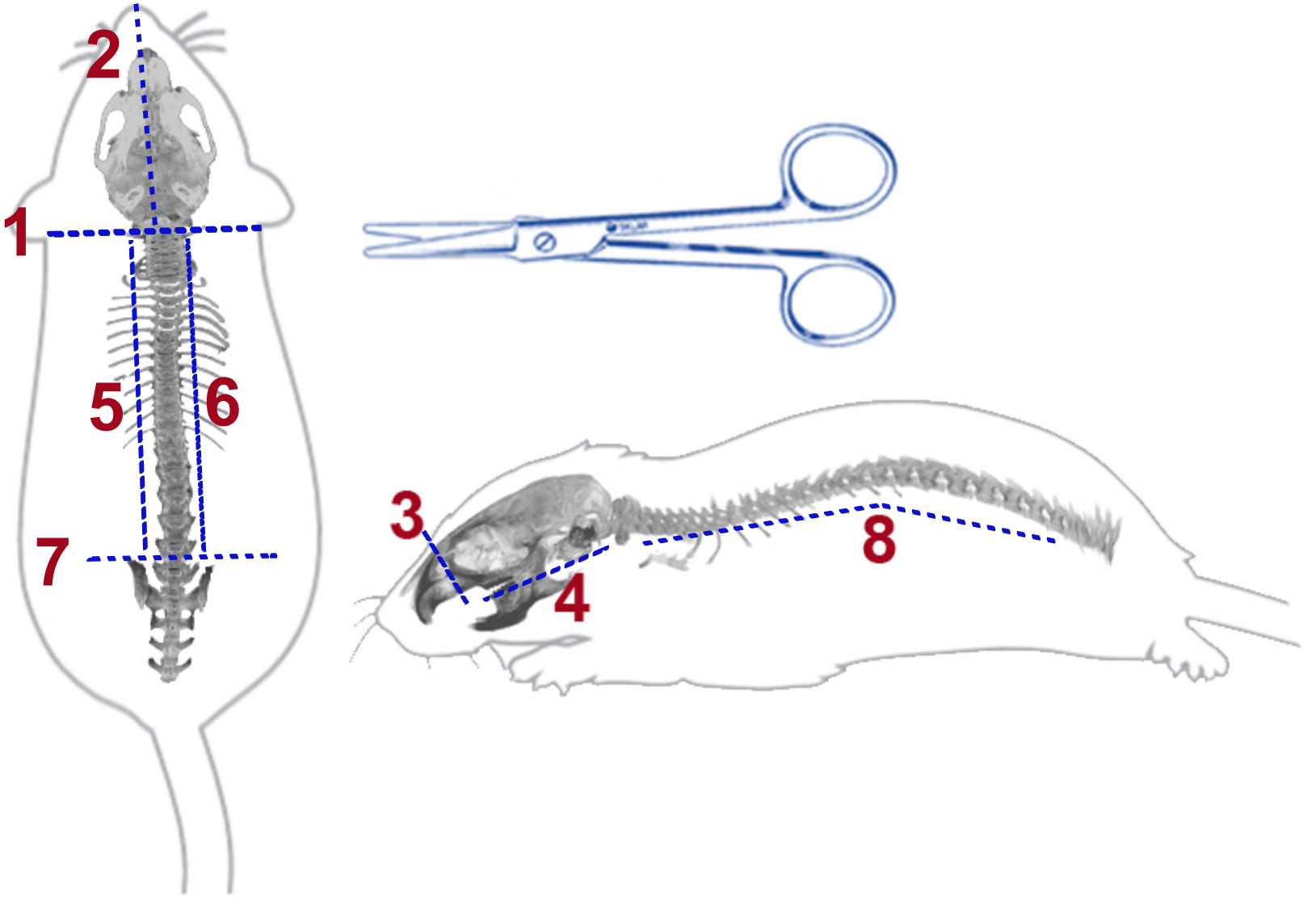

Figure 2

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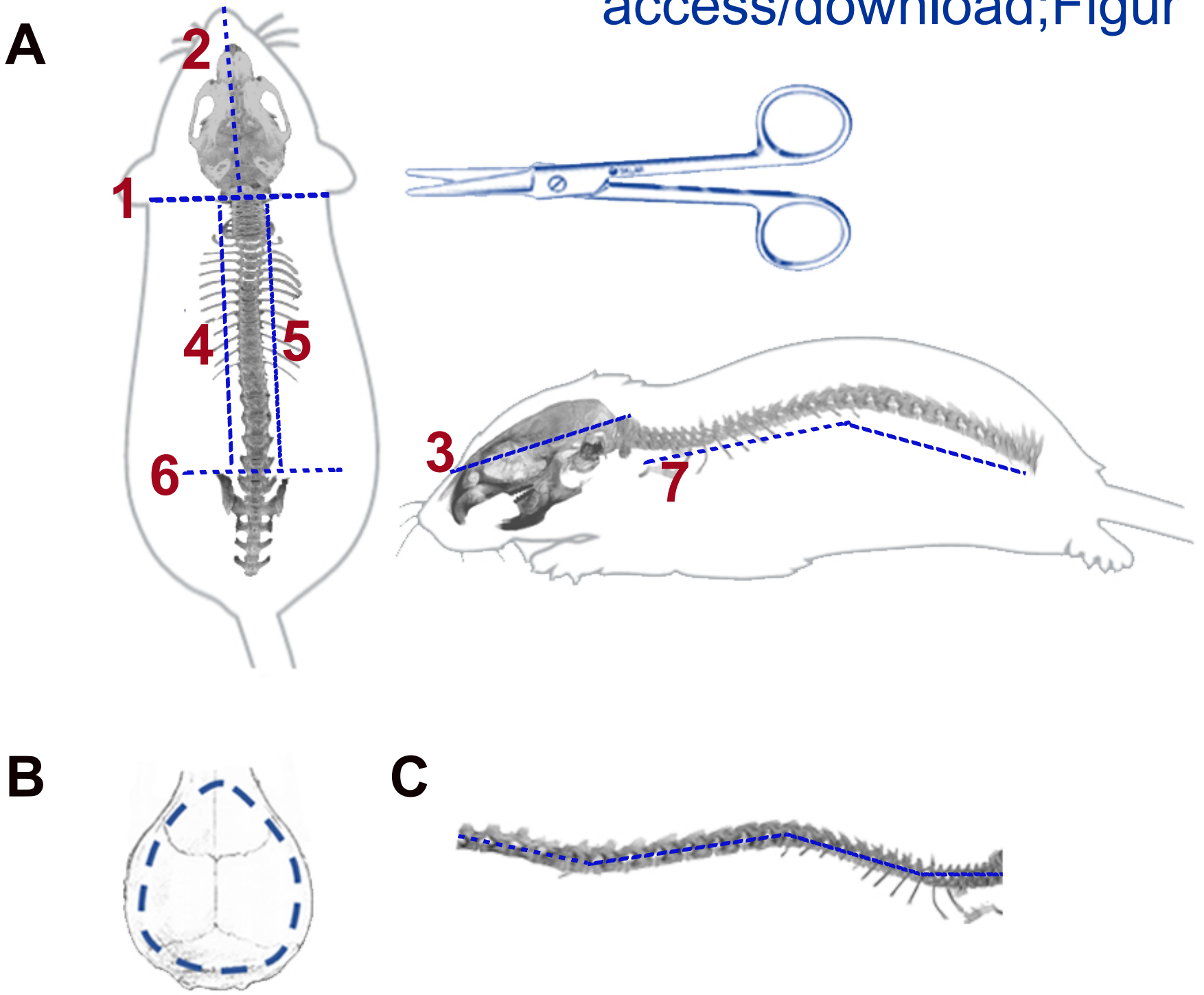

