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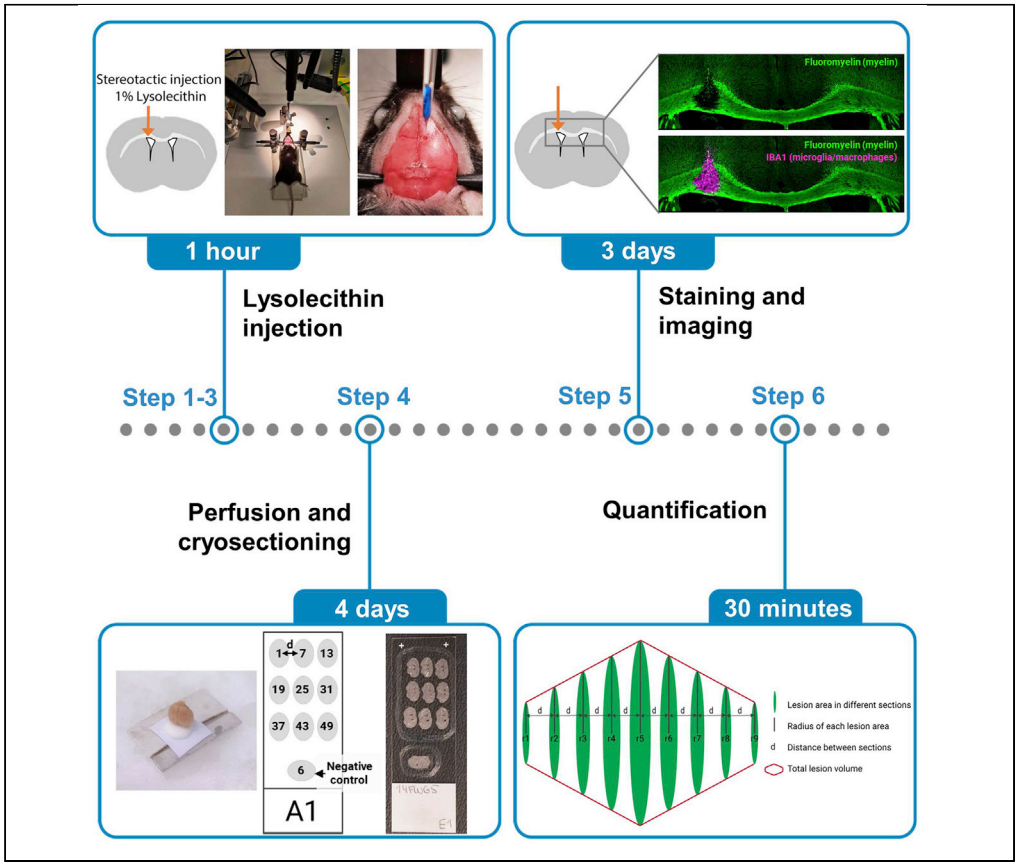
A fluorescence microscopy-based protocol for volumetric measurement of lysolecithin lesion-associated de- and re-myelination in mouse brain



Lysolecithin injections into the white matter tracts of the central nervous system are a valuable tool to study remyelination, but evaluating the resulting demyelinating lesion size is challenging. Here, we present a protocol to consistently measure the volume of demyelination and remyelination in mice following brain lysolecithin injections. We describe serial sectioning of the lesion, followed by the evaluation of the demyelinated area in two-dimensional images. We then detail the computation of the volume using our own automated iPython script.

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Highlights

Stereotaxic brain injections of lysolecithin cause focal demyelinated lesions

Serial sectioning of the lesion allows for control of the distance between sections

3D lesion volume is computed from lesion area measurements in two-dimensional images

Consistent and precise comparison of the size of de- and remyelination

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A fluorescence microscopy-based protocol for volumetric measurement of lysolecithin lesion-associated de- and re-myelination in mouse brain

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SUMMARY

Lysolecithin injections into the white matter tracts of the central nervous system are a valuable tool to study remyelination, but evaluating the resulting demyelinating lesion size is challenging. Here, we present a protocol to consistently measure the volume of demyelination and remyelination in mice following brain lysolecithin injections. We describe serial sectioning of the lesion, followed by the evaluation of the demyelinated area in two-dimensional images. We then detail the computation of the volume using our own automated iPython script.

