

Scoring Central Nervous System Inflammation, Demyelination, and Axon Injury in Experimental Autoimmune Encephalomyelitis

Carmen C. Ucciferri¹, Annette Gower², Nuria Alvarez-Sanchez^{1,2}, Heather Whetstone³, Valeria Ramaglia¹, Jennifer L. Gommerman¹, Koroboshka Brand-Arzamendi², Raphael Schneider^{2,4}, Shannon E Dunn^{1,2,5}

¹ Department of Immunology, University of Toronto ² Keenan Research Centre for Biomedical Science of St. Michael's Hospital ³ Sickkids Research Institute, The Hospital for Sick Children ⁴ BARLO MS Centre, St. Michael's Hospital ⁵ Women's College Research Institute, Women's College Hospital

Corresponding Author

Shannon E Dunn

dunn.shannon14@gmail.com

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is a common immune-based model of multiple sclerosis (MS). This disease can be induced in rodents by active immunization with protein components of the myelin sheath and Complete Freund's adjuvant (CFA) or by the transfer of myelin-specific T effector cells from rodents primed with myelin protein/CFA into naïve rodents. The severity of EAE is typically scored on a 5-point clinical scale that measures the degree of ascending paralysis, but this scale is not optimal for assessing the extent of recovery from EAE. For example, clinical scores remain high in some EAE models (e.g., myelin oligodendrocyte glycoprotein [MOG] peptide-induced model of EAE) despite the resolution of inflammation. Thus, it is important to complement clinical scoring with histological scoring of EAE, which also provides a means to study the underlying mechanisms of cellular injury in the central nervous system (CNS).

Here, a simple protocol is presented to prepare and stain spinal cord and brain sections from mice and to score inflammation, demyelination, and axonal injury in the spinal cord. The method for scoring leukocyte infiltration in the spinal cord can also be applied to score brain inflammation in EAE. A protocol for measuring soluble neurofilament light (sNF-L) in the serum of mice using a Small Molecule Assay (SIMOA) assay is also described, which provides feedback on the extent of overall CNS injury in live mice.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is the most common murine model for the human demyelinating disease, Multiple Sclerosis (MS)¹. Classic MS inflammatory

pathology, including the infiltration of IFN- γ (gamma) and IL-17-producing T helper cells², the infiltration of inflammatory monocytes³, the formation of perivascular

and sub-meningeal inflammatory demyelinating lesions⁴, and the occurrence of axon injury⁴ in the central nervous system (CNS), is also observed in EAE^{5,6,7,8,9}. The similarity in immune mechanisms between EAE and MS has made EAE a suitable pre-clinical model for testing the efficacy and mechanisms of action of a number of approved immune-based therapies for MS, including natalizumab, fingolimod, dimethyl fumarate, and glatiramer acetate (reviewed in^{1,5}). Certain EAE regimens model other aspects of progressive MS pathology beyond axonal injury, including the development of sub-meningeal inflammation in the brain, chronic demyelination, spinal cord atrophy, synapse, and neuron loss^{6,10,11,12}. Thus, EAE has utility for screening the efficacy of neuroprotective therapies for MS.

EAE is induced in rodents in a number of ways. Active immunization is the most common induction method and involves immunization of rodents with myelin antigens (either whole proteins or peptides) emulsified in CFA supplemented with heat-killed *Mycobacterium tuberculosis*¹³. Depending on the strain of mouse, pertussis toxin (PTX) is also administered on day 0 and day 2 of immunization to increase the penetrance of disease¹³. EAE can also be induced by adoptively transferring myelin-specific T cells obtained from myelin/CFA-primed mice into healthy mice¹⁴ or can develop spontaneously in mice that overexpress T cell receptors specific for the major myelin antigens⁵.

EAE disease severity and progression are commonly scored using a discrete 5-point clinical scale: 1 – tail limpness, 2 – hindlimb or foot weakness, 3 – complete paralysis in one or both hindlimbs, 4 – forelimb weakness, 5 – moribund or dead¹³. This clinical scoring system is sound in documenting the progression of ascending paralysis that occurs at disease onset but is less sensitive at capturing

the extent of recovery from CNS inflammatory attacks. For example, both mice that ambulate with difficulty and mice that ambulate easily but exhibit foot-grasping weakness are assigned a score of 2 on the EAE scale. Scores can remain high in the post-acute phase of EAE due to the presence of permanent axon injury or loss, even despite the resolution of the inflammatory response⁹. There have been a variety of attempts at developing more refined scoring systems, behavioral tests, measures of hindlimb and grip strength, and infrared monitoring systems to better capture differences in clinical deficits in EAE^{9,16,17,18}; however, these more intricate scoring measures do not distinguish the contribution of inflammation versus tissue injury to the underlying neurological deficits. Thus, the gold standard approach to score the severity of EAE is to conduct both clinical and histological scoring.

Here, a protocol is described for how to dissect and embed mouse spinal cord and brain specimens in paraffin in a way that captures the stochastic process of lesion formation that occurs in EAE. A protocol is also presented of how to stain sections with Luxol fast blue (LFB), originally created by Kluver and Barrera¹⁹, which detects myelin in the CNS. Sections are either stained with LFB alone (for demyelination analysis) or are counter-stained with hematoxylin and eosin (H&E) to help visualize and score inflammatory lesions. Protocols are also provided to quantify the presence of total leukocytes (CD45), the loss of myelin, and the number of injured axons (SMI-32) in the spinal cord using commercially available antibodies, immunohistochemical (IHC) techniques, and publicly accessible software. The protocol used to quantify leukocytes in the spinal cord can also be applied to quantify leukocytes in the brain.

Histological evaluation of axonal loss and injury in the brain is comparatively more difficult than in the spinal cord since brain white matter tracts do not run in parallel to one another. The measurement of serum neurofilament light (sNF-L) has emerged as a promising biomarker for neuronal injury in MS^{20,21}. Recent studies have extended this technology to EAE^{22,23,24}. Here, a method is presented to measure serum neurofilament light (sNF-L) in living mice using a Small Molecule Assay (SIMOA) assay. This method requires only a small quantity of serum and can be done in live mice in just half a day, providing rapid feedback on how a tested therapy is affecting overall CNS injury. All of the methods described here can be applied to mice of any sex or strain.

Protocol

All experiments conducted with mice were performed under animal use protocols approved by the Unity Health Toronto Animal Care Committee, following the guidelines set forth by the Canadian Council on Animal Care. Ensure to wear a lab coat, protective gloves, and eyewear throughout the laboratory procedures.

1. Harvesting and fixing the brain and spinal cord

1. Euthanize the mouse according to institutional policies. Lay the mouse prone on a dissecting table and decapitate using surgical scissors (cutting downwards).
2. Using Adson forceps (in non-dominant hand), grasp the skin on the top of the mouse's head. Then make a 2.5 cm incision in the skin on the top of the head using surgical scissors.
3. Use fingers to push the skin on the head laterally to visualize the underlying skull.

4. Stabilize the mouse's head by grasping eye sockets with standard Adson forceps.
5. Using fine scissors (dominant hand), make small snips in the skull along the midline from the cervical spine to the olfactory bulbs.

NOTE: Embed only a few millimeters of scissor tips below the skull at a time to avoid damaging the underlying brain.

6. Use Adson forceps with teeth to reflect the skull laterally to reveal the underlying brain.
 7. Hold the head using the non-dominant hand. Holding scissors closed (dominant hand), scoop the spinal cord out from the cervical spine and gently nudge the brain out from the skull, snipping the cranial nerves.
 8. Place the brain in a conical tube containing 10 mL of 10% neutral buffered formalin that is labeled with the animal's ID.
 9. Incise the fur along the midline of the mouse's torso from the neck to the tail.
 10. Use fingers to push the skin laterally to visualize the spine.
 11. Using surgical scissors, cut downwards through the spine at the level where the femurs attach to the hip.
 12. Use surgical scissors to cut the body wall on each side of the spine from the hip to the neck. Trim away any attached organs.
 13. Place the spine containing the spinal cord in the same tube of formalin that contains the brain. Allow the brain and spinal column to fix for 5–7 days.
- NOTE:** The timing of fixation is important. Some antibodies will not work if tissue is over-fixed. If tissue is under-fixed it is difficult to extrude the spinal cord from the spinal column in step 2.

2. Grossing and processing of the spinal cord and brain

NOTE: The following steps take place in a fume hood. Before starting, prepare 2 x 10 cm clean Petri dishes, an Erlenmeyer flask fitted with a funnel lined with filter paper, two scalpels (one for cutting bone and one for cutting CNS tissues), lens paper, a pencil, embedding cassettes, and specimen jars pre-filled with 10% formalin.

