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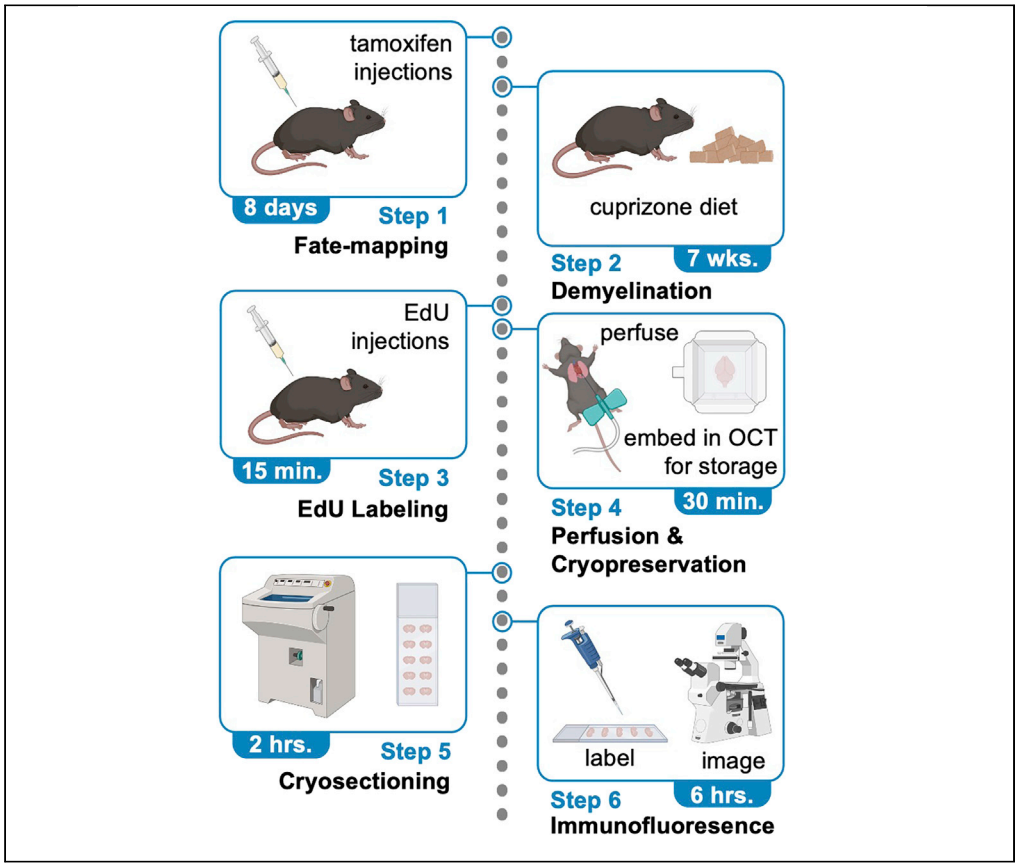
Immunofluorescence assay for demyelination, remyelination, and proliferation in an acute cuprizone mouse model



Here, we present a protocol to assess demyelination in the corpus callosum of an acute cuprizone mouse model, which is routinely used to induce demyelination for studying myelin regeneration in the rodent brain. We describe the tracing of neural stem cells via intraperitoneal injection of tamoxifen into adult Gli1CreERT2;Ai9 mice and the induction of demyelination with cuprizone diet. We also detail EdU administration, cryosectioning of the mouse brain, EdU labeling, and immunofluorescence staining to examine proliferation and myelination.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Administering tamoxifen for lineage tracing and EdU for labeling proliferating cells

Inducing demyelination in adult mice with cuprizone diet

Harvesting and cryosectioning the brain

Detecting proliferation with EdU labeling and demyelination with immunofluorescence

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Immunofluorescence assay for demyelination, remyelination, and proliferation in an acute cuprizone mouse model

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SUMMARY

Here, we present a protocol to assess demyelination in the corpus callosum of an acute cuprizone mouse model, which is routinely used to induce demyelination for studying myelin regeneration in the rodent brain. We describe the tracing of neural stem cells via intraperitoneal injection of tamoxifen into adult Gli1CreERT2;Ai9 mice and the induction of demyelination with cuprizone diet. We also detail EdU administration, cryosectioning of the mouse brain, EdU labeling, and immunofluorescence staining to examine proliferation and myelination. For complete details on the use and execution of this protocol, please refer to Radecki et al. (2020).[1]

BEFORE YOU BEGIN

Breed the Gli1$^{CreERT2/CreERT2}$ mice with Ai9$^{Fx/Fx}$ reporter mice to generate Gli1$^{CreERT2/WT}$;Ai9$^{Fx/WT}$ (Gli1^{CreERT2};Ai9) mice. In these mice, intraperitoneal administration of Tamoxifen to 8–12 week old male or female mice permanently labels the Gli1 expressing cells with tdTomato. The Gli1^{CreERT2} mice have CreERT2 knocked into the Gli1 locus such that the homozygous mice lack all Gli1 expression but are viable and fertile. In the Ai9 mice, a CAG promoter driving TdTomato fluorescent protein is inserted into the Gt(ROSA)26Sor locus. A *loxP*-flanked stop cassette prevents transcription of TdTomato in the absence of Tamoxifen. This protocol describes the steps for inducing demyelination with cuprizone diet, and harvesting the brain after intracardiac perfusion with paraformaldehyde, followed by cryosectioning of the brains for immunofluorescent staining to detect demyelination and remyelination, and EdU labeling to detect proliferation.

Institutional permissions

The use of mice in this protocol was approved and performed according to the regulations of the University of Wisconsin-Madison's Institutional Animal Care and Use Committee. Before beginning, approval must be obtained from each researcher's own institution.

Preparation for cuprizone diet and tamoxifen and EdU injections

Timing: 2–4 weeks

1. Order 0.2% Cuprizone diet in non-irradiated pelleted form, packaged in 1 kg vacuum sealed bags, at least 2 weeks prior to starting the experiment to ensure sufficient time for delivery.

Note: Store the unopened Cuprizone diet bags at −20°C until the expiration date on the package, which is usually 6 months from the manufacturing date. Refrigerate the opened bags at 4°C, and use within 4 weeks.

2. Prepare 20 mg/mL Tamoxifen solution.
   a. Dissolve 100 mg of Tamoxifen into 5 mL corn oil in a 50 mL conical tube.
   b. Shake for 4–6 h at 250 rpm in a 37°C incubator until the solution is clear and all the particles have dissolved.

Note: Keeping the solution at 37°C for extended periods may degrade the drug.

⚠ CRITICAL: Store Tamoxifen at −20°C in the powder form until the expiration date on the vial. After dissolving in corn oil, store the Tamoxifen solution at 4°C for a maximum period of four weeks.

3. Prepare 25 mg/mL EdU (5-ethynyl-2′-deoxyuridine) stock solution.
   a. Dissolve 250 mg EdU in 10 mL DMSO by vigorous mixing on a vortex mixer.
   b. Make 1 mL aliquots in microfuge tubes and store at −80°C until the manufacturer's expiration date.