For complete details on the use and execution of this profile, please refer to Bosch-Queralt et al. (2021).

BEFORE YOU BEGIN

This protocol describes the application of the volumetric quantification of lysolecithin lesions in the corpus callosum of mice. We have also successfully applied this protocol to evaluate the volume of demyelination in lysolecithin lesions in the spinal cord of mice, but we have not tested the protocol in other species or injection sites.

Prepare lysolecithin 1% solution

Timing: 1 h (30 min hands-on)

Perform all steps under a sterile laminar flow hood to prevent contamination.

1. Add 2.5 mL of sterile 1× PBS to the original bottle containing 25 mg of lysophosphatidylcholine (lysolecithin) (L4129, Sigma). Mix with the pipette and transfer to a 15 mL tube.
2. Sonicate lysolecithin for 30 min. After this step, the lysolecithin should be completely dissolved, rendering the solution fully transparent. If a sonicator is not available, incubate the solution at 50°C for 30 min. This should also allow full dissolution of the lysolecithin.

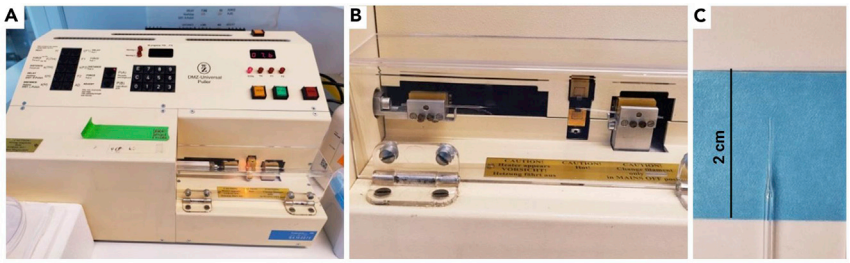

Figure 1. Pulling glass capillaries
(A and B) DMZ-Universal Puller used in our experiment. C. Capillaries are pulled with specific settings to achieve a long, thin tip for the injection capillary.

3. Immediately aliquot the solution into 20 µL aliquots and keep at −20°C for up to 2 years.

⚠ CRITICAL: Avoid repeated freeze-thaw cycles of the lysolecithin solution. Once the solution is prepared and aliquoted, freeze immediately and only thaw when needed for the experiment. Once an aliquot is thawed and used, discard the leftovers.

Prepare 3% monastral blue solution

Timing: 1 day (20 min hands-on).

4. Add 1.5 g of Monastral blue powder (274011, Sigma) to 30 mL of distilled H_2O.
5. Filter solution through a 0.45 µm filter or, alternatively, through a whatman filter paper to remove big particles.
6. Autoclave the resulting solution.
7. Aliquot and keep at room temperature (20°C–25°C) for up to 10 years.

Note: The 274011 product has been discontinued and we are still working on finding a suitable alternative.

Pull glass capillaries

Timing: 20 min hands-on.

Use a puller of your choice to pull the glass capillaries in order to achieve a capillary tip smaller than 1 µm and a taper of 6–8 mm. To achieve this, the conditions vary depending on the puller used; therefore, you will need to calibrate the settings of your puller to achieve the mentioned parameters.

As an example, we use a DMZ Universal Puller with the following parameters: Heat 400, Force pre-pull 025, Distance threshold 028, Delay heatstop Distance R-polish 006, Distance heatstop distance L-polish 000, Delay pull 1 250, Force pull 1 400, Distance pull 2 010, Force pull 2 200, Adjust 000 (Figure 1).