1. Using scissors, cut a small piece of lens paper (the same width but twice the length of the cassette) and place it in one Petri dish.
2. Label a plastic cassette with the specimen ID using a pencil.
3. Pour the tube containing the fixed brain and spine into the funnel. Transfer the spine and brain to the empty Petri dish.

NOTE: The used formalin will filter through into the Erlenmeyer flask and can be re-used at step 2.13.

4. Grossly divide the brain into six coronal pieces using a scalpel. Make one cut caudal to the cerebellum, one in the middle of the cerebellum, one just rostral to the cerebellum, and two cuts in the remaining rostral brain, creating 3 additional coronal slices of equal thickness.
5. Using forceps, transfer brain specimens onto one half of the lens paper in the Petri dish.
6. Cut the spinal cord into three pieces using the scalpel: the first cut is made at the bottom of the rib cage, and the second cut is made just below the curvature in the cervical spine.
7. Using the same scalpel, trim the sacral spine piece at the caudal end until the spinal cord can be visualized.

8. Pick up the thoracic spine (non-dominant hand). Hold Adson forceps with teeth tightly closed (dominant hand) and gently push the end of the forceps into the smaller opening of the spinal column using a gentle twisting motion. The cord should emerge from the other end.

NOTES: Any round-ended instrument that is the size of the spinal cord will work for this purpose. If the spinal cord does not pop out naturally, do not force it. Instead, use fine scissors to clip the bones along the side of the spine and reflect it open to reveal the spinal cord. Alternatively, fix the spinal cord and brain for a few additional days in formalin; however, the same fixation time should be applied to all specimens to avoid introducing variability in antibody staining.

9. Pick up the standard Adson Forceps (dominant hand). Still holding the spinal cord piece with the less-dominant hand, use forceps to gently pull the emerged cord out of the column. Place the spinal cord piece in the Petri dish containing the lens paper.
 10. Repeat this process for the lumbar/sacral and cervical column pieces.
 11. Divide the three spinal cord pieces (cervical, thoracic, lumbar/sacral) into smaller cross-sectional pieces using a scalpel. Cut at least 15 segments, each of which should be less than 2 mm thick.
- NOTE:** Ensure that the segments are shorter than they are wide, which will make the sections fall more easily in cross-section during the embedding process in step 3.
12. Arrange the spinal cord pieces on the same half of the lens paper containing brain pieces. Fold the lens paper over to sandwich the tissue pieces and place this in the labeled cassette.

NOTE: The lens paper prevents small pieces of tissue from escaping the cassette during processing.

13. Transfer the cassette into a specimen jar containing recycled or fresh formalin.
14. Repeat steps 2.1–2.13 for remaining specimens.
15. After 5-7 days of fixation, transfer cassettes from the specimen container into the first formalin bath in the automatic tissue processor (see **Table of Materials**). Run the tissue processor overnight according to the program described in **Supplementary Table 1**. Specimens are held in warm paraffin wax until embedding.

3. Embedding and cutting brain and spinal cord sections

1. Transfer cassettes from the processor to the warm holding chamber of the paraffin embedding station (see **Table of Materials**).
2. Embed spinal cord and brain cross-sections from each mouse in a single paraffin block as follows (**Figure 1**):
 1. First, pour paraffin wax just to cover the bottom of the mould. Using fine forceps, place brain coronal and spinal cord cross-sectional pieces into the paraffin in the bottom of the mould.
 2. Transfer mould to cooling surface for several seconds to fix brain and spinal cord pieces in place. Move the mould back to the heated surface and fill it to the top with hot paraffin.
 3. Place cassette lid (that is labeled with specimen ID) on top of the mould. Pour more paraffin on top of the cassette lid. Transfer the mould to the cooling station to allow wax to set (cool for 30–60 min).

NOTE: Embedding spinal cord sections requires practice. To improve success, use shorter lengths of spinal cord (<2 mm) as these are more likely to fall in cross-section. Surgical eye loupes can be worn to help distinguish if spinal cord pieces are in cross-section.

4. Mount paraffin block on rotary microtome. Trim block until tissues of interest appear in paraffin section.
5. Cut 5 μ M ribbons of sections of each block and transfer them into a 42 °C water bath.
6. Collect sections on slides and place slides in a glass slide rack.
7. Bake sections at 37 °C in a dry oven overnight. Cool slides before proceeding to staining.

4. De-paraffinization and rehydration of sections in preparation for staining

NOTE: Steps are performed in a fume hood. Before starting, prepare baths of solvents. Prepare 5 L of 1x PBS (1 L ddH₂O, 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄; pH = 7.4) with 0.05% Tween-20 (PBS-T) for all washing steps.

1. De-paraffinize by placing slides in two consecutive baths of xylene or a non-xylene based solvent on a shaker for 5 min each with gentle agitation.
2. Rehydrate tissues by transferring slides through successive baths of descending percentages of ethanol: 2 x 100% ethanol (5 min each), 2 x 95% ethanol (3 min each), and 1 x 70% ethanol (3 min). Hold in 95% ethanol for LFB staining, and rehydrate to 70% ethanol, and hold in PBS-T for immunohistochemistry (IHC).

5. LFB for myelin with H&E

1. Prepare a solution of 0.1% LFB (0.2 g LFB, see **Table of Materials**, 200 mL 95% ethanol, 0.5 mL glacial acetic acid). Mix and filter into an Erlenmeyer flask. Store in a dark bottle until use.
2. Transfer sections from 95% ethanol into a glass slide rack that is placed in a staining dish containing LFB. Cover the dish and seal with with paraffin film to prevent evaporation.
3. Incubate sections at 56 °C oven overnight (maximum 16 h).
4. The following morning, transfer slides into a ddH₂O bath and hold.
5. Meanwhile, prepare (1) fresh 0.05% lithium carbonate solution (0.05 g lithium carbonate, 100 mL ddH₂O); (2) Eosin Y solution (add 2 g of eosin salt to 40 mL ddH₂O and mix until dissolved and then mix with 160 mL of 95% ethanol).
 1. Prepare the following baths: 1 x lithium carbonate, 3 x 70% ethanol, 3 x 95% ethanol, 2 x 100% ethanol, 3 x ddH₂O. Place baths in order of use (see **Supplementary Table 2**).
6. Follow the steps as outlined in **Supplementary Table 2**.
7. After the slides are dry, visualize the demyelinating lesions under the microscope.

6. LFB for myelin without H&E

1. Perform this staining procedure for the analysis of myelin.

NOTE: The procedure is identical to step 5 (see **Supplementary Table 2**), but has an abbreviated workflow. After step 4, continue procedure starting at step 10.

7. Antigen retrieval and peroxidase quenching for immunohistochemical (IHC) stains

NOTE: Before starting, prepare 100 mL of hydrogen peroxide in methanol (1 part 30% hydrogen peroxide solution in 9 parts 100% methanol, in a fume hood). Prepare 1 L of 10 mM citrate buffer with Tween-20 (2.94 g of trisodium citrate, dissolved in 1 L of ddH₂O in a beaker on stir plate, bring pH to 6.0, and add 500 µl of Tween-20). Prepare PBS-T (see step 4). All washes are done in baths of PBS-T with gentle agitation (on a shaker) unless otherwise indicated.

1. Quench endogenous peroxidase by placing slides in 3% hydrogen peroxide in methanol for 15 min (in a fume hood). Wash slides twice in PBS-T (2 min each).
2. Transfer slides to a metal slide holder and place in 1 L of citrate buffer in a pressure cooker. Seal the lid and add a rubber stopper on the top of the steam escape vent.
3. Cook on high in the microwave until the yellow tab on the pressure cooker pops up, indicating that maximum pressure has been reached. Cook for an additional 5 min at maximum pressure, and then remove the pressure cooker from the microwave using protective gloves.
4. Depressurize by removing the stopper. Remove the lid and let the slides cool in the citrate buffer for 20 min and then proceed to desired staining method.

CAUTION: Stand back when releasing the steam as it can cause a scalding injury.

8. CD45 immunohistochemistry

NOTE: This IHC method is used to visualize infiltrating leukocytes. The avidin/biotin blocking steps are combined with the blocking and primary antibody incubation steps.