Note: Store EdU at −80°C in the powdered form until the expiration date on the vial.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse IgG2b anti-APC (CC-1), (1:100)	Thermo Fisher Scientific	Cat# OP80, RRID: AB_2057371
Rabbit anti-NG2, (1:200)	Sigma-Aldrich	Cat# AB5320, RRID: AB_2314937
Rat anti-RFP, (1:1000)	ChromoTek	Cat# 5F8-100, RRID: AB_2336064
Mouse IgG2b anti-MBP (SMI99), (1:100)	BioLegend	Cat# 808402, RRID: AB_2564742
Alexafluor Goat anti-Rabbit 568, (1:1000)	Thermo Fisher Scientific	Cat# A11011, RRID: AB_143157
Alexafluor Goat anti-mouse IgG2b 488, (1:1000)	Thermo Fisher Scientific	Cat# A21141, RRID: AB_2535778
Alexafluor Goat anti-rat 568, (1:1000)	Thermo Fisher Scientific	Cat# A48262, RRID: AB_2896330
Chemicals, peptides, and recombinant proteins		
32% paraformaldehyde solution	EMS	Cat# 15714-S
FatalPlus (390 mg/mL pentobarbitol sodium solution) euthanasia	Vortech Pharmaceuticals	V.P.L. 9373 N.D.C. No. 0298-9373-68
Tamoxifen	Cayman Chemical	Cat# 13258
Corn oil	Sigma-Aldrich	Cat# C8267-500mL
Goat serum	Thermo Fisher Scientific	Cat# 10000C
EdU (5-ethynyl-2′-deoxyuridine)	Cayman Chemical	Cat# 20518
10× phosphate buffered aaline (PBS)	IBI Scientific	Cat# IB70165
Bovine serum albumin (BSA)	Thermo Fisher Scientific	Cat# BP9703-100
Triton X-100	Thermo Fisher Scientific	Cat# 85111
Sucrose	Dot Scientific	Cat# DSS24060
Hoechst 33258	Thermo Fisher Scientific	Cat# H3569
Fluoromount-G	Southern Biotech	Cat# 0100-01
2016, Teklad Global 16% Protein Rodent Diet with 0.2% Cuprizone [bis(cyclohexane)oxaldihydrazone]	Envigo	TD.140800
Covergrip Coverslip Sealant	Biotium	Cat# 23005
Critical commercial assays		
Click-iT Plus EdU Alexa Fluor 647 Imaging Kit	Thermo Fisher Scientific	Cat# C10640

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Gli1^{CreERT2} Mouse: strain-C57bl/6J, genotype- *Gli1*$^{CreERT2/+}$, sex- Male or Female, Age- 8 to 10 weeks	The Jackson Laboratory	Stock# 007913, *Gli1*$^{tm3(cre/ERT2)Alj}$/J
Ai9^{FX} Mouse: strain-C57bl/6J, genotype- Ai9$^{FX/FX}$, sex- Male or Female, Age- 8 to 10 weeks	The Jackson Laboratory	Stock# 007909, *ROSA26Sor*$^{tm9(CAG-tdTomato)Hze}$
Software and algorithms		
GraphPad Prism	Dotmatics	https://www.graphpad.com/scientific-software/prism/
NIH ImageJ Fiji	Schindelin et al.[2]	https://imagej.net/software/fiji/
Other		
Gilson MINIPULS 3 peristaltic pump	Gilson	Cat# F155001
Vortex mixer	Gilson	Cat# 36110740
1 oz feeding jar set (individual mouse) (alternate for powdered diet)	Dyets Inc	Cat# 910019
Cuprizone (alternate for powdered diet)	Sigma-Aldrich	Cat# C9012-25G
50 mL conical polypropylene tubes	Celltreat	Cat# 229421
Cork board for dissection	VWR	Cat# 100498-390
Scalp vein set butterfly 27G	VWR	Cat# MSPP-EXL-26709
Nalgene 3-way Stopcock	Thermo Fisher Scientific	Cat# 6470-0002
Std pattern tissue forceps	Braintree Scientific	Cat# FC1 10
Supercut iris scissors, straight	Braintree Scientific	Cat# SCT-I 528
SuperCut surgical scissors, curved	Braintree Scientific	Cat# SCT-S 511
Std pattern dressing forceps	Braintree Scientific	Cat# FC100 2
Dumont forceps	Braintree Scientific	Cat# FC-5043
Flat blade membrane forceps	Thermo Fisher Scientific	Cat# 09-753-50
Fluoromount-G	Southern Biotech	Cat# 0100-01
Superfrost Plus Microscope Slides	Thermo Fisher Scientific	Cat# 12-550-15
Nutating mixer	Midwest Scientific	Cat# NS-10A
Tissue-Tek OCT media	Sakura	Cat# 4583
Peel-A-Way Embedding Molds (Square-S22)	Polysciences, Inc.	Cat# 1864A-1
Kimwipes disposable wipes	Millipore Sigma	Cat# Z188956
Tissue-Tek Accu-Edge Low Profile blades (tissue slicer blades)	Sakura	Cat# 4685
Cryostat	Leica	Cat# CM3050 S
Golden nylon brushes for cryo microtomy	Ted Pella	Cat# 11842A
A-PAP pen	Ted Pella	Cat# 22309
Gauze sponges	Thermo Fisher	Cat# 22-362178
Tuberculin syringe with 28G needle	BD Biosciences	Cat# 329461
22 × 50 mm glass coverslip	Corning	Cat# 2975-225
Precision glide needles 16G	BD Biosciences	Cat# 305198
Precision glide needles 27G	BD Biosciences	Cat# 305109
Fluorescent ,icroscope	Keyence	Cat# BZ-X710
Confocal microscope	Leica	Cat# SP8

MATERIALS AND EQUIPMENT

20 mg/mL tamoxifen solution

Reagent	Final concentration	Amount
Tamoxifen	20 mg/mL	100 mg
Corn oil	N/A	5 mL
Total	**N/A**	**5 mL**

Note: Store Tamoxifen solution at 4°C for up to four weeks.

⚠ CRITICAL: Tamoxifen is a carcinogen and teratogen. Weigh the tamoxifen powder inside a fume hood. Use appropriate PPE consisting of nitrile gloves, surgical mask, safety goggles and lab coat while handling tamoxifen.

4% paraformaldehyde solution		
Reagent	Final concentration	Amount
32% paraformaldehyde (PFA)	4%	4.4 mL
10× PBS	1× PBS	3.5 mL
Distilled water	N/A	27.1 mL
Total	**N/A**	**35 mL**

Note: Prepare fresh for each set of perfusions or store at 4°C and use within 24 h.

⚠ CRITICAL: PFA is a suspected carcinogen and an irritant. Perform all perfusions in a fume hood and use appropriate PPE consisting of nitrile gloves, surgical mask, safety goggles and lab coat while handling PFA.