Institutional permission and oversight information for the animal study

In this study, all mouse procedures were carried out with approval and according to the District Government of Upper Bavaria. All C57BL/6J mice were imported from Janvier Laboratories. All mice were housed at the mouse facility in the German Centre for Neurodegenerative Diseases (DZNE) in Munich in a standard, pathogen-free, 12-h light/12-h dark cycle. The temperature in the housing facility was kept between 20°C and 22°C with 40–60% humidity.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-IBA1, working dilution 1:1000–1:2000	Wako	Cat# 019-19741
Anti-rabbit Alexa Fluor 555, working dilution 1:1000	Thermo Fisher Scientific	Cat# A-21428
Chemicals, peptides, and recombinant proteins		
Fluoromyelin	Invitrogen, Thermofisher Scientific	Cat# F34651
DAPI	Thermo Fisher Scientific	Cat# D1306
1× PBS (sterile)	Thermo Fisher Scientific	Cat# 10010-056
Lysophosphatidylcholine	Sigma-Aldrich	Cat# L4129
Monastral blue	Sigma-Aldrich	Cat# 274011
Medetomidine 1 mg/mL	Zoetis	Dorbene
Midazolam 5 mg/mL	B. Braun	Midazolam
Fentanyl 0.05 mg/mL	Piramal	Fentanyl
Buprenorphine	Indivior	Cat# IND00979
Atipamezole 5 mg/mL	Zoetis	Alzane
Flumazenil 0.1 mg/mL	B. Braun	Flumanezil
Naloxone 0.4 mg/mL	Inresa	Naloxon
Ketamine 10%	WDT	Ketamine
Xylazine 2%	Bayer	Rompun
Bepanthen	Bayer	n/a
Mineral oil	Sigma-Aldrich	Cat# M5904 Sigma
Paraformaldehyde	Sigma-Aldrich	Cat# 158127
Sucrose	Sigma-Aldrich	Cat# S0389
10× PBS	AppliChem	Cat# A0965.9010
NaCl	Carl Roth	Cat# 3957.2
KCl	Carl Roth	Cat# 6781.1
Na2HPO4●2H2O	Merck Millipore	Cat# 1.06580.1000
KH2PO4	Carl Roth	Cat# 3904.1
Bovine calf serum (BCS)	GE Healthcare Life Sciences	Cat# SH30073.03HI
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A2153
Fish gelatine	Sigma-Aldrich	Cat# G7765
Mowiol	Carl Roth	Cat# 0713.2
Glycerol	Sigma-Aldrich	Cat# 65518
Tris-HCl	Merck	Cat# 10812846001
Tissue-Tek O.C.T	Science Services	Cat# SA62550-01
Triton X	Sigma-Aldrich	Cat# T8787
Experimental models: Organisms/strains		
C57BL/6J mice, 2–12 months old, male and female	Janvier	C57BL/6JRj
Deposited data		
Lesion area ImageJ Macro	This paper	GitHub and Zenodo
IPython code	This paper	GitHub and Zenodo
Software and algorithms		
IPython 2.7	Pérez and Granger (2007)	https://www.python.org/download/releases/2.7/
FIJI ImageJ	Schindelin et al. (2012)	https://imagej.net/software/fiji/downloads
Microsoft Office Excel	Microsoft	Excel
Other		
Whatman filter paper	Merck	Cat# WHA1001325
O-rings	World Precision Instruments	Cat# 300521
Glass capillaries	World Precision Instruments	Cat# 504949
Fine forceps	Fine Science Tools	Cat# 11251-35
Fine scissors	Fine Science Tools	Cat# 14060-09

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
1 mL syringe	B. Braun	Cat# 9166017V
MicroFil needle	World Precision Instruments	Cat# MF25G67-5
Suture (USP 4/0, DSS13)	SERAG-WEISSNER	Cat# HAN 6O15131L
Cotton pad sticks	n/a	n/a
Venofix needles 25G	B. Braun	Cat# 4056337
Steritop Filter system 0.22 µm	Merck Millipore	Cat# SCGPT01RE
Superfrost glass slides	Thermo Scientific	Cat# J1800AMNZ
ImmEdge Hydrophobic Barrier PaP Pen	Vector Laboratories	Cat# H-4000
Laminar Flow Hood	Thermo Fisher Scientific	Cat# 51026908
Warm chamber	MediHeat	n/a
Far Infrared Warming Pad	Kent Scientific Corporation	Cat# RT-0520
Nanoliter 2010* with Micro4 Controller	World Precision Instruments	Cat# NL2010MC4
MicroDrill	World Precision Instruments https://www.wpiinc.com/var-503598-omnidrill35-micro-drill	Cat# 503599
Stereotaxic device, digital and portable, SGL M	World Precision Instruments	Cat# 504926
Dissection microscope	World Precision Instruments	Cat# PZMIII-BS
Fiber Optic Illuminator 100W	World Precision Instruments	Cat# PL2000
Bifurcated light guides	World Precision Instruments	Cat# 595-20-167
Peristaltic pump	World Precision Instruments	Cat# PERIPRO-4LS
Cryostat	Thermo Scientific	Cat# CryoStar NX70
Fluorescence microscope	Leica Microsystems	SP5
Template for analyzed areas	This paper	File S1
DMZ Universal Puller	Zeitz	n/a