1. Prepare blocking buffer (2% BSA, 2% rabbit serum in 1x PBS).
2. Transfer slides to glass slide tray and wash twice with PBS-T (2 min each). Prepare avidin/blocking solution (4 drops/mL of avidin in 2% BSA/2% rabbit serum in 1 x PBS, see **Table of Materials**).
3. Dry off excess PBS-T around tissue using a laboratory tissue paper. Using a hydrophobic pen, draw a circle around the tissue and place slide in the humid chamber.
4. Apply avidin/blocking solution onto each section (400 μ L/slide).
5. Cover humid chamber and incubate for 30 min at room temperature. During this step, prepare anti-CD45 antibody (**Supplementary Table 3**) in blocking buffer containing biotin (4 drops/mL biotin, 2% BSA/2% rabbit serum in 1x PBS).
6. Tap off blocking solution from slide onto a lint-free laboratory wipe. Dab around tissue using a laboratory wipe to remove excess liquid.
7. Place the slide back into a humid chamber. Add 400 μ L of CD45 antibody solution (see **Table of Materials**) onto the section. Incubate overnight at 4 °C in a covered humid chamber.
8. The next day, drain off primary antibody and wash slides 3 x in PBS-T (5 min each).
9. Dry area around section using a lint-free laboratory and then add 400 μ L of secondary antibody (1:200 dilution in blocking buffer) onto each section. Incubate at room temperature for 1 h.
10. Meanwhile, prepare ABC reagent (see **Table of Materials**) by adding 2 drops of reagent A to 5 mL of 1x PBS and mix. Add 2 drops of reagent B to the same solution and mix (prepare ~30 min before use).
11. Wash the slides in 3 changes of 1x PBS-T (5 min each) and place the slides in a humid chamber.
12. Add 400 μ L ABC reagent to sections. Cover humid chamber and incubate for 30 min at room temperature.
13. Wash the slides in 3 changes of 1x PBS-T (5 min each). Meanwhile, prepare an appropriate amount of DAB solution in a foil-covered 15 mL centrifuge tube according to the manufacturer's instructions (see **Table of Materials**).
14. Take one slide and focus on a spinal cord section under a microscope. Add 400 μ L DAB to the slide and start lab timer.
15. Visualize the section while it is developing and stop timer when leukocytes are brown. Transfer the slide into a ddH₂O bath to stop the reaction. Hold in water for 5 min. The same development time is used for the remaining slides.
CAUTION: DAB is a carcinogen. Dispose of DAB waste and post-DAB ddH₂O as hazardous waste.
16. Counterstain slides with Mayer's Hematoxylin for ~4-10 min (see **Supplementary Table 2**). Rinse slides under running tap water for 10 min.
17. Dehydrate in 95% ethanol (1 x 3 min), followed by absolute 100% ethanol (2 x 3 min each).
18. Moving into a fume hood, transfer the slides into xylene or a xylene-substitute solvent for 5 min. Coverslip with mounting medium, and allow the slides to dry for 1-2 days in the fume hood.

CAUTION: If using xylene, use double gloves and forceps to handle slides when cover-slipping, since it is toxic and can dissolve gloves.

- Clean the slides using xylene and scan using a slide scanner at 20 x magnification.

9. SMI-32 IHC for axonal damage

NOTE: This protocol uses a mouse SMI-32 antibody, which reacts against non-phosphorylated neurofilament heavy, which can accumulate in injured axons²⁵. Since this antibody has been raised in mouse and detects a mouse antigen, it is recommended that a Mouse on Mouse (MOM) kit be utilized. In this procedure, the avidin/biotin blocking step is done as a separate step from the primary antibody incubation. Before starting this protocol, de-paraffinize, rehydrate, quench endogenous peroxidase activity, and conduct antigen retrieval as described in step 4 and step 7.

- Wash sections twice with 2 x PBS-T (2 min each). Remove extra liquid around sections using a laboratory tissue and draw a circle around tissues using a hydrophobic pen.
- Add 400 μ L blocking buffer (2% (w/v) goat serum in 1x PBS-T) with avidin (4 drops/mL) to the sections. Incubate for 15 min at room temperature.
- Dip the slides twice in 1x PBS-T. Add 400 μ L of blocking buffer with biotin (4 drops/mL) to the slide and incubate for 15 min at room temperature.
- Wash the slides in a 1 x PBS-T bath for 2 min. Meanwhile, prepare MOM blocking reagent by adding 2 drops of stock solution (see **Table of Materials**) to 2.5 mL of 1x PBS.
- Add 400 μ L of MOM reagent onto the section. Incubate for 1 h at room temperature.

- Wash the slides twice in a 1x PBS-T bath for 2 min. Meanwhile, prepare MOM diluent by adding 300 μ L of protein concentrate stock solution to 3.75 mL of 1x PBS.
- Add 400 μ L of MOM diluent and incubate for 5 min at room temperature. Meanwhile dilute the SMI-32 antibody (see **Table of Materials**) in the MOM diluent.
- Tap off the diluent from slides and add 400 μ L of SMI-32 antibody solution onto section. Cover the humid chamber and incubate for 30 min at room temperature.
- Wash slides twice in a 1x PBS-T bath for 2 min, each with gentle agitation. Meanwhile, dilute MOM anti-mouse IgG working reagent in the diluent (10 μ L of stock in 2.5 mL of diluent).
- Add 400 μ L of anti-mouse IgG working reagent per slide. Incubate for 10 min.
- Wash slides twice in a 1x PBS-T bath for 2 min each. Continue staining following the steps outlined in CD45 staining protocol (step 8.11 – 8.19).

10. LFB and H&E scoring for the presence of demyelinating lesions

NOTE: The following is an analysis approach that can be applied to gain rapid insights into the severity of inflammatory demyelination. This analysis is conducted in sections of the cord sampled at different levels (cervical, thoracic, and lumbar, at least 3 sections per/level). Refer to the Allen Brain Atlas for Mouse spinal cord²⁶ to help identify the anatomical level of the spinal cord. This analysis requires TIFF files. If scanned images are in .czi format, follow instructions in **Supplementary Table 4** to convert czi files to TIFF files.

- Open the TIFF image of the section to be analyzed by dragging and dropping the file into the ImageJ. Observe

the spinal cord section in 4 quadrants: dorsal, left lateral, right lateral, and anterior (**Figure 2A,B**).

- Score for the presence of demyelinating lesions in each quadrant as depicted in **Figure 2**.

NOTE: The presence of a lesion results in a score of 1 in that quadrant, for a total of 4 possible points per section. A score of 1 is assigned even if there are multiple lesions within a quadrant.

- Add up all points for each mouse and divide by the total number of quadrants sampled to obtain the fraction of quadrants with sub-meningeal lesions.

11. Calculating the area fraction of LFB-staining in spinal cord white matter

NOTE: This analysis measures the percent area fraction of spinal cord white matter that is stained with LFB.

- Open an LFB-stained section saved as a TIFF file by dragging/dropping the file into ImageJ.
- Click on **Image > Type > 8-bit** to produce a grey scale image. Click on **Image > Adjust > Threshold**. Adjust the bottom slider in order that the red overlay captures all of the dark regions (myelin) seen by eye (**Figure 2C–E**). Click on **Apply**.
- Click on **Analyze > Tools > ROI Manager** to open the ROI Manager.
- Select the **polygon** drawing tool on the ImageJ tool bar. This drawing tool allows one to outline a region of spinal cord with the computer mouse.
- Outline the dorsal region of spinal cord and add the polygon to the ROI Manager by clicking **t** on the keyboard.

- Outline the anterior-lateral region of the spinal cord white matter and add the polygon to the ROI manager.

NOTE: When tracing, exclude large blood vessels, tears in the tissue, artifacts, and areas adjacent to the dorsal horn (**Figure 2A,B** arrow), which are normally less myelinated. Be as accurate as possible when tracing since including too much white background will skew the results.

- Click on **Analyze > Set Measurements** and select **Area Fraction**.
- Click on **Measure** in the ROI Manager. This will give the fraction of the outlined area that is stained with LFB.
- From the newly produced results box, copy the values into Excel and close the image window. Calculate the average % fraction area stained for the dorsal and ventro-lateral regions. Average these to get a value for that section.
- Repeat steps 11.1–11.9 for cervical, thoracic, and lumbar sections (N = 3/level/mouse).
- Calculate the average percent myelin fraction for all sections for each mouse. Percent demyelination is estimated by subtracting the % area staining from 100.