30% sucrose solution		
Reagent	Final concentration	Amount
sucrose	30%	30 g
10× PBS	1×	10 mL
Distilled water	N/A	90 mL
Total	**N/A**	**100 mL**

Note: Store sucrose solution at 4°C for up to 6 months. However, discard the solution if cotton ball like fungal growth is observed before 6 months.

BSA-Triton X-100 solution		
Reagent	Final concentration	Amount
Bovine Serum Albumin	0.1%	0.1 g
Triton X-100	0.25%	25 µL
10× PBS	1×	1 mL
Distilled water	N/A	8.975 mL
Total	**N/A**	**10 mL**

Note: Prepare fresh and keep on ice or store at 4°C and use within 24 h.

Blocking buffer		
Reagent	Final concentration	Amount
Normal goat serum	10%	50 µL
BSA-Triton X-100 solution	N/A	450 µL
Total	**N/A**	**500 µL**

Note: Prepare fresh and keep on ice or store at 4°C to be used within 24 h.

PBS-Triton X-100 solution		
Reagent	Final concentration	Amount
10% Triton X-100	0.5%	500 μL
10× PBS	1×	1 mL
Distilled water	N/A	8.5 mL
Total	**N/A**	**10 mL**

Note: Store at room temperature for up to two months.

3% BSA-PBS solution		
Reagent	Final concentration	Amount
Bovine Serum Albumin	3%	0.3 g
10× PBS	1×	1 mL
Distilled water	N/A	9 mL
Total	**N/A**	**10 mL**

Note: Make fresh and keep on ice or store at 4°C and use within 24 h.

EdU stock solution		
Reagent	Final concentration	Amount
EdU	25 mg/mL	250 mg
DMSO	N/A	10 mL
Total	**N/A**	**10 mL**

Note: Make 1 mL aliquots and store at −80°C for a year. Avoid repeated freeze thaw cycles.

EdU working solution		
Reagent	Final concentration	Amount
EdU stock solution (25 mg/mL)	2.5 mg/mL	1 mL
10× PBS	1×	1 mL
Distilled water	N/A	8 mL
Total	**N/A**	**10 mL**

Note: Prepare fresh and use within 2 h at room temperature.

Alternatives: Substitute PBS with Hanks Balanced Salt Solution (HBSS) or 0.9% Saline.

1× Click-iT EdU reaction buffer		
Reagent	Final concentration	Amount
Component D in Click-iT EdU imaging kit	1×	4 mL
Deionized water	N/A	36 mL
Total	**N/A**	40 mL

Note: Store at 4°C for 6 months.

10× Click-iT EdU buffer additive		
Reagent	Final concentration	Amount
Component F in Click-iT EdU imaging kit	10×	400 mg
Deionized water	N/A	2 mL
Total	N/A	2 mL

Note: Store at ≤20°C for one year. Discard if the solution develops a brown color.

1× Click-iT EdU buffer additive		
Reagent	Final concentration	Amount
10× Click-iT EdU buffer additive	1×	5 μL
Deionized water	N/A	45 μL
Total	N/A	50 μL

Note: Prepare fresh and keep on ice or store at 4°C and use within 24 h.

Click-iT Plus reaction cocktail (add the reagents in the order below for one slide):		
Reagent	Final concentration	Amount
1× Click-iT EdU reaction buffer	N/A	440 μL
copper protectant (component E)	N/A	10 μL
Alexa Fluor picolyl azide (component B)	N/A	1.2 μL
1× Click-iT EdU buffer additive	0.1×	50 μL
Total	N/A	501.2 μL

Note: Prepare fresh and use within 15 min at room temperature (20°C–25°C).

STEP-BY-STEP METHOD DETAILS

Administration of cuprizone, tamoxifen, and EdU

Timing: 7 weeks

In this step, the Gli1 expressing cells are fate-mapped by intraperitoneal administration of Tamoxifen to Gli1CreERT2;Ai9 mice and demyelination is induced 1 week later by feeding 0.2% cuprizone diet for 5 weeks. In addition, the proliferating cells are labeled by intraperitoneal administration of EdU a day prior to euthanasia.

1. Inject 5 mg tamoxifen per day intraperitoneally on alternate days for 4 injections (total 20 mg/mouse).
   a. Fill the 1 mL tuberculin syringe with Tamoxifen and wipe the tip of the filled syringe with a kimwipe, before attaching a 27G needle to the syringe.
   b. Administer 250 μL/day (5 mg/day) of 20 mg/mL Tamoxifen by intraperitoneal (i.p.) injection to each mouse, on alternate days, for a total of 4 i.p. injections.
      i. Scruff the mouse and insert the needle into the lower left or right quadrant of the abdomen, perpendicular to the surface of the skin.
      ii. After insertion, aspirate to check for the presence of fluid prior to injecting the drug.
      iii. Alternate between the left and right side for i.p. injections.

⚠ CRITICAL: Ideally there should not be any fluid aspirate before injecting the drug. If a blood vessel is punctured by the needle, the aspirated fluid will be red blood and if the urinary bladder is punctured by the needle, the aspirated fluid will be clear or yellow urine. Injecting Tamoxifen into the bladder can lead to urinary tract inflammation and death if not treated promptly. In case fluid is aspirated, remove and discard the needle properly. Using a clean syringe, perform the i.p. injection again at a different site. If the bladder is punctured, watch for turbid white discharge from the urethra for about 2 days, which indicates a urinary tract infection.

Note: The typical dose of Tamoxifen is 100 mg/kg live weight. However, we examined the recombination efficiency with i.p. injections of 1–6 mg/day tamoxifen on 4 alternate days and found that 20 mg Tamoxifen ensures recombination of the floxed allele in adult mice of C57bl/6 and swiss webster background strains with weights ranging from 20 g to 60 g, without any toxic effects on neural stem cells. We have also used this dosage in multiple CreER and floxed mouse lines.[1,3,4]

2. One week after the last Tamoxifen injection, change the diet to 0.2% cuprizone pellets.
   a. Transfer the mice to a clean cage before starting cuprizone diet to prevent the mice from eating any leftover regular chow buried in the bedding.
   b. Provide the cuprizone diet and water ad libitum for 5 weeks.
      i. Discard the pellets in the cage, every 2–3 days and replenish with fresh cuprizone pellets, since the drug degrades gradually with time.

⚠ CRITICAL: Feeding 3 g per mouse per day is sufficient to avoid discarding excessive amounts of food.

   c. After 5 weeks of cuprizone diet, change the diet to regular chow to allow for remyelination.

Note: Analyze remyelination from 2–10 weeks after cessation of cuprizone diet.

Note: Store the vacuum sealed bags of Cuprizone pellets at −20°C. Refrigerate the opened bag at 4°C, away from light and use within 4 weeks.

Alternatives: Instead of the pellets, provide 0.2% Cuprizone diet in the powdered form by mixing 0.2 g of cuprizone with 99.8 g of powdered chow. For the healthy control mice, feed regular chow in the powdered form. Place the powdered diet in a feeding jar on the floor of the cage and change the diet every day to minimize consumption of food contaminated with urine and feces.