MATERIALS AND EQUIPMENT

MMF solution

To prepare MMF solution, mix the following together:

Reagent	Final concentration	Amount
1 mg/mL Medetomidine	0.2 mg/mL	0.5 mL
5 mg/mL Midazolam	2 mg/mL	1 mL
0.05 mg/mL Fentanyl	0.2 mg/mL	1 mL

Store the solution at 4°C. It should be stable and safe to use for 3 months.

AFN solution

To prepare AFN solution, mix the following together:

Reagent	Final concentration	Amount
5 mg/mL Atipamezole	0.29 mg/mL	0.5 mL
0.1 mg/mL Flumazenil	0.06 mg/mL	5 mL
0.4 mg/mL Naloxon	0.14 mg/mL	3 mL

Store the solution at 4°C. It should be stable and safe to use for 3 months.

Ketamine 10%/Xylazine 2% solution

Mix the following:

Reagent	Final concentration	Amount
Ketamine 10%	1.25% mg/mL	1.2 mL
Rompun 2% (Xylazine)	0.08% mg/mL	0.4 mL
PBS 1×	n/a	8 mL

Prepare the mix freshly and store at 4°C for 1 week.

Paraformaldehyde 4%

Prepare paraformaldehyde (PFA) 16% stock solution by dissolving 80 g PFA in 450 mL dH_2O at 65°C, while stirring for 15–20 min. Then, add 6–7 pellets of 5 N NaOH (6771.1, Roth, Germany) until the solution becomes clear. Afterwards, add 50 mL 10× PBS and adjust the pH to 7.4. Finally, add dH_2O until the final volume of 500 mL (to compensate for evaporation). Store 50 mL aliquots of the solution at −20°C for up to 6 months. The working solution of 4% PFA in PBS (35 mL required per mouse) is prepared by diluting 50 mL of PFA stock solution in 150 mL of 1× PBS, filtering the resulting solution with a Steritop filter system and storing it at 4°C for up to 24 h.

⚠ CRITICAL: PFA is toxic, thus, always use under a laminar flow hood to avoid breathing its gas. Discard in special aldehyde waste.

Sucrose solution

To cryoprotect tissues before freezing, a 30% sucrose solution should be prepared by mixing 15 g of sucrose in 50 mL of 1× PBS (10 mL required per mouse). Sucrose solution should be prepared each time freshly to avoid contamination. If required, the sucrose solution can be stored at 4°C for up to 1 month.

1× phosphate buffer solution (PBS)

Prepare 1× PBS by dissolving the following in 1L ddH2O:

Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na2HPO4●2H2O	10.14 mM	1.805 g
KH2PO4	1.76 mM	0.24 g

Adjust the pH to 7.4 and store the solution at room temperature (RT) for up to 6 months. Alternatively, you can use premixed 10× PBS powder (A0965, 9010, Applichem).

Blocking and staining solution for immunohistochemistry

The blocking solution for immunohistochemistry is prepared by mixing the following in 500 mL of 1× PBS:

Reagent	Final concentration	Amount
Bovine calf serum (BCS)	2.5 %	12.5 mL
Bovine serum albumin (BSA)	2.5 %	12.5 g
Fish gelatine	2.5 %	12.5 mL

The solution should be aliquoted and kept at −20°C for up to 6 months. To prepare the staining solution, dilute the blocking solution 1:4 in 1× PBS.