12. Analysis of the number of CD45⁺ cells and SMI-32⁺ axon ovoids

- Drag and drop a CD45 or SMI-32 stained TIFF image into ImageJ. Click on **Analyze > Set Scale > Global > Ok** to set the scale bar across all images. Note that this is done only for the first image.
- Select the **polygon** drawing tool and trace around the grey matter using the computer mouse. Click on **Analyze > Measure** and record the results in Excel as “Grey Matter Area”.

3. With the drawn polygon still on the image, remove the grey matter from the image by clicking on the **Delete** key (keyboard). This action leaves only the white matter to be analyzed.
4. Outline the entire spinal cord section using the **polygon** drawing tool. Exclude any regions of tissue that are missing or damaged.
5. Click on **Analyze > Measure** and record the results as “Total Tissue Area” in the Excel file.
6. Click on **Image > Colour > Colour Deconvolution**. From the vectors drop-down window select **H DAB**. Three new windows will appear – keep the brown hued window (DAB channel) and delete other image windows.
7. Click on **Image > Adjust > Threshold**. Adjust the bottom slider to threshold the image so that the red overlay captures the same amount of brown staining detected by eye. Click on **Apply**.
8. Click on **Process > Binary > Watershed**. At this step compare the original image and the binary image to assure that they are in agreement.
9. Click on **Analyze > Analyze Particles**. Select **Show Overlay** and modify the settings: Size = 5 - 150 μm^2 , Circularity = 0.4 – 1. Click on **OK**.
NOTE: These settings allow for individual cells and small groups of cells that were not split with the Watershed function to be included as opposed to being excluded in the analysis.
10. In the results window, record the last number in the left-most column in Excel, which represents the total particle count. Then, calculate the white matter area (total tissue area – grey matter area in mm^2). Express total particle count per white matter area (count/ mm^2).

11. Repeat steps for each saved RGB image. Once all spinal cord sections have been analyzed for one mouse, average the particle count per mm^2 tissue for that mouse.

NOTE: The workflow used to analyze spinal cord leukocytes can also be applied to brain regions.

13. Measurement of sNF-L using a SIMOA assay

1. Collect 100–200 μL blood from live mice by saphenous bleed using capillary microtainer tubes or *via* cardiac puncture using a syringe and 25 G needle (terminal procedure). For the latter, transfer blood into a 1.5 mL microcentrifuge tube.
2. Allow the blood to clot at room temperature for 30–60 min.
3. Centrifuge samples at 2660 x *g* for 5 min at 4 °C and pipette the upper fraction (serum) into a new microcentrifuge tube. Store the serum at -80 °C until ready to use.
4. Preparation: Allow calibrators and controls (NF-light assay kit, see **Table of Materials**) to warm to room temperature for 1 h prior to use. Take the enzyme substrate (RGP) out of the refrigerator and place in a 30 °C waterbath for at least 30 min, vortexing every 10 min.
5. Thaw the serum samples on ice. Once thawed, gently vortex samples and centrifuge at 10000 x *g* for 5 min to pellet any debris.
6. Load the plate: Vortex the calibrators and the controls. Load the calibrators in duplicate, controls in duplicate, and serum samples in duplicate onto the 96-well plate provided with the kit. Serum samples are diluted in a 1:3 ratio with diluent provided in the kit. Seal the plate.

7. Turn on the SIMOA machine (see **Table of Materials**) in the order of computer, program, and then machine. Allow the machine to initialize and run **Start of Day** maintenance.
8. Vortex the magnetic beads for 30 s.
9. **Load Reagents** tab on the screen, double clicking the rack position where the reagent bottle is to be placed, and then scanning the barcode of the bottle.
10. Place magnetic beads in the shaking position on the rack (positions 1–3).
11. Continue, loading the detector, the SBG reagent, and RGP reagent into the machine.
12. Click **setting** on the software. Ensure sample mode is on plate. In the setup run tab, name the experiment.
13. Assign wells on the plate for the calibrators as follows: Click the well, then select the assay. Select **calibrate A** then click on **ascending**. Highlight wells A1-8 then click **replicates (2)** to assign the coordinates for the calibrators to be run in duplicate. Run using the neat protocol.
NOTE: Refer to the certificate of analysis (manufacturer website) to get the concentrations of each calibrator for each particular lot number. Once wells are assigned, it is not possible to navigate away from this screen without losing the work, until the plate is locked in place.
14. Assign the samples following the steps below:
 1. From the bottom right of the screen, click **button** to assign the samples. Highlight wells where the samples are to be located.
 2. Check off the assay to be run from the list, chose the number of replicates, and specify the onboard dilution of 4x.
 3. With the wells still highlighted, enter a prefix for the sample ID and the starting number and then click on **generate** to produce sequential IDs for the samples. Repeat steps for control samples.
15. Load plate into the plate holder (A1 to A12).
16. On the screen interface, click on **done programming samples** and proceed to system resources tab. Check that all reagent containers are full and the waste containers are empty.
17. Click on **Run**.
18. When the run is complete, check the calibration curve under “Data Reduction” tab by selecting the assay and plate name.
19. Go to “Run History” and use the filters to find the most recent run. Select **run** and then **all results**. Click on **export**, and save the .csv file. Click on **report** and then select **batch calibration report** and select the recent run. Preview the report and export it.
20. Run the end of day maintenance recommended by the manufacturer. Turn off the program, the machine, and then the computer.

Representative Results

Figure 3 shows representative IHC and histochemical staining, with examples of both acute (left) and older EAE lesions (right). Representative CD45 staining with hematoxylin counterstaining is shown in **Figure 3A,B**. **Figure 3C–F** show examples of LFB staining with (**Figure 3C,D**) or without (**Figure 3E,F**) the H&E counterstain. Though hematoxylin is not specific to immune cells, the nuclei of the immune cells stain more darkly and can be distinguished from CNS resident cells. **Figure 3G,H** show representative staining

of SMI-32⁺ axons, counterstained with hematoxylin. Notice the increased appearance of this stain in older EAE lesions.

Damage of myelinated tracks is most prevalent in the spinal cord in active murine EAE and this is the main driver of paralysis in this disease^{7,9}. Thus, scoring for the presence of inflammation and tissue damage in the spinal cord is prioritized in histological analyses. EAE lesions occur sporadically at different regions (anterior, lateral or dorsal) (**Figure 2A,B**) and at different levels (sacral, lumbar, thoracic, cervical) of the spinal cord. The described embedding method ensures good sampling of lesions throughout the cord. More sections are embedded than analyzed, since some sections can become damaged in the processing or sectioning process. To ensure representative sampling, a minimum of 3 representative sections are analyzed at cervical, thoracic, and lumbar levels of the spinal cord for each mouse. The identity of each specimen is blinded in order that the person conducting the analysis is not biased when selecting representative sections for analysis.

To gain quick insights into differences in the histological severity of EAE, one can score for the presence of sub-meningeal demyelinating lesions in spinal cord quadrants in selected sections (**Figure 2A,B**). This is a rapid method that can be performed on scanned images or using a light microscope. This analysis is sensitive enough to detect differences in histological EAE severity between groups when EAE is severe in one group and mild in another. For example, in the experiment in **Figure 4**, EAE was induced in female wild type (WT) and mice with a deletion in OGR1 (OGR1 KO) using MOG p35-55/CFA plus PTX. Mice in the WT group developed severe EAE with complete paralysis, whereas the OGR1 knockout group developed mild disease. This difference in

clinical score corresponded to a difference in the fraction of quadrants that had sub-meningeal lesions (**Figure 4C**).

It is important to complement the scoring of demyelinating lesions with percent area fraction of myelin staining to capture the extent of loss of myelin and/or myelinated axons during the autoimmune attack. In the example in **Figure 4**, the percent myelin fraction also differed significantly between the OGR1 and WT mice (**Figure 4D**). The percent myelin fraction also significantly correlates with the cumulative EAE score in mice with EAE (**Figure 4E**) and therefore serves as a good measure of overall tissue damage in this disease. Note that this protocol does not distinguish the intensity of myelin stain. If this is the desired outcome, one should conduct immunofluorescence staining for myelin proteins such as proteolipid protein or myelin basic protein and measure the intensity of this staining.

In the case where EAE is severe in both comparator groups, a higher fraction of spinal cord quadrants will contain inflammatory/demyelinating lesions. In this case, a more sensitive approach to score inflammation is to count the number of CD45⁺ leukocytes per mm² white matter (see representative staining in **Figure 3A**). The CD45 antibody clone described here detects all infiltrating leukocytes, and only stains occasional microglia that upregulate CD45 expression in EAE (see open arrow in **Figure 3B**) and therefore is useful at capturing peripheral immune cell infiltration.