3. Administer EdU by i.p. injection, 24 h prior to euthanasia.
   a. Prepare working solution of EdU (2.5 mg/mL).
   b. Administer 20 µL of 2.5 mg/mL working solution per gram body weight of mouse, or 50 mg/kg body weight, by a single i.p. injection following the precautions mentioned in step 1.

Intracardiac perfusion and harvesting the brain

Timing: 30 min per mouse

In this step, Gli1CreERT2;Ai9 mice are perfused by intracardiac administration of 4% Paraformaldehyde (PFA) and their brains are cryopreserved by embedding in OCT.

4. Set up the peristaltic pump.
   a. Connect the scalp vein set tubing to the outlet tube of the peristaltic pump.

b. Insert one tube of the pump into a container with 1× PBS (30 mL/mouse) and another tube into a container with 4% PFA solution (30 mL/mouse).
c. Attach the tubing from the two containers to a 3-way stopcock.
d. Set the pump speed at 8 mL/min and fill the main outlet line with 1× PBS until there is a steady stream without bubbles from the needle.
e. Turn off the peristaltic pump and turn the stopcock to allow the flow of 4% PFA.
f. Pump 4% PFA until the fluid has reached the stopcock but not entered the main line.
g. Turn off the peristaltic pump and turn the stopcock to allow the flow of 1× PBS.

5. Anesthetize the mouse.
   a. Administer 120 mg/kg of Fatal Plus (390 mg/mL Pentobarbitol sodium), via i.p. injection using a tuberculin syringe with 27G needle.

⚠ CRITICAL: Fatal-Plus is a controlled substance. A DEA license is required to purchase it.

   b. Lay the mouse on its back on a cork board or a flat piece of styrofoam placed in a dissecting tray, after the mouse is completely unresponsive to painful stimuli, such as a sharp toe pinch, but the heart is still beating.
   c. Pin all four limbs of the mouse to the cork board using dissection pins or needles.
6. Expose the heart.
   a. Incise the skin over the abdomen and chest with a large forcep (std. pattern tissue forceps) and dissecting scissors (supercut surgical scissors).
   b. Incise the diaphragm with small dissecting scissors (supercut iris scissor), being careful not to puncture the beating heart.
   c. Cut the ribcage on both sides of the sternum with a small scissor (supercut iris scissor).
   d. Lift the sternum with a forcep (std. pattern dressing forceps) to expose the heart.
   e. Pin the xiphoid process of the overturned sternum to the dissection board with a needle.
7. Intracardiac perfusion.
   a. Gently lift off the white membrane like thymus covering the upper part of the heart with a forcep (std. pattern dressing forceps).
   b. Make a nick in the right auricle which is the dark appendage attached to the right atrium, using the tip of small pointed scissors (supercut iris scissor) (Figure 1A).

Note: This allows the blood and perfusion fluids to drain into the dissection tray.

   c. Insert the butterfly needle, connected to the main line of the peristaltic pump, into the apex of the heart, which is formed by the left ventricle (Figure 1A).
   d. Turn on the peristaltic pump and perfuse with 30 mL of 1× PBS at approximately 8 mL/min.
   e. Switch the stopcock to perfuse with 30 mL of 4% PFA at the same rate.

Note: Curling and stiffening of the tail is the earliest indication of a good perfusion. Subsequently the liver becomes pale and the body stiffens when perfused well. (See troubleshooting Problem 1) Ideally, the lungs should not inflate and fluid should not ooze out of the nose or mouth during perfusion. (See troubleshooting Problem 2).

⚠ CRITICAL: If bubbles appear in the main line, remove the needle from the heart to expel the bubble and reinsert it into the heart.

8. Collect the brain.
   a. Remove the needle from the heart and the pins holding the mouse to the dissection board.
   b. Cut the head at the base of the skull with large scissors (supercut surgical scissors) (Figure 1Bi).

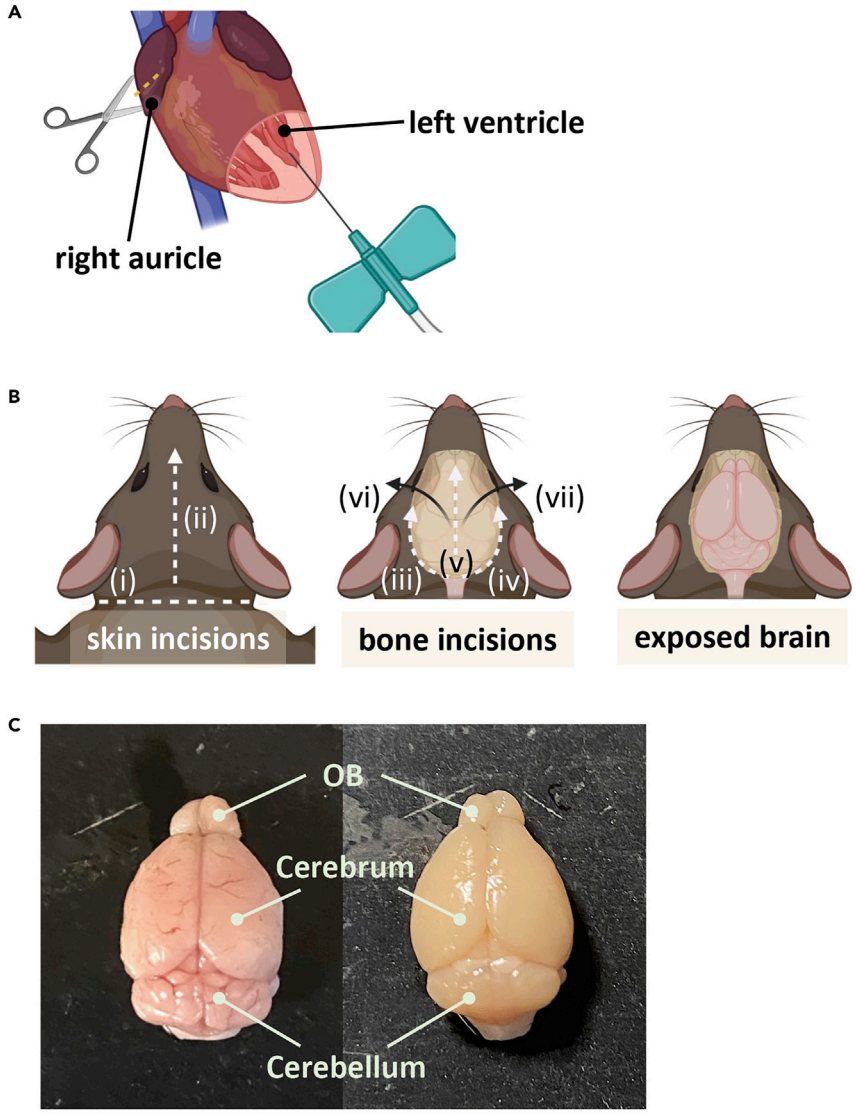

Figure 1. Perfusion and brain harvest

(A) Illustration of the incision point for creating an outlet for the blood and perfusion fluids, and the insertion point for the butterfly needle, demonstrating the position of the needle in the left ventricle.