Mowiol solution

Mowiol was used as the mounting medium after immunohistochemistry. The solution is prepared by stirring 2.4 g mowiol (0713.2, Roth, Germany), 6 g glycerol (65518, Sigma-Aldrich, Germany) and

6 mL ddH_2O for several hours at RT. After addition of 12 mL 0.2 M Tris-HCl (pH 8.5), the solution was incubated at 60°C for 10 min and then centrifuged at 4000 g for 15 min. Finally, mowiol should be aliquoted and kept at −20°C for up to 6 months until further use.

Reagent	Final concentration	Amount
Mowiol	13.33 %	2.4 g
Glycerol	33.33 %	6 g
Tris-HCl 0.2M	66.67 %	12 mL

STEP-BY-STEP METHOD DETAILS

Mouse anesthesia and nanoliter injector preparation

Timing: 30 min

These steps will ensure that the equipment, solutions and experimental animals are ready for the surgery and lysolecithin injection.

1. Prepare the lysolecithin - Monastral blue solution:
   a. Thaw one 20 μL aliquot on lysolecithin 1% and warm to 37°C. If precipitates are formed, sonicate for 15 min.
   b. Add 0.2 μL of Monastral blue solution to 20 μL of lysolecithin.
   c. Keep the solution at 37°C.

   Note: the final concentration of Monastral blue in the lysolecithin – Monastral blue solution is very low (0.03%) but sufficient to recognise the lesions while cryosectioning. In our experience, a very small amount of Monastral blue might be phagocytosed by microglia/macrophages at the central core of the lesion; otherwise, the Monastral blue is inert to the cells in the CNS. The very low concentration further ensures that the Monastral blue has no effects on microglia/macrophages or on myelin. The final concentration of Monastral blue in the lysolecithin – Monastral blue solution can be further decreased, as long as it can be observed during cryosectioning.

2. Weigh the mouse and inject the corresponding amount of MMF anaesthesia intraperitoneally (0.5 mg/kg BW Medetomidine, 5 mg/kg BW Midazolam, 0.05 mg/kg BW Fentanyl). Keep mice in a dark, warm space to ensure correct full anesthesia.

3. Prepare the Nanoliter injector (Figure 2):

   Note: these are instructions for using the Nanoliter 2010. By the time this protocol was written, the Nanoliter 2020 was released, which offers extended options of injection volumes – see manufacturer's manual for more information.

   Note: use gloves or tweezers when handling the injector and O-rings to avoid hand-fat deposition onto them.

   a. Loosen the collet covering the gasket and plunger and the O-rings. There are three O-rings: a black plunger sealing O-ring with a small hole, a white plastic sleeve called "spacer" and a conical shaped O-ring with a large hole (Figure 2).
   b. Make sure the O-rings are completely clean and dry.
   c. Take a glass capillary and break the tip by pressing it with fine forceps. Break approximately 1.5 mm off the tip. This should ensure that the capillary has a sharp tip (Figure 3B). We

recommend performing this procedure under a magnification stereoscope to achieve consistent breaking.

d. Pass the capillary through the collet, with the conical part of the collet facing the tip of the capillary.
e. Next, slide the capillary through the red O-ring from its blunt end, so that the conical part of the O-ring points towards the collet and the tip of the capillary.
f. Fill the glass capillary with mineral oil from the large, non-pulled end. The oil in the capillary allows the collet to easily slide in and out of the capillary during the injection or withdrawal of the lysolecithin – Monastral blue solution.
    i. Fill in a 1 mL syringe with mineral oil, attach a MicroFil needle to the syringe and ensure that the oil is being ejected through the MicroFil needle.
    ii. Place the MicroFil needle into the glass capillary from the blunt end, so that the needle's tip is pushed all the way into the front part of the glass capillary, near the pulled end.
    iii. Eject the liquid into the glass capillary and simultaneously slowly withdraw the MicroFil needle as the liquid fills up the glass capillary until the glass capillary is completely full with oil.
g. Install the black O-ring onto the collet so that the larger hole is facing towards the injector. Then, slide in the white spacer. The flat side of the spacer should face towards the injector. The other side has a recessed part which receives the capillary. The black O-ring should hold onto the plunger, but the white spacer needs to be held so that it stays in place (Figure 2).
h. Slide the filled glass capillary onto the plunger until the glass capillary contacts the recessed part of the white spacer. This will cause some oil to leak out of the glass capillary; this should be cleaned away with a cotton swab. Keep on pressing the glass capillary towards the injector to avoid bubbles forming within the oil. Push the collet towards the aluminum barrel and screw it until it is finger tight.
i. Fill the glass capillary with lysolecithin – Monastral blue solution (Figure 3C):
    i. Set and run the Micro4Pump to inject 4000 nL at a rate of 2000 nL/min so that the plunger pushes mineral oil out of the glass capillary. Clean the excessive oil from the tip of the glass capillary.