Figure 3

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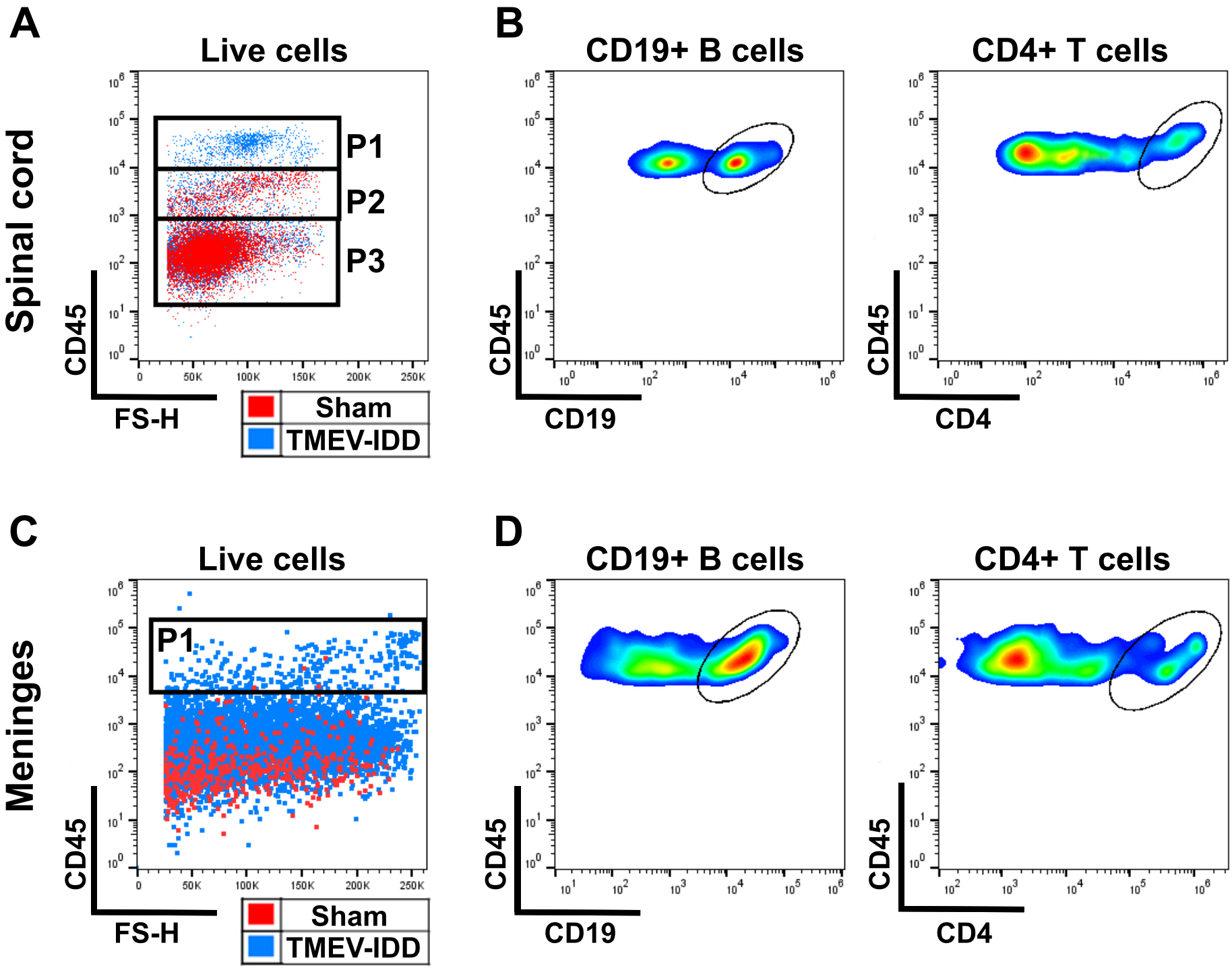