(B) Illustration of mouse brain harvest. Incisions for separating the skin (i-ii), and the bone (iii-vii) to expose the brain.

(C) Examples of incompletely perfused (left) vs. completely perfused brains (right).

c. Remove the skin from the dorsal side of the head (Figure 1Bii) using a smaller pair of scissors (supercut iris scissor).

d. Insert the blade of a small scissor (supercut iris scissor) through the foramen magnum and cut the skull bone laterally up to the orbit on both sides, taking care not to nick the brain (Figure 1Biii-iv).

e. Make another midline incision through the skull bone, along the sagittal suture, anteriorly past the eyes with a small scissor (supercut iris scissor) (Figure 1Bv).

f. Carefully remove the skull bone from around the brain using a pair of thin pointed forceps (Dumont forceps), starting by gently pulling the bone laterally, away from the midline on either side (Figure 1Bvi-vii).

g. When reaching the area of the skull encasing the olfactory bulbs, it may be necessary to make a few more small snips to release the olfactory bulbs.

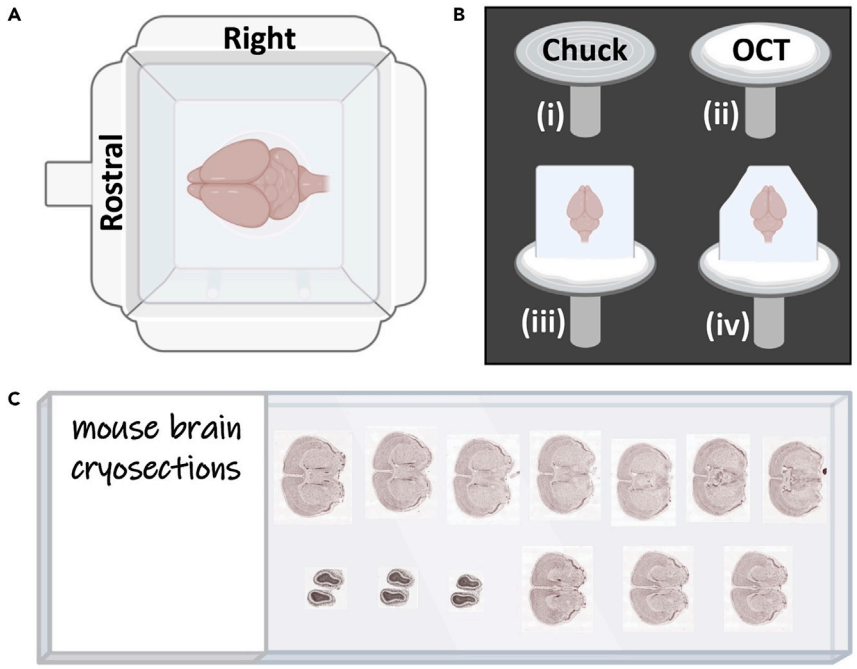

Figure 2. Cryopreservation and cryosectioning

(A) Illustration of the brain embedded in OCT in a tissue mold.

(B) Illustration of mounting the frozen tissue block for cryosectioning coronal sections. The chuck is coated with a layer of OCT (i-ii), and the frozen tissue block firmly pressed into the OCT on the chuck (iii). Excess OCT can be carefully trimmed before sectioning (iv).

(C) Illustration of olfactory bulb and forebrain sections (from Allen Brain Atlas) on a slide.

Note: Ensure that the olfactory bulbs are no longer attached to the bone before removing the brain.

h. Separate the brain along with the olfactory bulbs from the skull using a pair of flat forceps (flat blade membrane forceps), and drop it into a 15 mL conical tube containing 5 mL of 4% PFA.

Note: A well-perfused brain appears pale and does not have a pinkish or red color (Figure 1C) (See troubleshooting Problem 3).

i. Incubate the brain in 4% PFA for 4–6 h at 4°C.

9. Cryoprotect the brain.
   a. Gently remove the brain from the 4% PFA solution by emptying the tube in a petri dish.
   b. Drop the brain into a 15 mL conical tube containing 15 mL of 30% sucrose solution using flat forceps (flat blade membrane forceps).

Note: The brain should float to the top of the solution.

c. Incubate at 4°C on a nutating mixer at 20–30 rpm, overnight for 12–16 h.

Note: After incubation, the brain should be at the bottom of the tube due to increase in density.

⚠ CRITICAL: Minimize the amount of air space in the tube containing 30% sucrose to ensure that the brain remains submerged in the solution and the surface does not dry.

Pause point: Brains can be incubated in 30% sucrose solution for up to 24 h at 4°C.

10. Embed brains in OCT media and freeze.
    a. Label the tissue mold and add OCT media into the mold.
    b. Remove the brain from sucrose solution by gently emptying the tube into a petri dish.
    c. Pick the brain with flat forceps (flat blade membrane forceps) and roll it on lint free paper napkin like kimwipe to remove the excess sucrose from the surface.
    d. With minimal pressure, gently lift the brain with flat forceps (flat blade membrane forceps) and place it into the mold with the ventral surface facing the bottom of the mold (Figure 2A).

Note: Add more OCT on top if needed, to submerge the brain completely in OCT.

    e. Immediately place the mold on a level surface in the −80°C freezer for at least 24 h prior to cryosectioning.

Pause point: Brains can be stored in OCT at −80°C for years.

Cryosectioning

Timing: 3 h per brain for beginners

In this step, the brain is sectioned into 20 μM thick sections.

11. Equilibrate the temperature of the embedded frozen brain to that of the cryostat by incubating the brain at −20°C, either in a freezer or in the cryostat, at least 1 h prior to sectioning.

Pause point: The frozen brain can be kept in a −20°C freezer for 12–16 h before sectioning.

12. Place a fresh cryostat blade, a 25 mm diameter metal specimen holder or chuck, and a brush inside the cryostat, at least 30 min prior to sectioning, to equilibrate their temperature to −20°C.
13. Mount the brain to the specimen holder or chuck.
    a. Remove the plastic mold from the embedded brain by cutting the 4 corners with a razor or a used cryostat blade.
    b. Pour OCT medium on the pre-cooled chuck (Figure 2Bi) inside the cryostat; quantity should be enough to cover the surface (~500 μL) (Figure 2Bii).
    c. Immediately place the embedded brain on the OCT before it freezes (Figure 2Biii).

Note: For coronal sections, position the brain so that the caudal end is facing the chuck and the rostral end is facing you. For sagittal sections, place the brain on its side.

    d. Allow the brain to freeze on the chuck for 10–20 min before sectioning.
14. Collect 20 μM sections of the brain.
    a. Clamp the chuck firmly into the object head of the cryostat.
    b. Turn on the cryostat and trim the block until the brain tissue is visible.
    c. Trim the excess OCT from the sides of the block with a razor or a used cryostat blade (Figure 2Biv).
    d. Collect about 3–5 sections of the olfactory bulb and 8–10 sections of the forebrain, starting from the genu of the corpus callosum to the hippocampal commissure, on each slide (Figure 2C).