Note: If the glass capillary is fully pushed out of the injector and falls down, refer to troubleshooting 1.

    ii. Set and run the Micro4Pump to withdraw 400 nL at a rate of 2000 nL/min. This will create a 1 mm-thick air bubble within the glass capillary.
    iii. Immerse the glass capillary into the lysolecithin – Monastral blue solution by holding the tube with the solution below the injection device and withdraw 3600 nL of the solution at a rate of 1000 nL/min into the glass capillary.

Note: if the lysolecithin – Monastral blue solution is not being correctly pipetted into the glass capillary, see troubleshooting 2.

    iv. Use a cotton swab wet with saline solution to slightly stroke the tip of the glass capillary in order to keep the tip of the glass capillary moist. Repeat this step every 5–10 min to prevent drying and clotting of the lysolecithin within the glass capillary.

⚠ CRITICAL: The correct orientation of the O-rings and the correct assembly of the glass capillary in the injector are essential (Figure 2). Otherwise, leakage may occur. After long periods of regular use of the O-rings, there might be leakage around the seals. To correct this, replace the O-rings with new ones. Please, refer to the manufacturer instructions for more detailed information on how to assemble the injector correctly.

4. Position the mouse into the stereotaxic device:

a. Transfer the mouse from the dark, warm cage onto a heating pad. Check for full anasthesia by testing the interdigital reflex on the paw.
b. Protect the eyes from drying out by covering them with a small drop of Bepanthen eye cream.
c. Cut hair from the head with scissors or alternatively, using a trimmer.
d. Using fine scissors or a thin scalpel, cut a 1 to 1.5 cm-long incision on the head skin. This exposes the mouse skull above the cortical hemispheres, the most caudal part of the olfactory bulbs and the most cranial part of the cerebellum. You should be able to visualize both the Bregma and Lambda intersections (Figure 3D).
e. Place the mouse in the stereotaxic apparatus (Figure 3E):
    i. Fix the left ear bar to a predefined scale (e.g., 4 mm).
    ii. Fit the left ear canal of the mouse onto the left ear bar so that the tip of the ear bar pulls the skin down and exposes the skull.
    iii. Keep on holding the mouse head in this position while you slide the right ear bar into the right ear canal so that the tip of the ear bar pulls the skin down and exposes the skull. The right ear bar should be fixed to the same scale as the left ear bar.

⚠ CRITICAL: Correct stereotaxic fixation of the mouse is essential to successfully target the brain region of interest. When pressing the ear bar onto the mouse ear, you should feel a hard bone, and no soft tissues. When the mouse head is in the right position, the head is straight and symmetrical to the ear bars. The investigator should be able to move the mouse snout up and down, but the head should not be able to be moved laterally.

    iv. Insert the incisor adapter into the mouse's mouth until its incisors fit into the small opening in the adapter. Make sure to gently pull out the tongue of the mouse slightly to allow for correct breathing.
    v. Slightly pull back the incisor adapter to check for its correct position. If the incisors are correctly placed within the hole, the adaptor should not be able to move back.
    vi. Fix the incisor adapter and carefully place the snout clamp just below the eyes using low pressure.
    vii. At this point, the mouse's head should be completely fixed in the stereotaxic apparatus and no movement should be possible.

Note: from Step 5, you require visualization through a dissection microscope using 20× to 40× magnification.

5. Align Bregma and Lambda: these steps ensure that the top of the animal's skull is in the horizontal plane. This step is essential to be confident that the coordinates you follow will hit the target region. To adjust the horizontal position of the head, you can use the screw at the incisor adapter (Figure 3F).