Figure 4

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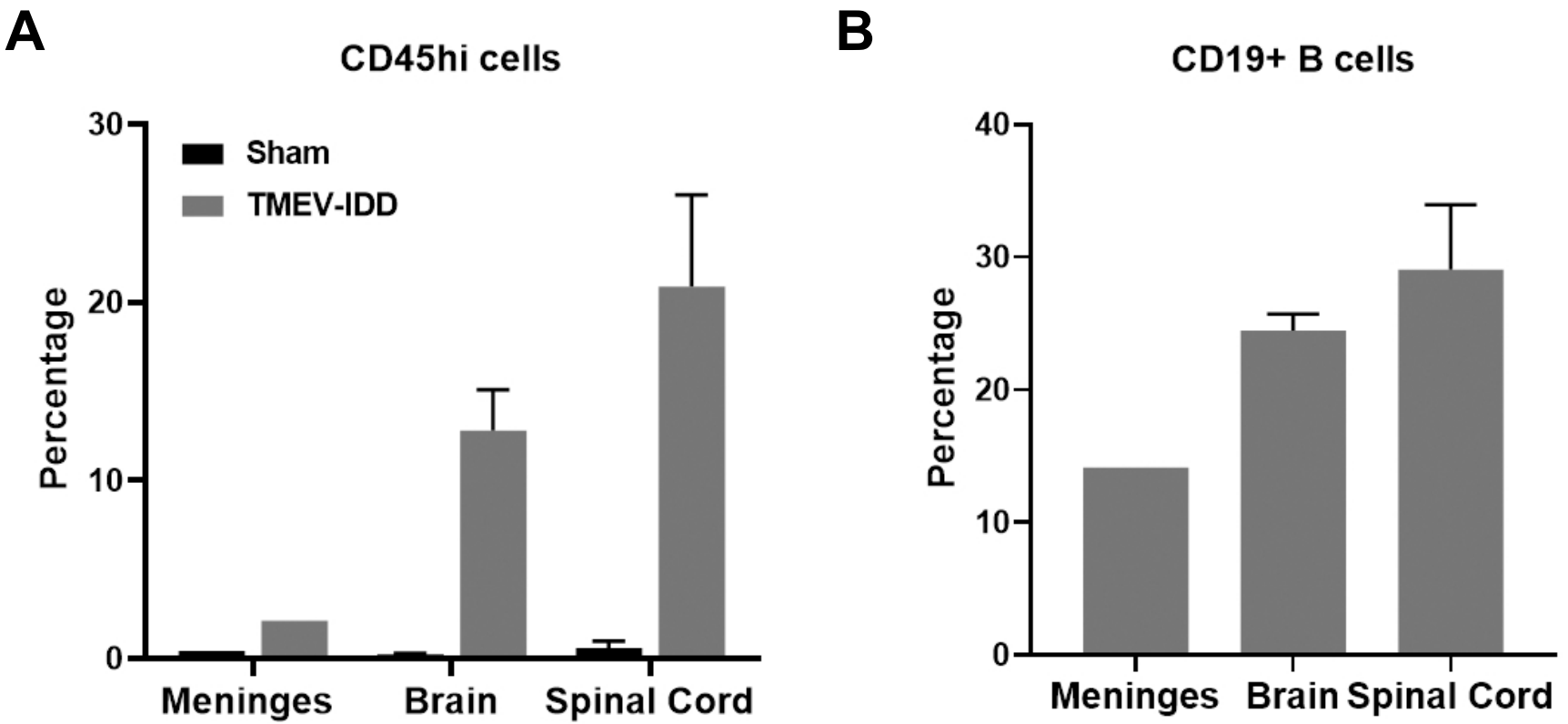