⚠ CRITICAL: To avoid wrinkling of the tissue section, apply a drop of PBS on the slide with a brush and immediately collect the section on the PBS.

(See troubleshooting Problem 4).

(See troubleshooting Problem 5).

Note: Use a gauze sponge dipped in PBS to remove unwanted tissue sections and OCT from the slide. To avoid detachment of the section from the slide in subsequent procedures like immunohistochemistry, prevent OCT from collecting between the tissue and the surface of the slide (Figure 2C).

15. To dry the sections, keep the slides on a flat surface at room temperature, overnight or for 12–16 h.
16. After drying, keep the slides in a slide storage box at −80°C.

Pause point: The cryosections on the slides can be stored at −80°C for years.

Immunofluorescent staining and EdU labeling

Timing: 18–22 h

In this step, EdU labeling is followed by immunofluorescence of the brain cryosections to examine proliferation and myelination respectively.

17. Equilibrate the frozen slides to room temperature for at least 15 min.
    a. Place the slides in a moist chamber prepared with a paper towel soaked in distilled water in a tray.

Note: Balance the slides on two rods or serological pipets glued/taped to the tray (Figure 3A).

    b. Draw a line with a PAP pen along the periphery of the slide to form a hydrophobic barrier for minimizing the amount of reagents used per slide.

Note: If needed, gently wipe the condensation on the edge of the slide without touching the cryosections, with a kimwipe before using the pap pen.

    c. After 15 min, wash the sections once with 500–800 µL 1× PBS for 5 min at room temperature (20°C–25°C).
    d. After the wash, remove the PBS either by tilting the slide to pour the liquid into the tray or aspirate the liquid with vacuum suction (Figure 3B).

Note: To prevent the cryosections from lifting off the slide during suctioning, attach a 200 µL pipet tip to the tip of the glass pipette attached to the suction tube for slowing the aspiration (Figure 4B).

18. EdU labeling with Click-iT Plus EdU imaging kit.

Note: EdU labelling kits are available for many wavelengths depending on the microscope filter or laser used for imaging. We have used the Click-iT Plus Alexa-647 kit. The following steps are modified from the manufacturer's directions in Life Technologies MAN0009885 MP10637 Revision A.0.

    a. Add 500 µL of 0.5% PBS-Triton X-100 solution on each slide to permeabilize the tissue.
    b. Incubate for 20 min at room temperature (20°C–25°C).
    c. Meanwhile, prepare the 1× Click-iT EdU buffer additive and use it to prepare the Click-iT Plus reaction cocktail.

d. Remove the PBS-Triton X-100 and wash the slide twice with 3% BSA-PBS solution.
e. Add 500 μL Click-iT Plus reaction cocktail to the slide and incubate for 30 min at room temperature (20°C–25°C).

Note: Protect from light by covering the transparent lid of the tray with aluminum foil.

⚠ CRITICAL: From this point onward, protect the tissue from light in all the steps.

f. Remove the Click-iT Plus reaction cocktail and wash once with 3% BSA-PBS solution for 5 min at room temperature (20°C–25°C).
g. Remove the 3% BSA-PBS solution.
h. Wash 3 times with 500–800 μL of 1× PBS for 10 min each at room temperature (20°C–25°C) before continuing with immunofluorescence.

19. Immunofluorescence.
    a. **Optional**: This step is specifically needed for immunofluorescence of Myelin Basic Protein (MBP). For all other proteins, you can start from step 19c.
        i. Fix the tissue with methanol to enhance detection of myelin proteins by adding 500 μL of cold methanol (stored at −20°C) on each slide.
        ii. Incubate at room temperature (20°C–25°C) for 15 min (See troubleshooting Problem 6).
        iii. Remove the methanol.
        iv. Wash the slide 3 times with 500–800 μL of 1× PBS per slide for 10 min/wash at room temperature (20°C–25°C).
    b. To minimize non-specific binding of the primary antibody to the tissue, add 500 μL of blocking buffer per slide.
    c. Incubate for 45 min to 1 h at room temperature (20°C–25°C).
    d. Remove the blocking solution.
    e. Add 500 μL of primary antibody per slide.

Note: Dilute the primary antibody in the BSA-Triton X100 solution to obtain the desired concentration.

f. Incubate with the primary antibody for 2 h at room temperature (20°C–25°C) or overnight at 4°C.

⏸ **Pause point:** Slides can be incubated in primary antibody for up to 24 h at 4°C.

g. Remove the primary antibody.
h. Wash 3 times with 500–800 μL of 1× PBS per slide for 10 min/wash at room temperature.
i. Remove the PBS.
j. Add 500 μL of the secondary antibody with Hoechst stain per slide.

Note: Dilute the Alexafluor secondary antibodies 1:1000 fold and the Hoechst nuclear stain 1:5000 fold in the BSA-Triton X100 solution.

k. Incubate with the secondary antibody plus Hoechst for 1 h at room temperature (20°C–25°C), protected from light.

Note: We do not recommend longer incubation periods since they can result in higher background staining.

⚠ CRITICAL: For performing immunofluorescence without EdU labelling, the slides should be protected from light from this step onwards.

l. Remove the secondary antibody.

m. Wash 3 times with 500–800 μL of 1× PBS per slide for 10 min/wash at room temperature (20°C–25°C).
n. Wash the slide once with 500–800 μL of distilled water per slide at room temperature (20°C–25°C) for 5 min.

20. Mount coverslips with Fluoromount G mounting media.
    a. Gently aspirate the distilled water from around the tissue, taking care not to aspirate the tissue.
    b. To avoid creating bubbles, dip a 500 μL pipet tip into Fluoromount G mounting media and add 4–5 drops of the medium on each slide by touching the pipet tip to the slide.
    c. Gently place the coverslip on the slide by hinging it from one end (Figure 3C).
    d. Allow Fluoromount G to solidify overnight or for 12–16 h in the dark at room temperature (20°C–25°C) before imaging.
    e. Seal the coverslips on the slides using a coverslip sealant.