In longer-term EAE studies (>20 days), it is recommended that one also carry out an analysis of axon injury. SMI-32 staining in spinal cord sections is a sensitive method to detect damaged axons. Though inflammation in the spinal cord subsides with time and spared axons can re-myelinate, surviving axons exhibit a differential extent of residual

injury⁹ (**Figure 3G,H**). For example, in the MOG p35-55-induced model of EAE in C57BL6/J mice, the extent of axonal injury and loss is a driver of clinical scores after the inflammatory process has subsided⁹. **Figure 5** shows an example of this in an EAE experiment in male and female mice WT mice and mice that are deficient in a gene called peroxisome proliferator-activated receptor-delta (PPAR-delta) in the myeloid compartment (*LysM^{Cre}: Ppard^{fl/fl}*). In the males, the WT mice regained hindlimb function, yet the clinical scores remained high in male *LysM^{Cre}: Ppard^{fl/fl}* group. By contrast, in the experiment in the females, both experimental groups had high scores throughout. At first glance, this result suggested that PPAR-delta had a sex-specific effect in EAE; however, pathological scoring of the spinal cord revealed that mice of both sexes in the *LysM^{Cre}: Ppard^{fl/fl}* group had increased axonal injury compared to WT counterparts (**Figure 5B**). A genotype effect on clinical scores was likely not observed in females because WT female mice tended to exhibit increased axonal injury, which manifested into chronic neurological deficits.

In this same experiment, female *LysM^{Cre}: Ppard^{fl/fl}* female mice were found to have more extensive T cell infiltration in the cerebellum, providing an example of how scoring brain inflammation may be useful in EAE. In EAE, inflammation in the brain is predominantly found in the cerebellum and brain stem (**Figure 6A,D,G**), but can also be found in the meninges (seen under hippocampus in **Figure 6C**), near the

ventricles (**Figure 6F**), and other white matter tracts including the optic nerve and corpus collosum (**Figure 6B,E**). Scoring brain inflammation is done in a specific brain region (e.g., cerebellar white matter) by counting the number of CD45 cells per mm² tissue region using the same methodology as outlined for the spinal cord protocol. In the grossing method outlined here, a cut is in the middle of the cerebellum, which provides the perspective of the cerebellum and brain stem as shown in **Figure 6A**.

Measuring sNF-L using a SIMOA assay has become a useful biomarker for assessing ongoing axonal injury and responses to therapy in relapsing-remitting MS^{20,21,27,28}. The same SIMOA assay kit used to measure of sNF-L humans can be applied to measure mouse sNFL^{22,23,24}. To explore how well this assay performs is detecting axonal injury in EAE, sNF-L was measured in female C57BL6/J mice at the endpoint of an EAE experiment and levels were compared to those in sex-matched healthy control mice that did not have EAE. It was found that mice with EAE had much higher levels of sNF-L than in healthy mice (**Figure 7A**) and these levels correlated with the density of SMI-32⁺ axons in the spinal cord (**Figure 7B**). Compared to histological scoring of axonal injury, the SIMOA assay is faster (from bleeding mice to results can be achieved in just over half a day) and therefore provides rapid feedback of how a treatment is working in living mice. This assay also has the advantage that it reflects axonal injury in both the spinal cord and the brain.



Figure 1: Representative paraffin block of brain and spinal cord sections. The 5 coronal brain sections and spinal cord cross sections (1.5–2 mm thick) are embedded in the same block in order that they can be cut in one section. At least 15 sections of spinal cord should be embedded, allowing for adequate selection of sections for analysis. [Please click here to view a larger version of this figure.](#)

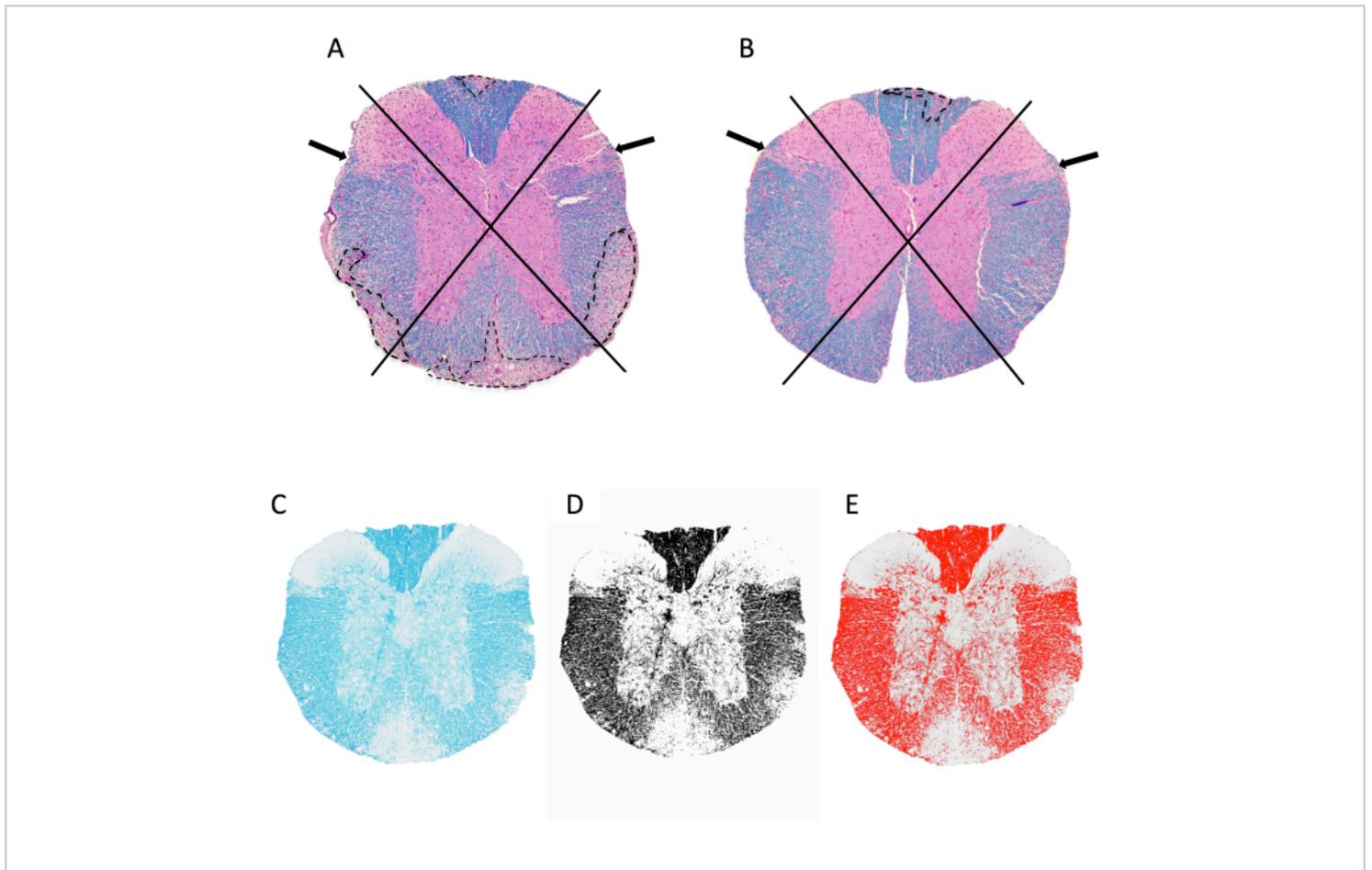


Figure 2: Scoring meningeal inflammation and percent myelin area at the level of the thoracic spinal cord. (A,B) show images of the thoracic spinal cord from a female C57BL6/J mouse with MOG p35-55-induced EAE stained with LFB/H&E. Shown is the approach used to visualize quadrants and examples of demyelinating lesions (traced in dotted line). The mouse in A has 4 of 4 quadrants with confluent demyelinating lesions, while the mouse in B has 1 of 4 quadrants affected. The mouse in B does have some inflammation in other quadrants, but this has not manifested into a confluent lesion and therefore is not scored. (C–E) Example of LFB image, and the greyscale and thresholded image in image J. [Please click here to view a larger version of this figure.](#)