Figure 5

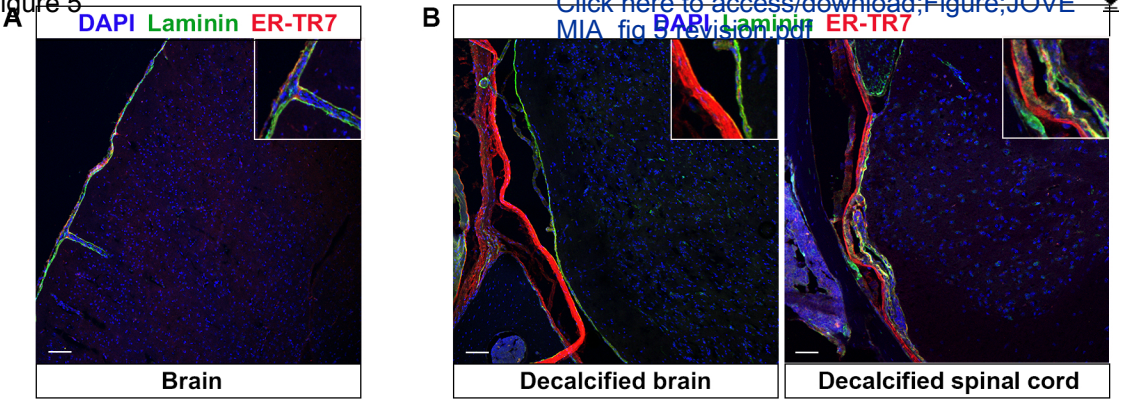

Figure 6

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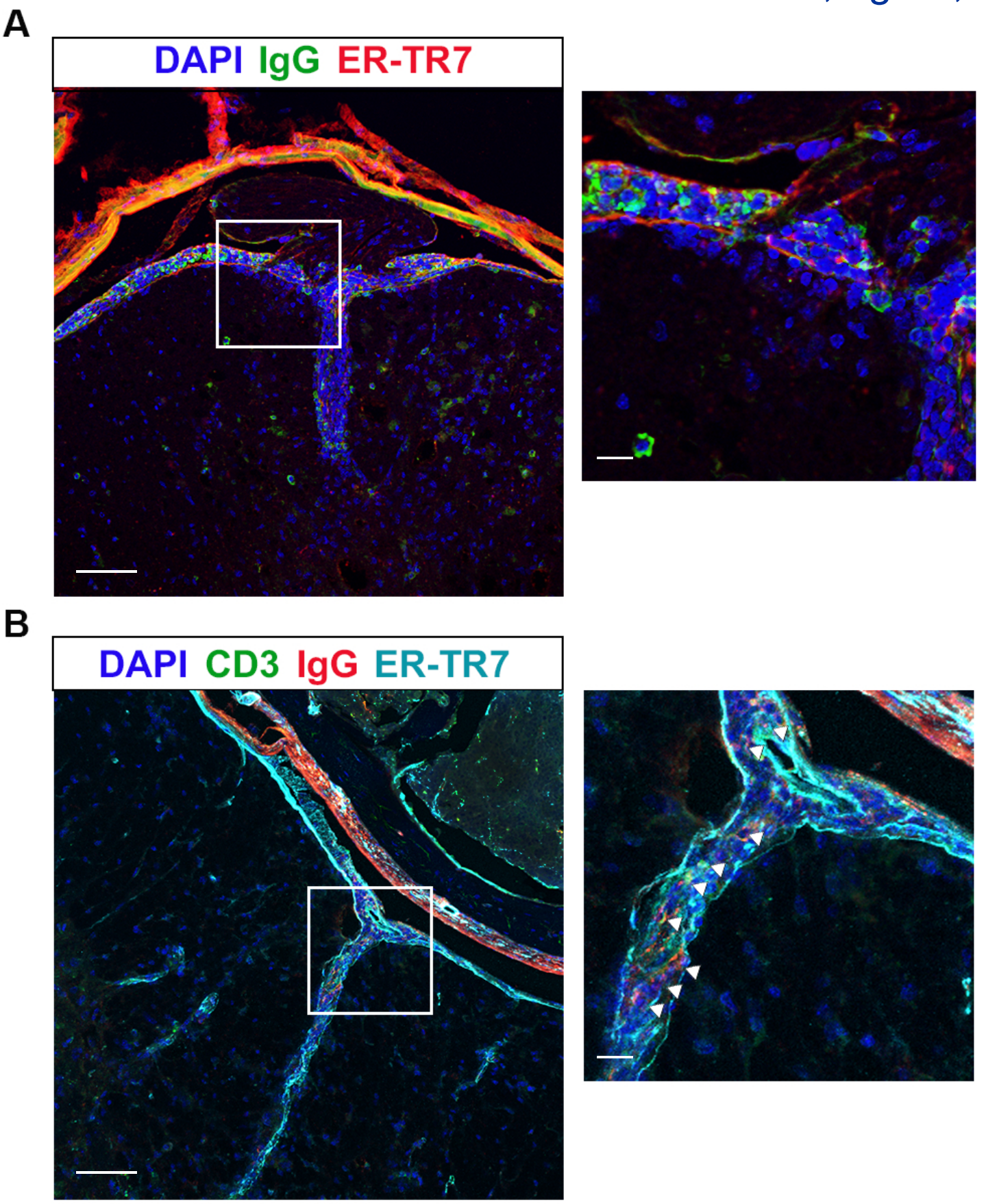

Name of Material/ Equipment	Company
Aluminum foil	any
Bovine Serum Albumin	ThermoFisher Scientific
Centrifuge	Beckman Coulter
Collagenase I	Worthington
Conical tube, 15 mL	VWR
Conical tube, 50 mL	VWR
Cover glass	Hauser Scientific
Cryomold	VWR
Curved forceps	Fine Science Tools
Disposable polystyrene tube, 14 mL	Fisher Scientific
Disposable Scalpel	Fisher Scientific
DNAse I	Worthington
Dry ice	Airgas
Durmont #7 Forceps	Fine Science Tools
EDTA disodium salt dihydrate	Amresco
Ethanol, 100%	any
Fetal Bovine Serum (FBS)	Hyclone
Filter top tube, 5 mL	VWR
Fixable viability stain 780	Becton Dickinson
Flow cytometer	Beckman Coulter
Glucose	Fisher Chemical
Goat anti-mouse IgG (488 conjugate)	Jackson immunoresearch
Goat anti-mouse IgG (594 conjugate)	Jackson immunoresearch
Goat anti-rabbit 488	Jackson immunoresearch
Goat anti-rat 594	Jackson immunoresearch
Goat anti-rat 650	Jackson immunoresearch
Hank's Balnced Salt Solution (HBSS)	Corning
Hemacytometer	Andwin Scientific
Hemostat	Fine Science Tools
HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid)	ThermoFisher Scientific
KCl	Fisher chemical

KH_2PO_4 (anhydrous)	Sigma Aldrich
Liquid Nitrogen	Airgas
Mouse FC block (CD16/32)	Becton Dickinson
Na_2HP0_4 (anhydrous)	Fisher Chemical
NaCl	Fisher chemical
Needle, 25 gauge	Becton Dickinson
Normal mouse serum	ThermoFisher Scientific
Nylon mesh strainer	VWR
OCT	Sakura
Paraformaldehyde, 20%	Electron Microscopy Sciences
Pasteur pipette, 9 inch, unplugged	Fisher Scientific
PBS (1x)	Corning
PE Rat Anti-Mouse CD4	Becton Dickinson
PE-CF594 Rat Anti-Mouse CD19	Becton Dickinson
Percoll density gradient media	GE healthcare
PerCP-Cy5.5 Rat Anti-Mouse CD45	Becton Dickinson
Petri dish, 100 mm	VWR
pH meter	Fisher Scientific
Pipet-Aid	Drummond Scientific Corporation
Pipette 200 µl	Gilson
Pipette tips, 1 mL	USA Scientific
Pipette tips, 200 µl	USA Scientific
Pipette, 1 mL	Gilson
Prolong Diamond mountant with DAPI	ThermoFisher Scientific
Purified Rat Anti-Mouse CD16/CD32	Becton Dickinson
Rabbit anti-mouse CD3 (SP7 clone)	Abcam
Rabbit anti-mouse laminin	Abcam
Rat anti-mouse ERT-R7	Abcam
RPMI 1640	Corning
Serological pipet, 1 mL	VWR
Serological pipet, 10 mL	VWR
Serological pipet, 5 mL	VWR