EXPECTED OUTCOMES

In this protocol, cuprizone diet is fed to mice for 5 weeks, which results in acute demyelination (Figure 4A). Remyelination is observed as early as 2 weeks following cessation of cuprizone diet (Figures 4A and 4B), but complete remyelination may take about 6–10 weeks. The number of fate-mapped Gli1 cells labeled by RFP, NG2 positive oligodendrocyte progenitor cells, and CC1 positive mature oligodendrocytes, observed in the corpus callosum, are lower at peak demyelination i.e., 5 weeks of cuprizone diet, and gradually increase by 2 weeks following cessation of cuprizone diet (Figure 4B). EdU labeled proliferating cells do not show significant changes at these time points in the corpus callosum (Figure 4B). The proliferating cells in the corpus callosum are known to be a mix of oligodendrocyte progenitor cells, astrocytes and microglia.[5–7] Higher proliferation of cells following cuprizone diet, is also observed in the subventricular zone lining the lateral ventricles.[3,8] The late myelin proteins like MOG appear after 4 weeks of cuprizone withdrawal,[1] and myelinated segments of axons with mature nodes and paranodes are observed at 6–10 weeks following cessation of cuprizone diet.[3] Feeding the cuprizone diet for 12–15 weeks however, results in chronic demyelination with very little remyelination after withdrawal of cuprizone.[9,10]

LIMITATIONS

The extent of demyelination caused by cuprizone diet depends on the strain, age, and weight of the mice along with the cuprizone content and duration of the diet. 0.2% cuprizone diet produces demyelination in young adult mice of inbred strains like C57bl/6 but outbred strains like swiss webster mice need 0.4% cuprizone to produce similar levels of demyelination.[3,11] Mice older than 8 months age need higher cuprizone content in the diet to produce demyelination, compared to young adult mice of the same strain.[12–14] In addition, mice with low body weight at the start of cuprizone diet demyelinate to a greater extent than those with higher starting weights.[15] Finally, increasing the cuprizone content of the diet of young adult mice, from 0.2 to 0.5% increases the extent of demyelination with 0.5% cuprizone diet leading to status spongiosus in inbred strains, along with higher mortality.[16] In addition to mice, the cuprizone diet can be used to induce demyelination in rats[17,18] but it does not cause demyelination in non-human primates.[19]

Remarkably, different areas of the brain are demyelinated depending on the cuprizone content, duration, and strain of mice. Thus, in young adult C57bl/6 mice, 0.2% cuprizone diet for 5 weeks consistently produces complete demyelination of the corpus callosum, along with some demyelination in the cerebral cortex, basal ganglia, and cerebellar nuclei.[1,20–24] Within the corpus callosum, there are strain related differences with C57bl/6 mice showing more complete demyelination in the medial and caudal corpus callosum, CD1 mice exhibiting partial demyelination, and SJL/J mice showing extensive demyelination in the rostral and lateral areas of the callosum.[25–27] However, the spinal cord remains unaffected in both C57bl/6 and SJL/J mice.[28] In the cerebral cortex, 6 weeks of the diet causes complete demyelination in C57bl/6 mice, but partially affects the young adult

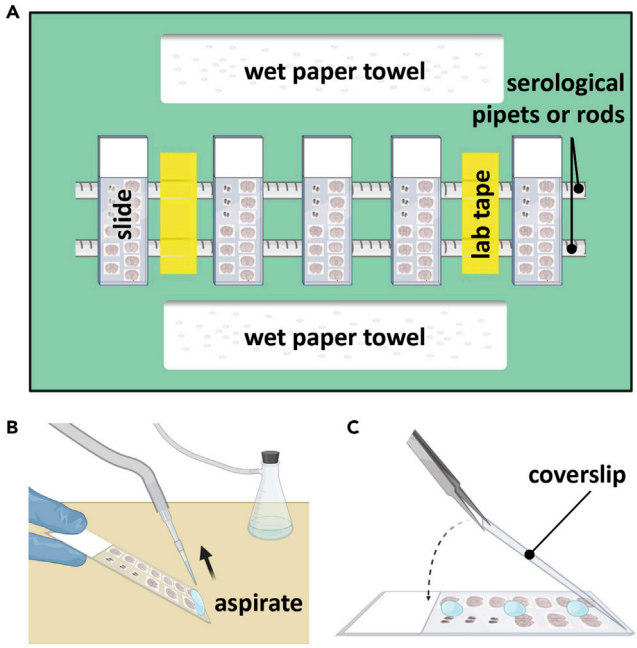

Figure 3. Immunolabeling of tissue sections

(A) Diagram of a humidity chamber for immunolabeling tissue slides.

(B) Tilting the slide for aspirating during washes, as well as using a 200 μL pipet tip on the end of the glass pipet attached to the suction tube to decrease the force of aspiration.

(C) Hinging the coverslip onto the slide during mounting.

BALB/cJ mice.[20] A 0.2% cuprizone diet for 7 weeks demyelinates the optic nerve[29] and for 6–12 weeks, results in demyelination of the hippocampus in C57bl/6 mice.[30–32] In the cerebellum, feeding 0.2% cuprizone diet for 12 weeks results in demyelination of the cortex and white matter in adult C57bl/6 mice,[33] while the superior cerebellar peduncle has been reported to be demyelinated when 0.5% cuprizone is fed to weanling mice of the outbred ICI strain.[34,35]

In the current protocol, we have reliably induced demyelination in the corpus callosum of young adult C57bl/6 mice by feeding 0.2% cuprizone diet for 5 weeks. Although we prefer to use pelleted diet, there are reports of the powdered diet being more potent at inducing demyelination, with the caveat that the control mice fed with powdered diet showed demyelination in one of the studies.[36–38] We switched to pelleted diet when the powdered diet significantly increased the mortality rate in both control and 0.2% cuprizone groups, after moving to a different facility. This was later found to be due to a change in the air circulation within the cages in the new facility, which increased the inhalation of powdered diet causing inflammation in the lungs. Hence, powdered diet should be used with caution.

TROUBLESHOOTING

Problem 1

The liver does not appear pale and the body and tail are relaxed after perfusion.

The perfusate did not flow through the systemic circulation (step 7).

Potential solution

The tip of the butterfly needle may have pierced through the interventricular septum into the right ventricle, and the perfusate may be flowing into the right ventricle. Gently pull out the needle and reposition it until the tip is in the left ventricle. The brain can be perfused well even if the perfusate flows partially through the systemic circulation i.e., from the left ventricle to the aorta.