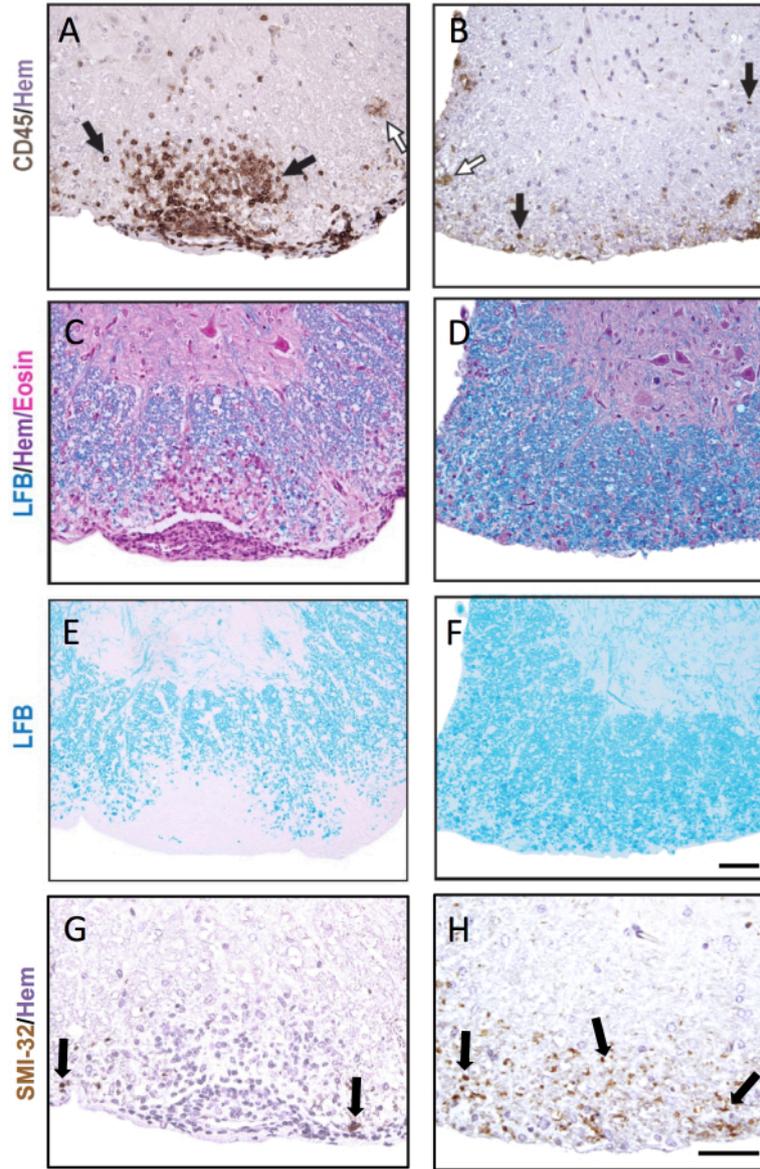


Figure 3: Spinal cord sections stained with CD45, LFB H&E, LFB and SMI-32. Examples of an early (A,C,E,G) and late (B,D,F,H) sub-meningeal lesion in the spinal cord stained for CD45 antibody (A,B), LFB/H&E (C,D), LFB alone (E,F), and SMI-32 antibody (G,H). Black arrows show examples of cells stained with each respective antibody. White arrows show putative microglia that have been stained as CD45⁺. Scale bar = 50 μ m. This figure shows representative staining of lesions in the spinal cord of a female C57BL6/J mouse during EAE and highlights how pathology can be different across different spinal cord sections. [Please click here to view a larger version of this figure.](#)

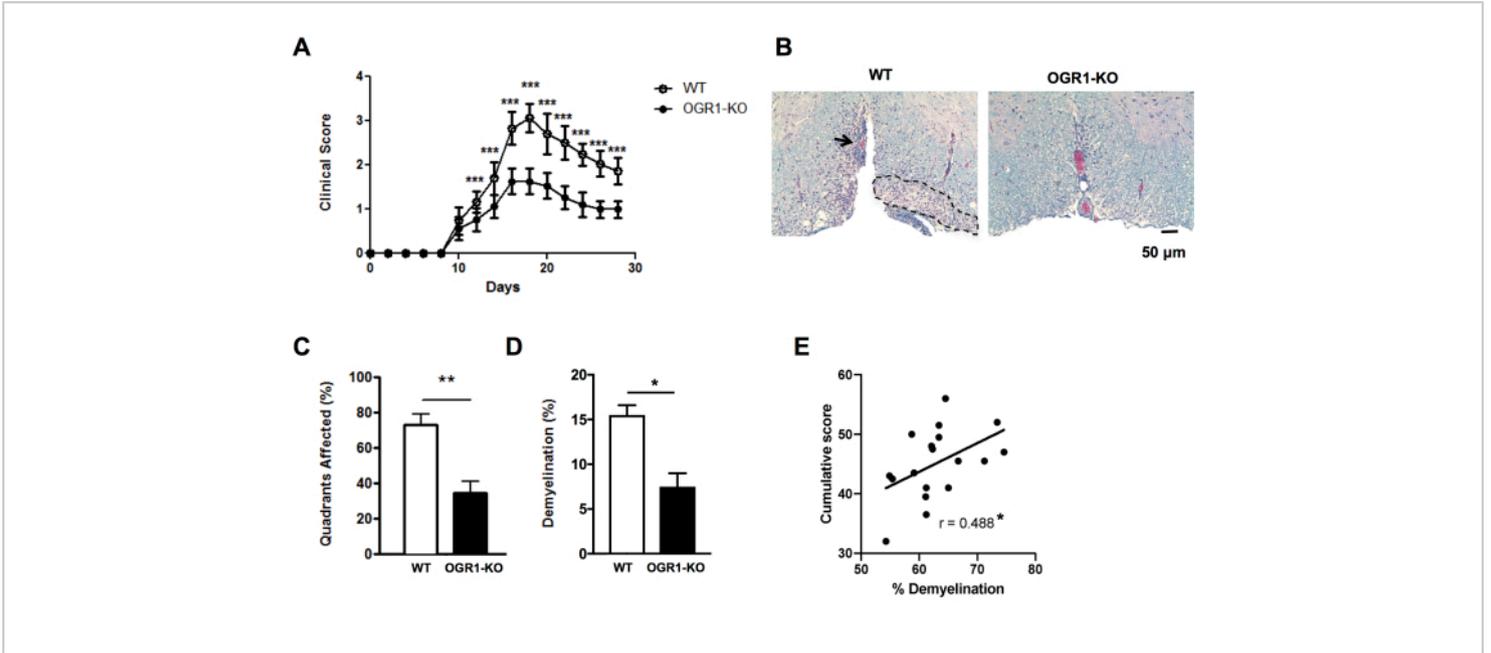


Figure 4: Application of scoring for lesions and percent demyelination in EAE. Shown is an example of an EAE experiment where female mice deficient in Ovarian cancer G-protein coupled receptor 1 (OGR1) gene on the C57BL6/J background developed less severe clinical EAE than wildtype (WT) female C57BL6/J mice. EAE was induced by immunization with MOG p35-55/CFA plus PTX and mice were scored according to the following clinical scale: 1 = tail paralysis, 2 = hindlimb and foot weakness, 3 = hindlimb paralysis, 4 = forelimb weakness, 5 = moribund. (A) Mean + SEM clinical scores of mice over time. (B) Shown is an example of LFB/H&E staining in the ventral spinal cord. Scale bar = 50 μ m. (C) Mean + SEM percent quadrants that contained demyelinating lesions. (D) Mean + SEM percent demyelination in each group. (E) shows result from another experiment in MOG p35-55-induced EAE in C57BL6/J mice where EAE scores of individual mice were summed over the 30 days of observation and were correlated with the percent demyelination in the spinal cord. Correlations were performed using a Spearman test. Panels in (A–D) are adapted from Souza C et al.²⁹. Data in (E) are original data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. [Please click here to view a larger version of this figure.](#)