Sodium hydroxide	Fisher Scientific
Sucrose	Fisher chemical
Surgical scissors	Fine Science Tools
Surgical scissors, extra fine	Roboz
Syringe, 10 mL	Becton Dickinson
Syringe, 5 mL	Becton Dickinson
Trypan blue	Gibco
Vacuum filter system	Millipore
Vacuum flask	Thomas Scientific
Vacuum in-line filter	Pall Corporation
Vacuum line	Cole Palmer
Water bath	ThermoFisher Scientific

Catalog Number	Comments/Description
N/A	
37002D	
Allegra X-12R centrifuge	
LS004196	
525-1069	
89039-658	
5000	
18000-128	
11003-14	
14-959-1B	
NC0595256	
LS002139	
N/A	
11271-30	
0105-500g	
N/A	
SH30910.03	
352235	
565388	
Gallios	
D16-500	
115-546-146	
115-586-146	
111-545-144	
112-585-167	
112-605-167	
21-020-CV	
02-671-51B	
13004-14	
15630080	
BP366-500	

P5655-100G	
N/A	
553141	
S374-500	
S671-500	
305122	
31881	
352350	
4583	
15713-S	Diluted to 4% using 1 x PBS
13-678-20C	
21-040-CV	
553730	
562329	
17-0891-01	
550994	
353003	
13-636-AB150	
4-000-101	
FA10005M	
1111-2831	
1111-1816	
FA10006M	
P36962	
553141	
ab16669	
ab11575	
ab51824	
10-040-CV	
357521	
357551	
357543	

S318-100
S5-500
14001-16
RS-5882
302995
309646
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5340-2L
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EW-06414-20
Versa bath

Dear Sir/Madam

Thank you for the opportunity to submit a revised version of the manuscript mentioned above. We have revised the manuscript per the editor and reviewers' suggestions.

We have addressed each point separately in the enclosed point-by-point reply. Revisions to the body of the manuscript were also emphasized with track changes.

The comments were constructive, and we are appreciative of this constructive feedback on our manuscript. After addressing the issues raised, we feel the quality of the manuscript is much improved. We hope that the revised manuscript will now be acceptable for publication on JoVE.

Sincerely,

Krista DiSano, MS, PhD

encl. point-to-point reply

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response:
The text has been reviewed for spelling/grammar issues and all modifications have been made.

2. Please provide at least 6 keywords or phrases.

Response:
Additional keywords have been added.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Percoll, ELISPOT, etc.

Response:
All commercial names have been removed. Percoll has been changed in sections 2.4.9-2.4.14 to density gradient medium/media. ELISpot will remain the same as it is a name of a technique, and it not a commercial assay.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response:
All actions now contain the imperative tense and only Notes are using "should".

5. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

Response:
All actions now contain 2-3 action steps.

6. Please ensure you answer the "how" question, i.e., how is the step performed?

Response:
Steps have been further clarified where needed to address the "how" question.

7. 1.1.: Age, sex, strain of the mouse used for the study.

Response:

Age, sex, and strain of mice have been added to the results section (see lines 431)

8. 2.3.3: What is the minimum number of cells required for flow cytometric analysis?

Response:
The minimum number of cells required for flow cytometric analysis depends on the target population to be analyzed. We have discussed this in the results section (lines 585-592). We have also added estimates of cells isolated per brain, spinal cord, and pooled meninges on lines 299-300 and 421-423.

9. Line 284-292, 315-323: Some of the details can be converted to numbered action steps.

Response:
Lines 284-292 were split into action steps and a note (see new 2.3.7-2.3.9). In lines 315-323 (now lines 337-345), these lines describe suggestions for optimizing enzymatic digestion and should be worked out by the user before conducting this protocol. Since enzyme optimization is not normally part of the final protocol outlined, we would prefer to keep this section as a note.

10. Only one note can follow one step.

Response:
The notes are now divided under two separate steps.

11. Please include citation for the cell surface markers tested.

Response:
Cell surface markers and product numbers have been included in the results (lines 447-450 and table of materials.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less 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.

Response:
To our knowledge, 2.75 pages of filmable content have been highlighted. Please let us know if the filmable content should be further reduced. The protocol is under the maximum 10 page limit.

1**3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."**

Response:
All figures are new and have not been previously published.

14. Please do not abbreviate the journal title in the reference section.

Response:
All references have been updated to include the full journal title.

15. For images with microscope, please include a scale bar e.g., Figure 5, 6

Response:
Scale bars have been added to the remaining images in figure 5 and 6.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The paper describes two methods of preserving CNS tissue, while build on current methods, such that the meninges are better preserved, and thus immune system function in the CNS can be better studied. One method keeps the anatomical structure intact, while the other homogenizes the cells for quantification.

Major Concerns:
I have no major concerns. I think the paper is well done and broadly suitable for publication.

Response:
We are glad the reviewer appreciates the contributions of the current manuscript.

Minor Concerns:
I think the title should indicate that these methods are optimized for the recovery of immune cells and meningeal tissue so as to highlight the key advantages of the method.

Response:
The title has been altered to include the recovery of immune cells and meningeal tissue

The authors should make it clear somewhere in the keywords, summary, and/or abstract that this method is presented in mice. They may also want to include somewhere in the discussion whether they have validated their method in any other species.

Response:
The keywords, abstract (line 45), and introduction (line 88) have all been clarified to include the method is optimized on mice. In the results section, the age, sex, and strain of mice used has been added (line 431).

There are a lot of acronyms, not all of which are standard. I would suggest adding table of acronyms for easy reference.

Response:

We agree with the reviewer's comment, however, all acronyms have been fully described in the text. We contacted the editor regarding a table/summary of the acronyms, and the editor replied the acronyms should just be introduced within the text. We have verified that all acronyms were properly described in the text.

I find the introduction and discussion overly long for this type of paper. I suggest the authors may be able to cut them down by as much as half.

Response:
The introduction and discussion are designed to answer the journal's desired points to be addressed in each section of the text. We have removed any extraneous information from the introduction to shorten the paper. We feel the discussion points need to remain the same to address JoVE's desired points. If any point/topic in the discussion is seen as extraneous by the reviewer, we would be willing to exclude it from the discussion.