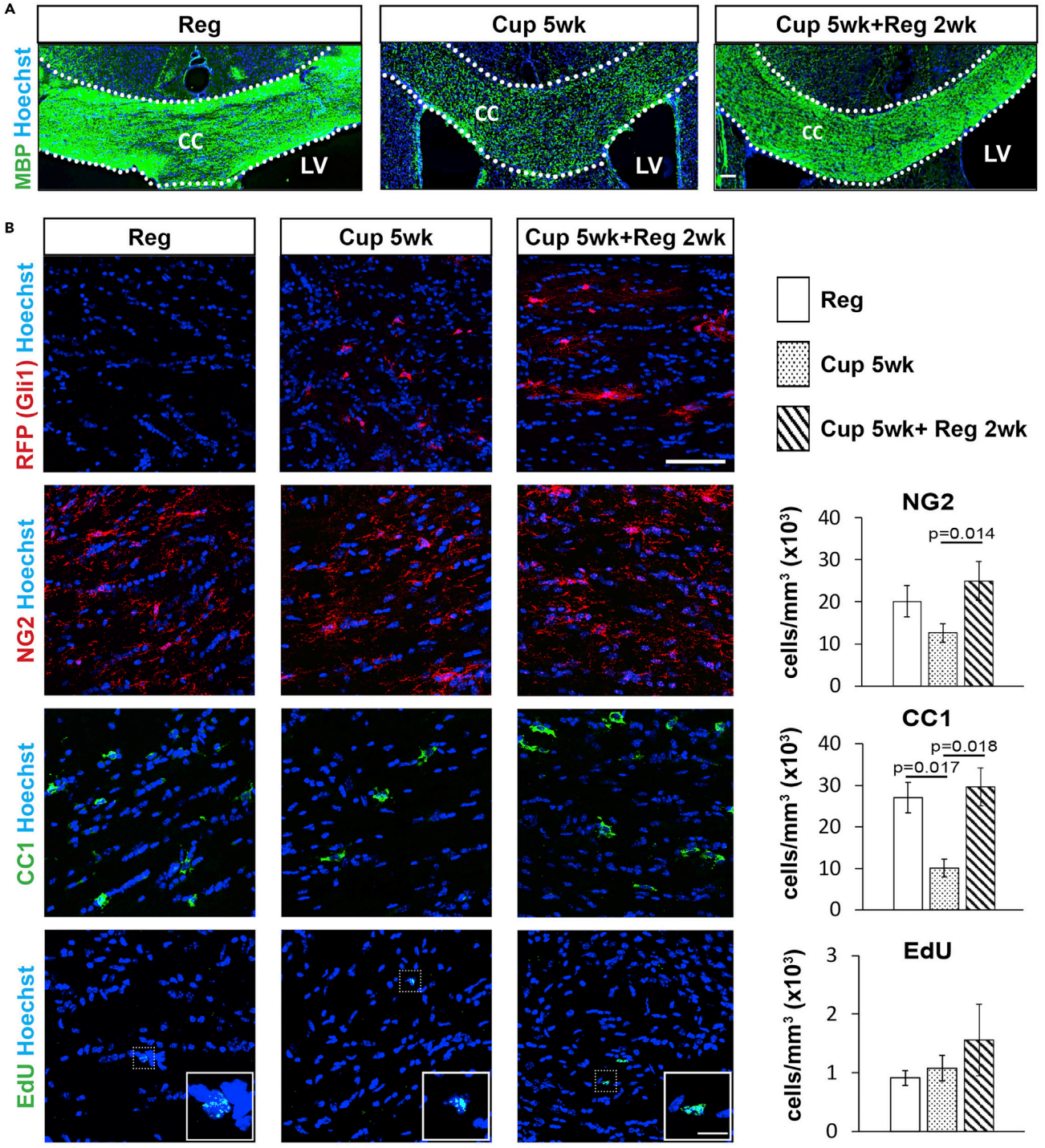

Figure 4. Immunofluorescence and EdU labeling

(A) Immunofluorescent labeling of brain cryosections from mice fed with regular diet (Reg), 0.2% cuprizone diet for 5 weeks (Cup 5wk), and 0.2% cuprizone diet for 5 weeks followed by two weeks regular diet (Cup 5wk + Reg 2wk), showing MBP in the corpus callosum (CC) outlined with white dotted lines. Scale bars = 50 μm, Subventricular zone (SVZ), Lateral ventricle (LV).

(B) Examples of immunofluorescent labeling of CC1 expressing mature oligodendrocytes, NG2 expressing oligodendrocyte precursor cells, RFP expressing Gli1 fate mapped cells, and EdU positive proliferating cells in the CC of mice fed with regular diet, cuprizone diet for 5 weeks, and cuprizone diet for 5 weeks followed by two weeks regular diet, with quantification of CC1, NG2, and EdU positive cells in the corpus callosum. N = 3, Data = Mean ± SEM, One-way ANOVA with Tukey's post-hoc t-test, Scale bars = 25 μm, inset scale bar = 5 μm.

Problem 2

Lungs inflate and/or fluid leaks through the mouth and nose during perfusion.

The perfusate is flowing through the pulmonary circulation (step 7).

Potential solution

If the entire perfusate flows through the right ventricle into the pulmonary arteries, the lungs inflate and the bronchial capillaries rupture leading to leakage of frothy fluid from the mouth and nose. Gently pull out the needle and reposition it until the tip is in the left ventricle. The brain can be perfused well even if the perfusate flows partially through the systemic circulation.

Problem 3

The olfactory bulbs are missing from the harvested brain.

Olfactory bulbs tear away when the brain is removed from the skull (step 8).

Potential solution

To prevent the olfactory bulbs from tearing off, ensure that they are no longer attached to the bone before attempting to remove the brain. Removing the bone around the olfactory bulbs may require some extra trimming of the bone with a small pair of scissors. Carefully cut the bone between the two bulbs to release them, as well as gently nudge them away from the bone by the closed tips of fine forceps.

Problem 4

Tearing of tissue during cryosectioning.

The brain is soft and the sections break apart while collecting them on the slide (step 14).

Potential solution

This is most often a result of incomplete perfusion especially if the brain looks bloody or pink after harvesting. During perfusion, ensure that the butterfly needle tip is fully inserted into the left ventricle and the perfusate flows through the systemic circulation. Shattering of tissue is also seen when either the brain or the cryostat temperature is too cold. Keeping the brain in the cryostat long enough to equilibrate its temperature with that of the cryostat and/or increasing the cryostat temperature by 1°C–2°C can make the sectioning smoother.

Problem 5

Stripes of torn tissue in the sections.

The sections show thin stripes perpendicular to the edge of the blade or thick stripes of irregular shape (step 14).

Potential solution

Nicks on the blade can result in thin stripes perpendicular to the edge. Move the blade or the stage to section the brain with an unused edge of the blade. When either tissue or OCT adheres to the edge of the blade, it can result in thicker stripes and sudden difficulty in cryosectioning. Clean the blade by wiping with the gauze sponge in perpendicular strokes away from the edge of the blade, taking care to avoid cutting your fingers.

Problem 6

No sections on the slide after immunofluorescence.

Tissue lifts off the slide and floats away during immunofluorescent labeling (step 19).

Potential solution

If the tissue section is not in direct contact with the glass slide, then the sections do not adhere to the slide during the immunofluorescent wash steps. This is often due to presence of a layer of OCT between the tissue and the glass surface. To prevent this situation, make sure you wipe off the OCT or unwanted sections from the slide with a gauze sponge dipped in PBS or water and dry the surface before collecting a section on the same surface, during cryosectioning.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jayshree Samanta (jayshree.samanta@wisc.edu).

Materials availability

No materials were newly generated in this course of this study.

Data and code availability

Not applicable.

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AUTHOR CONTRIBUTIONS

E.C.D. performed experiments using this protocol, fine-tuned the protocol, and wrote the manuscript. D.Z.R. performed experiments using this protocol, fine-tuned the protocol, and edited the manuscript. J.S. developed the original protocol, supervised the experiments, and edited the manuscript.

DECLARATION OF INTERESTS

J.S. is a co-inventor in the patent US 9,248,128 and a consultant for GliXogen Therapeutics.

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