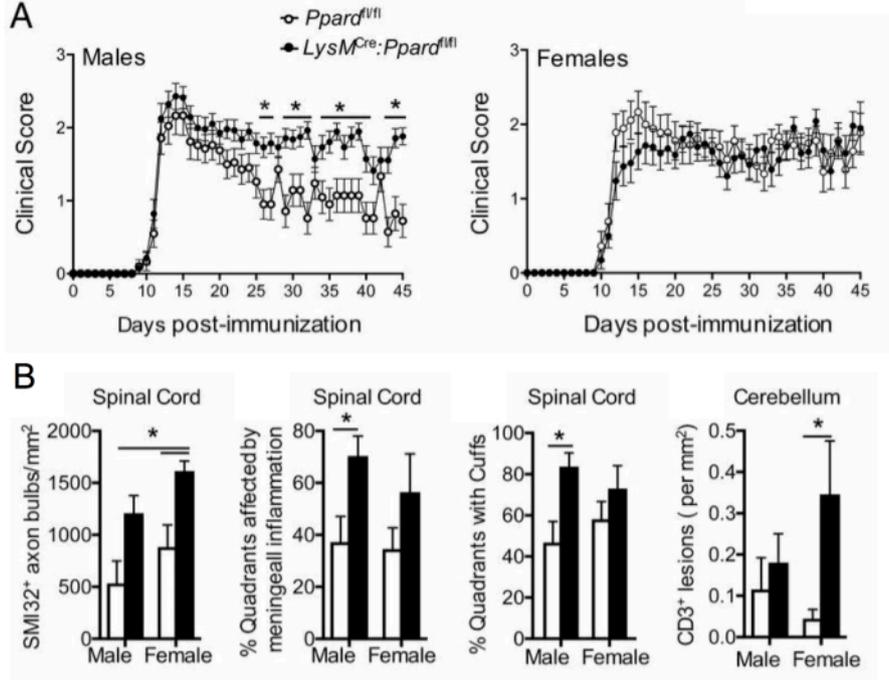


Figure 5: Application of SMI-32 staining to understand the effect of a genotype on clinical EAE phenotype. This figure shows an example of an EAE experiment where male and female wildtype (carry floxed allele of *Ppard*) and myeloid specific *Ppard* mutant mice (*LysM^{Cre}; Ppard^{fl/fl}*) on the C57BL6/J background were immunized with MOG p35-55/CFA and PTX and were followed for 45 days. **(A)** shows the mean + SEM clinical scores of mice. **(B)** shows mean + SEM results of histological scoring of the number of SMI-32⁺ axons in the spinal cord, %quadrants with submeningeal lesions, percent quadrants with perivascular cuffs, and #CD3 lesions in the cerebellum per mm² tissue. This experiment showed a genotype effect on SMI-32 staining. This figure is adapted from Drohomyrecky. et al.¹⁵. [Please click here to view a larger version of this figure.](#)

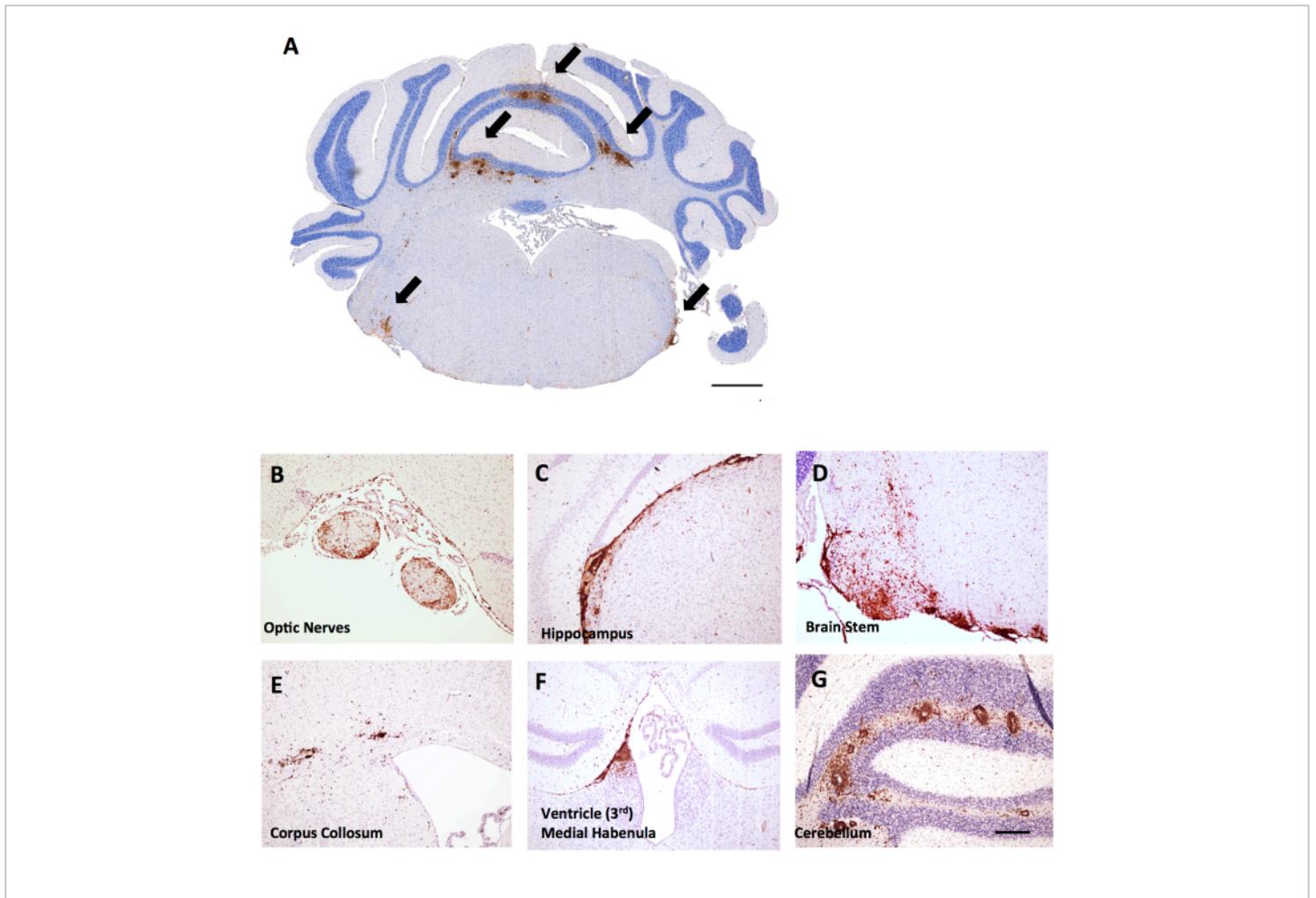


Figure 6: Examples of CD45⁺/hematoxylin staining in brain coronal sections in MOG p35-55-induced EAE in female C57BL6/J mice. CD45⁺ lesions are shown in brown. (A) CD45⁺ lesions in the brain stem of coronal sections. Scale bar = 150 μ m. (B–G) Examples of CD45⁺ lesions in the optic nerves (B), meningeal extensions under the hippocampus (C), the brain stem (D), the corpus collosum (E), the medial habenula near the ventricle (F), and the cerebellum (G). Scale bar: (B–G) = 50 μ m. [Please click here to view a larger version of this figure.](#)

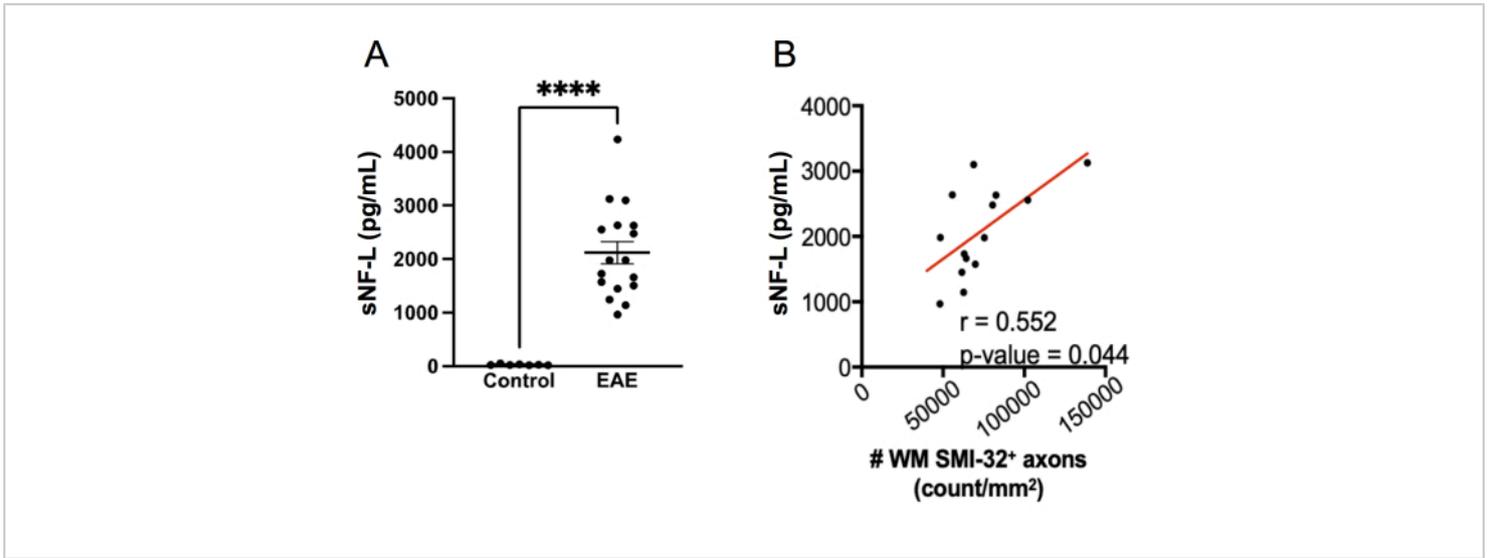


Figure 7: sNF-L levels in serum in MOG p35-55-induced EAE. (A) Serum NFL levels collected from female control and EAE mice at end-point of one experiment. Data was analysed using a two-tailed Mann Whitney test. (**** p value < 0.0001). (B) Spinal cord sections were harvested at end-point and stained with SMI-32. The number of positive cells per white matter tissue area was determined and correlated with serum NF-L at endpoint using a Spearman test. [Please click here to view a larger version of this figure.](#)