Note: this step is optional if your target region is neocortical or superficial. However, it becomes crucial if the Z coordinate of the target region is deeper than −2 mm.

a. Identify Bregma and Lambda (Figure 3F)
b. Move the capillary to Bregma so that the tip of the capillary lightly touches onto it. Reset all coordinates.
c. Move the capillary to Lambda. Do not change the X coordinate, but change the Y and Z coordinate so that the glass capillary is lightly touching Lambda. Check the Z coordinate at this point.
d. If the Z coordinate value has changed by more than +/- 50 μm, adjust the head position with the screw at the incisor adapter until the Z coordinate for Bregma and Lambda become equal.

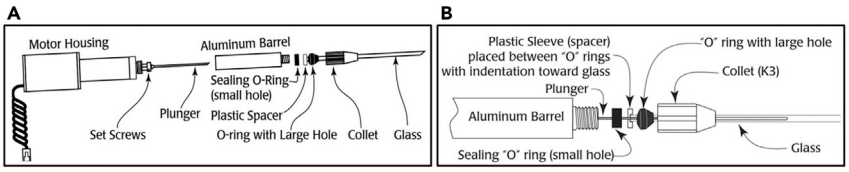

Figure 2. Correct assembly of the Nanoliter injector 2010

(A) Cartoon displaying the different components of the Nanoliter injector 2010.

(B) Cartoon showing the correct orientation and order of the O-rings. These orientations and the order are essential for the capillary to be correctly adjusted into the injector. *Image source: Nanoliter 2010 instruction manual.*

Lysolecithin injection

Timing: 30 min

Here we elucidate how to perform the intracranial injection of lysolecithin to achieve a focal demyelination in the corpus callosum.

6. Drill a small hole through the skull at the target position (Figure 3G):
   a. Reset all coordinates at Bregma so that Bregma is the reference.
   b. Using the different stereotaxic axis, move the glass capillary to the coordinates X: −1 mm and Y: −0.1 mm.
   c. Lower the glass capillary, and carefully mark on the skull the resulting position with a marker. Raise the glass capillary again to its highest position to protect it.
   d. Attach a 0.5 mm drill-bit to the Micro Drill and adjust the speed to 20,000 rpm.
   e. Make a single burr-hole in the skull at the marked site by applying gentle pressure on the skull while holding the Micro Drill steady.

   Note: The head should not move while drilling. Head movement would indicate an incorrect fixation of the mouse head.

   f. Once the Micro Drill has drilled through the skull, a small sudden drop will be noticed on the hand holding the Micro Drill. Stop immediately to prevent damaging the brain parenchyma.

   Note: To prevent damage to the brain parenchyma, another option would be to stop drilling when the drill-bit reaches the internal layer of the skull, where a thin layer of skull may remain. With a sharp object (e.g. sharp forceps, fine blade, or surgical hook), remove the small pieces of the remaining skull to expose the brain.

   g. Apply saline solution on the hole to keep the brain moist.
   h. Lower the glass capillary again just to the brain surface to confirm that the skull opening is in the correct position.

   Note: the tip of the glass capillary should not be diverted by the edges of the burr-hole. If it is, use the Micro Drill again to widen the hole.

7. Inject the lysolecithin into the desired position (Figure 3G):
   a. Make sure that the glass capillary is not clogged by injecting a small volume of fluid while visually controlling the tip of the glass capillary (e.g., 50 nL at a speed of 1000 nL/min). A drop of blue fluid should be formed at the tip.
   b. Set the settings of the Micro4Pump to inject 1000 nL at a speed of 100 nL/min.