Reviewer #2:

Manuscript Summary:
In this manuscript, the authors describe several methods that allow the analysis of meningeal, brain and spinal cord immunity, potentially the phenotype, function and localization of different immune cells that reside or were recruited to these different CNS tissues. Overall, the methodology is accurate. However, there are a few concerns that should be addressed to clarify certain methodological steps.

Major Concerns:

- In section 1.2.4, the authors describe that upon emersion in 30% sucrose, and sinking, the tissue can be stored in 30% sucrose at 4oC until embedding. I would strongly oppose storing the tissue in this solution for more than 24h, as this solution facilitates the growth of microorganisms (e.g. fungi). Preferably, the tissue should be embedded in 24h post sinking in the 30% sucrose solution.

Response:
The reviewer is correct that prolonged storage in sucrose solutions facilitates growth of microorganisms. We have clarified that mounting should proceed shortly after sinking of the tissue in 30% sucrose on lines (lines 185-186). We have not provided a 24 hour limit, as in our experience, decalcified brains or spinal cords may take more than 24 hours to sink. A note has been added to avoid prolonged storage in sucrose solutions to avoid microorganism growth on lines 190-192.

- The process of peeling the meninges (mostly dura and arachnoid layers) from the skull cap is not trivial and requires a lot of training and practice. Specially if the researchers are peeling "fresh", unfixed meninges for further processing and analysis of single-cell suspensions by flow cytometry or for single-cell RNA-seq experiments. This should highlighted, so that the readers are aware of the difficulty of this procedure. It is essential that the authors provide a video where it is evident how to collect a skull cap and peel meningeal dura+arachnoid, as well as how an acceptable "fresh" or fixed meningeal whole mount should look like.

Response:
We agree with the reviewer that the extraction of the meninges is a difficult process. We also would like to emphasize that because this is a difficult process, a video protocol is urgently needed to visualize the real-time extraction of the meninges. We have indicated in the Note in section 2.3.1 that meninges isolation for single cell suspensions may be difficult and a dissecting scope may be required (lines 257-259). The training/practice required for isolating meninges is also highlighted in the discussion on lines 553-562. The isolation of meninges is a key procedure for this protocol and is intended to be filmed as indicated by the highlighted text in section 2.3.

- The authors present representative flow cytometry data. However, there are no details about the antibodies used, or the solutions and procedures followed to perform the viability and extracellular stainings for cell phenotyping. Were live cells distinguished by flow cytometry by staining with viability dyes? The authors should provide alternative methodology and representative data, where staining using viability dyes (ZombieNIR or ZombieAQUA, BioLegend) are used in combination with other extracellular staining using fluorescently-labeled primary antibodies.

Response:
The cell viability dyes, antibodies, and protocol have been briefly described in the results section (lines 445-451). References have also been given for a more complete description of the staining protocol. Viability stains were used for initial live/dead gating and live cells were assessed as noted on line 450-451. Antibodies, dyes, and buffers for this procedure have been added to the table of materials.

In certain conditions, activated microglia can upregulate CD45 (becoming CD45high); the authors should point this out, otherwise the representative gating strategy can be misleading.

Response:
With regards CD45 expression, while activated microglia do express CD45 and it becomes upregulated during activation, we respectfully disagree with the reviewer that microglia upregulate CD45 to the level of CD45^{hi} infiltrating immune cells. Microglia, identified by TMEM119[1,2,3]and CXC3CR1[2-4], express CD45 at lo/int levels by flow cytometry in steady state and in inflammatory conditions[1-6].
References:

1 Li, Q., Lan, X., Han, X. & Wang, J. Expression of Tmem119/Sall1 and Ccr2/CD69 in FACS-Sorted Microglia- and Monocyte/Macrophage-Enriched Cell Populations After Intracerebral Hemorrhage. *Frontiers in Cellular Neuroscience.* **12** 520, (2018).
2 Sousa, C. *et al.* Single-cell transcriptomics reveals distinct inflammation-induced microglia signatures. *EMBO Reports.* **19** (11), (2018).
3 Li, Q. *et al.* Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. *Neuron.* **101** (2), 207-223 e210, (2019).
4 Mizutani, M. *et al.* The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. *Journal of Immunology.* **188** (1), 29-36, (2012).
5 Hammond, T. R. *et al.* Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity.* **50** (1), 253-271 e256, (2019).
6 Bennett, M. L. *et al.* New tools for studying microglia in the mouse and human CNS. *Proceedings of the National Academy of Sciences of the United States of America.* **113** (12), E1738-1746, (2016).

It is not possible to identify CD4 T cells just by expression of CD45 and CD4, as there is a population of CD4+ macrophages in the brain meninges of mice. In order to identify CD4 T cells, the authors have to present representative data showing a combination of CD45, TCRbeta and CD4 to identify conventional CD4 T cells (or CD8 for recruited/resident cytotoxic T cells).

Response:
To our knowledge, only CD4 T cells in the meninges express CD4[7] and CD4 is not expressed on macrophages[8]. If the reviewer has a reference showing CD4 expression on meningeal macrophages, we would include this as a caveat of identifying CD4 T cells by CD4. In a literature search, we did encounter CD4 expression on some subpopulations of dendritic cells found in the mouse spleen and thymus, although this has not been investigated in the CNS[9]. Moreover, TCRbeta has recently been found to be expressed on resident myeloid cells in the CNS[7]. Thus, if CD4 macrophages express CD4, the addition of TCRbeta may not aid in the further discrimination of CD4 T cells.

References:

7 Korin, B. *et al.* High-dimensional, single-cell characterization of the brain's immune compartment. *Nature Neuroscience.* **20** (9), 1300-1309, (2017).

8 Faraco, G., Park, L., Anrather, J. & Iadecola, C. Brain perivascular macrophages: characterization and functional roles in health and disease. *Journal of Molecular Medicine (Berlin, Germany).* **95** (11), 1143-1152, (2017).