Supplementary Table 1: Description of baths used in tissue processing. Cassettes are automatically moved through these series of baths using an automated processor. [Please click here to download this File.](#)

Supplementary Table 2: Steps in Luxol Fast Blue and Hematoxylin and Eosin staining. This table outlines the order of steps in the Luxol Fast Blue and Hematoxylin and Eosin staining protocol. [Please click here to download this File.](#)

Supplementary Table 3: Antibodies used for immunohistochemical staining. Described are the antibodies that are used in this protocol as well as those that can be used to further explore inflammation, microgliosis, and astrogliosis. [Please click here to download this File.](#)

Supplementary Table 4: How to convert .czi to TIFF files. Note that it is optimal to use a high-resolution image, but medium-resolution images can be saved instead if the working memory of the computer is limiting. It is imperative to use images of the same resolution across analyses. Also, note that the last image of the series is the slide label. Avoid reading the label to ensure that the analysis is blinded.^{30,31} [Please click here to download this File.](#)

Discussion

Histological staining of the spinal cord is an important tool in assessing EAE disease severity, particularly in instances where there are differences between treatment groups in the extent of disease recovery in the post-acute phase of disease. Staining for immune cell infiltration (CD45), myelin (LFB) and axonal injury (SMI-32) helps characterize the underlying cause of the altered clinical scores in mice. The histological

staining protocol described here provides a perspective of inflammation as well as the extent of myelin and axonal injury. Furthermore, the results shown validate sNF-L measurement as a method to assess the extent of overall neuronal damage in EAE.

The critical parameters for this analysis are to ensure that investigators are blinded to the identity of the sections and that there is equivalent sampling at each level of the spinal cord across the different mice. This is because the severity of inflammation can be greater at lower levels of the cord. Another critical parameter is the size of the experimental groups. Spinal cords and brains are typically harvested from 6–8 mice per group at endpoint to see significant differences between groups with treatments or genotypes having modest effect sizes. It is also important to ensure that selected mice, when averaged, have representative mean scores of the entire group. Regarding trouble shooting, a common problem encountered by those who are inexperienced with the protocol is that the spinal cord is fixed for an insufficient length of time and it is not easily extruded from the spinal column. If this is the case, the spinal cord can be manually dissected from the column by clipping along the spinous processes using fine scissors and opening the column to reveal the spinal cord. Alternatively, tissues can be fixed for a few additional days without interfering with the success of antibody staining. The antibody clones that are described here work in tissue fixed up to 2 weeks in formalin.

Embedding the spinal cord pieces requires skill and practice. It is recommended that eye loupes be worn and a lamp be directed over the embedding station to better visualize whether the sections are falling in cross-section or in longitudinal section. Keeping the lengths of the spinal cord pieces at less than 2 mm during grossing will help them fall

in cross-section. Another common problem encountered for less-experienced users is that the LFB evaporates during the overnight incubation, leaving half of the slide stained and half unstained. To avoid evaporation, the glass staining dish should be sealed with thermoplastic film and then plastic wrap. If evaporation occurs and sections are unevenly stained, it is recommended to completely de-blue the slides with lithium carbonate and re-stain them again in LFB overnight. Another common issue is that users do not fully de-blue the grey matter after LFB. It is critical to examine individual sections under the microscope to ensure that a sufficient amount of de-bluing has been reached before proceeding with other steps in the protocol. In addition, though the CD45 and SMI-32 IHC stains perform robustly, it is still important to trouble shoot antibody concentrations in preliminary experiments for each new antibody lot received. This can be done by testing a variety of concentrations of the antibody on a positive control section (EAE spinal cord). First-time staining should also include a negative control that consists of secondary antibody alone without primary antibody added. Finally, it is critical in the image analysis to threshold individual images as staining can be uneven across slides or sections.

This protocol uses freely available software. If one does not have access to a processor, an embedder, or microtome, these steps can be sourced to a hospital-based pathology core that offers these services. Also, if one does not have access to a slide scanner, one can use a light microscope that is fitted with a video camera to save TIFF images of the spinal cord or brain regions. For a microscope-based workflow, capture LFB or LFB/H&E sections at low power (40x magnification) and for CD45 and SMI-32 staining, image at least four windows that are centered in the ventral, dorsal, and lateral parts of the spinal cord (200x magnification for CD45

and 400x magnification for SMI-32). Image analysis can be performed on these images to quantify staining in a similar manner as described.

The decision of what histological approach to take to score EAE is dependent on how much the clinical scores differ between groups. For example, if there are drastic differences in EAE clinical score (one group got EAE and one did not), this usually relates to differences in peripheral-mediated inflammation. In this case, scoring for the presence of demyelinating lesions on LFB/H&E-stained sections is sufficient and will reveal differences between groups. If groups are more similar in clinical score at onset and there are instead differences in the extent of clinical recovery (e.g. experiment in **Figure 5A**), it is best to apply the full histological workflow that is outlined here, including scoring of brain inflammation in the brain stem and cerebellum, to distinguish whether the differences the disease chronicity relate to differences in inflammation or tissue damage. If differences in inflammation are found as assessed by CD45 counting, further IHC studies can be done to stain for T cells (anti-CD3), infiltrating monocyte/macrophages (Mac3) and microglia (Iba-1/TMEM119) (recommended antibody clones are in **Supplementary Table 3**). Microglia activation is reflected by an increase in the intensity of Iba-1 staining on double-labeled Iba-1⁺TMEM-19⁺ microglia and an increased retraction of microglia processes that can be assessed by Sholl analysis on sections³². Furthermore, techniques like flow cytometry or single cell RNA sequencing can be applied to conduct a deeper characterization of the frequency and phenotype of immune populations in the brain and spinal cord.

The counting of SMI-32⁺ axons is a sensitive method to detect axon injury in EAE^{32,33} and in MS³⁴. SMI-32, which detects the non-phosphorylated form of neurofilament heavy

or medium accumulates in end-bulbs of transected neurons. An alternative to detect injured axons is to stain with amyloid precursor protein (APP) that can accumulate in axons as a result of disrupted axon transport³³. The pattern of staining for SMI-32 and APP though both both reflective of axon injury, do not typically overlap, indicating they they are detecting different pathologies³³. One can also complement histological measures of axon injury by measuring sNF-L, which is a rapid and sensitive measure of ongoing axonal injury in both the spinal cord and the brain. It offers the advantage that it can be done in half a day in living mice. A drawback of this method is that the kits are expensive and the machine is highly specialized. The company that sells the sNF-L kit does offer a fee for service for those who do not have access to a SIMOA machine. An alternative to assessing axon injury is to score for axon loss by either counting axons in toluidine blue-stained sections of the spinal cord¹² or counting neurofilament bundles detected by SMI-31 in areas of the spinal cord white matter³². Both of these are more laborious approaches than SMI-32 or sNF-L measurement.

If EAE clinical scores differ between groups, but scoring for inflammation, demyelination and axonal injury does not reveal differences between groups, it may be useful to stain for astrocyte activation using GFAP (see **Supplementary Table 3** for recommended antibody clone). Astrocyte activation is associated with an increase in GFAP staining and this has been shown to correlate with EAE progression in some EAE models including chronic EAE in the DA rat³⁵.

In conclusion, this protocol describes methods and provides an analysis workflow to conduct histological scoring of EAE.

Disclosures

Shannon Dunn consults for FSD Lucid Psycheceuticals.

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