9 Vremec, D., Pooley, J., Hochrein, H., Wu, L. & Shortman, K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *Journal of Immunology.* **164** (6), 2978-2986, (2000).

- If the authors are presenting representative flow cytometry and immunohistochemistry/immunofluorescence data, then they have to describe the methods used to generate and analyze that data, or alternatively refer to studies/manuscripts from leading experts in the field that provide detailed descriptions of similar methodology.

Response:
Due to the length limitations of the JOVE protocol (i.e. 10 pages) and the current length of the protocol (~7 pages), we cannot describe both the isolation protocols and downstream analysis protocols. We find there is a greater need for efficient isolation protocols for CNS tissues. Moreover, there are a plethora of protocols (including video protocols) currently in the literature describing flow cytometry and IHC protocols and these protocols do not significantly differ from our protocols previously described. We have referred to our manuscripts describing example protocols for immunofluorescence (line 467) and flow cytometry protocols (line 445-451) in the results section. In the protocol section, cited references are given for example protocols for downstream analysis (lines 303-305), including available protocols for CNS tissues/meninges. We feel our protocols that would be described would highly overlap with protocols previously described in JOVE manuscripts and other previously published protocols utilizing these methods, so we have chosen not to include them.

Minor Concerns:
- Highlight study exploring meningeal lymphatic drainage and inflammation in Alzheimer's disease in the introduction: Da Mesquita et al, Nature 560, 185-191, 2018

- The authors should also mention recently published manuscripts, that describe similar (and in certain aspects complementary) techniques optimized to analyze single cell suspensions from the meninges, as well as brain and spinal cord: [10], 2018; Herz et al, Methods Mol Biol 1846, 141-151, 2018.

Response:
We thank the reviewer for the reference suggestions. The above suggested references have been added.

Reviewer #3:

Manuscript Summary:

In the current manuscript, the authors investigated two different methods of obtaining cells and tissues from the central nervous system (CNS) structures, such as brain parenchyma, spinal cord and meninges, for further analysis. These methods are supposed to facilitate the preparation of tissue for histological processes and the isolation of single-cell suspensions, especially focusing on the meninges and the identification of resident cells and immune cells derived from outside the CNS. Both experiments are performed as a murine model, one approach contained the preparation of the meninges and CNS tissues for flow cytometry staining to analyze and quantify the cell composition and the other experiment concentrated on processing brain and spinal cord samples for decalcification and further application of immunohistochemistry to describe B- and T-cell localization in the different CNS compartments. The authors claim, that both resident and derived immune cells residing in the meningeal compartment of the CNS are crucial for homeostasis in the CNS as well as for the development of CNS pathologies. However, according to this study, the meninges are often omitted from analysis since many conventional methods for preparing CNS tissue for further experiments do not enable the sampling of these meningeal tissues. Due to the careful extraction of the tissues in question, the proposed methods are supposed to allow high-quality tissue morphology.

This study addresses the important role of the meningeal structures in CNS homeostasis and pathology and particularly introduces two methods, which are essential for further analyses.

Response:
We are glad the reviewer appreciates the contributions of the current manuscript and views the methodologies introduced as essential.

However, minor objections have to be made:

(1) Abstract:
a. Both experiments include a murine model, which should be mentioned in the abstract.

Response:
The keywords, abstract (line 45), and introduction (line 88) have all been clarified to include the method is optimized on mice. In the results section, the age, sex, and strain of mice used has been added (lines 436-437).

b. There exists no structural subdivision. Please divide into e.g. Background, Aim, Methods, and Conclusion

Response:
The structure is based on the specifications and guidelines of JOVE. Subdivisions are indicated according to JOVE's template.

(2) Discussion:
a. Please describe other methods for obtaining cells and tissue as a reference; i.e. to which other methods are the two described experiments compared?

Response:
These comparisons are noted in the introduction (lines 74-85). We have outlined a comparison for isolating the spinal cord with meninges (i.e. laminectomy) for IHC. There is no current alternative to isolate the brain with intact meninges, as peeling of the skull cap leaves the dura/arachnoid behind. For single-cell suspensions from the meninges and CNS tissues, to our knowledge, there are no alternative protocols for obtaining the meninges and CNS tissues besides the previously described protocols by Dr. Kipnis and Dr. McGavern and the protocol described in this manuscript.

b. It is not quite clear what the exact benefits of the two experiments are - these should be more outlined.

Response:
The advantages of utilizing both methods are outlined on lines 101-104. Although the representative results are a simple example of information that can be obtained from analysis of single-cell suspensions and IHC, we have further expanded the advantages of the obtained results (see line 479-488).

c. Include a statement about the investigated immune cells (from the "representative results") and their impact in CNS homeostasis and pathology - this might underline the importance of further research about the role of the meninges and the need for such described methods for the assessment of future studies.

Response:
We thank the reviewer for the above suggestion to outline the impact of examining these cells. We have added a sentence outlining the potential role of these cells in the TMEV-IDD model and in MS on lines 479-488.

d. Divide the "Discussion" into 2-3 subsections e.g. "Discussion, Limitations, Conclusion" for better structure and clear traceability.

Response:
We have followed the JOVE template in formatting the manuscript which does not include subheadings. Each introductory sentence now emphasizes the topic of the paragraph (i.e. critical step, limitation,

essential advancements, future optimization).

Reviewer #4:

Manuscript Summary:
The authors describe two methodologies to study immune cells from the central nervous system. One method comprises a procedure of decalcification that preserves the cells and structures of the meninges (dura and arachnoid) to further perform histological analysis. The second method includes 2 procedures (one for the meninges and other for the brain and spinal cord) to obtain single-cell suspensions for further flow cytometry staining with potential other applications such as single-cell RNA-seq. The manuscript has a clear description of the methodologies, it is accurate to be applied to the isolation of immune cells (and only immune cells) of the CNS, focusing mostly in the meninges. Studies on the meningeal lymphatics and the role of immune cells in the CNS are growing, as such, the described methodologies will be very useful to the scientific community. Therefore, I recommend to accept this manuscript with minor revisions.

Response:
We are glad the reviewer appreciates the contributions of the current manuscript and recommends to accept this manuscript with minor revisions.

Major Concerns:
No major concerns

Minor Concerns:

General comments:
The title is not appropriate to the described methodologies which focuses in the isolation of immune cells only, no other CNS cells (glia and neurons) are described. It should be changed accordingly, for instance: "Two methodologies for processing immune cells from CNS tissues for downstream studies of histology and single-cell analysis"

Response:
The reviewer is correct that this technique focuses on immune cells from CNS tissues, though the protocol could be easily adapted for isolating other cells of interest including microglia (see lines 418-419 and lines 548-551). The gentle processing methods by collagenase I for brain/spinal cord tissue and filter grinding for the meninges allows the isolation of most infiltrating and resident cells in the CNS. We have altered the title to reflect the focus of the protocol.

The introduction is too long. It could be more clear and direct to the point with brief descriptions of: what is your method's purpose, why it is your method important to study the immune cells of the CNS, more specifically in the meninges, what has been done and the advantages/disadvantages of those methods and what does your method brings and how is it useful for the scientific community to study the immune cells in the CNS.

Response:
We thank the reviewer for the suggestion to clarify the introduction. We have streamlined the introduction to highlight the points noted above and the points JOVE requires the author to address.

Specific comments:
Abstract:
The sentence "the CNS is an immunologically-specialized site, and in steady- state conditions, immune privilege is most evident in the CNS parenchyma. In contrast, under homeostatic conditions..." it is not clear for me what was the message, in steady-state there is immune previledge and in homeostasis not? Maybe the authors could re-write it.

Response:
We thank the reviewer for the suggestion and have modified line 37 to clarify this concept.

Introduction:
lines 106-108 "Single-cell suspensions can be used in applications, including cell culture assays to perform in-vitro stimulation, enzyme-linked immunospot (ELISpot), flow cytometry, and single-cell and bulk transcriptomics." Do authors have references for it? Have they tested it for this applications?

Response:
We have expanded references listed for these methods in the protocol section (lines 303-305). We have also added them to the introduction to emphasize protocols are readily available for downstream techniques (lines 93-95). Single-cell suspensions from brain/spinal cord tissue have been widely tested in the mentioned applications. Protocols specifically focused on the meninges have been added as available (lines 303-305).

Protocol:
Line 126: It should be mentioned here how old are the mice used, I guess adults?

Response:
The age, strain, and sex of the mice used has been added to the results (line 431-432).

Line 243: The authors could mentioned that the scissors used are fine point and/or indicate which of the two scissors from the methods, this is important not to damage the spinal cord

Response:
The reviewer is correct that fine point scissors must be used for the methods. We can clarified the use of these scissors for removing an intact spinal cord (line 240).

Line 302: Since FCS can activate/alter the cells transcriptome for for single-cell RNA-seq, maybe an alternative to FBS can be presented? Have the authors tested 10% BSA for instance?

Response:

The reviewer is correct that certain lots of FBS/FCS may activate cells. We have added a note that 1x PBS with 0.04% BSA is a safe alternative for single-cell RNA seq analysis if FCS lots are not tested for cell activation (line 323-326).

Line 305: Is minced brain spin down to remove the media before the 3ml of RPMI with 10% FCS is added? If so, it could be written down, if not, the final volume would be different in the ext steps

Response:
To clarify, the tissue is first minced without any media on the petri dish. The minced tissue is then resuspended in 3 mL of media and transferred to a conical tube. The petri dish is then washed with 2 mL of media to remove remaining tissue and this is transferred to the conical tube for a final volume of ~5 mL. This process will be filmed, which will further clarify the mincing and resuspension process. Please let us know if further clarification is necessary in the text.

Line 331: It would be better to also have the final concentration of EDTA instead or in addition to the volume to be added.

Response:
We have added the final concentration to the text (see lines 359).

Line 338: The authors could also write here that this step is to remove the debris in order to get a clear cell suspension necessary for the single-cell analysis

Response:
We thank the reviewer for this suggestion. We have made an additional note that this step is essential for obtaining a pure single-cell suspension for single-cell analysis (lines 391-393)

Line 352: Is SIP solution (1 part of percoll dilution buffer and 9 parts of percoll media) considered the 100% stock of SIP solution?

Response:
The SIP solution (now stock isotonic density gradient medium) is considered the 100% stock. We have added this point (lines 366; 371-377; 387-391) to clarify this.

Line 361: 2 tubes means 1 brain or 1 spinal cord per tube? Can we pool 2 brains or 2 spinal cords in 1 tube?

Response:
The content of the tube (i.e. whether it has 1 or two spinal cords) does not matter. A note has been added that two spinal cords may be processed together for enzymatic digestion, but brains should be processed individually (lines 316-317). 2 tubes may be pooled together regardless of whether they contain spinal cord or brain tissue cell layers. A limit of two tubes should be pooled, as pooling more can increase the percoll concentration resulting is cells not pelleting to the bottom of the tube (line 403-404). Please let us know if further clarification is needed.

Line 369: It would be useful here to give an estimate of how many cells can we recover from 1 brain or spinal cord after this step, and if all cell types are represented or if there is a cell type preferentially lost or not surviving to the procedure. Otherwise, just mentioned that these are the conditions optimised to viable immune cells and other CNS cell populations were not further explored.

Response:
For the brain and spinal cord, we have added estimates of the cell numbers obtained on lines 421-423, although this will depend on the batch of collagenase and the degree of inflammation. Cell counts have also been added for meninges (lines 299-300). Titration of collagenase type I provides gentle enzymatic digestion, allowing for the isolation of glia cells also, as seen in figure 3. In this specific article, we only identified microglia but, collagenase-based digestion may be using for immune cells, astrocytes, neurons, microglia, as previously described (lines 418-419)

Line 674: Reference 49 is not enough to find the mentioned article. The title is needed for instance.

Response:
We apologize for the insufficient reference. All references have been reviewed and updated to ensure all articles mentioned are accessible.