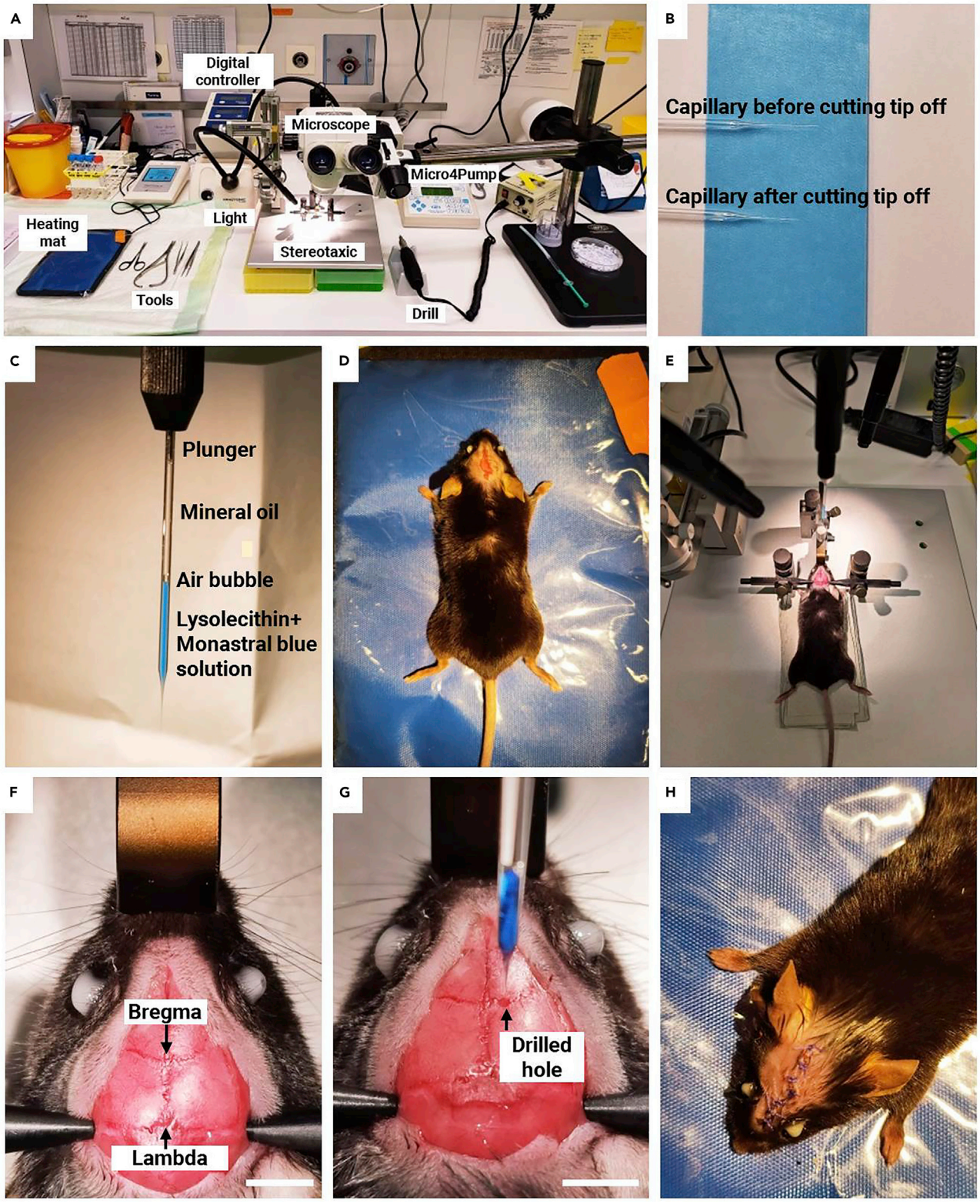

Figure 3. Mouse surgery

(A) Overview of all materials needed to perform the stereotaxic injections.

(B) Image of the glass capillary before and after cutting its tip off.

Figure 3. *Continued*
(C) Image showing the fully loaded glass capillary. There should be a small air bubble between the mineral oil and the lysolecithin-Monastral blue solution.
(D) Image of the skin incision above the brain skull.
(E) Image showing the mouse correctly fixed into the stereotaxic apparatus. Note that the ear holders are simultaneously holding the skull at a fixed position and the skin open.
(F) Image of anatomical Bregma and Lambda points. Bregma is used as a reference for all coordinates, while the alignment of Bregma and Lambda in the Z plane is essential to ensure a proper head positioning. Scale bar: 4 mm.
(G) Image of the hole drilled into the skull and the capillary inside the brain. Scale bar: 4 mm.
(H) Image of the sutured incisure.

c. Lower the glass capillary at a slow but steady speed into the brain parenchyma until the Z coordinate is −1.45 mm. Then raise the glass capillary to the Z coordinate −1.4 mm. This creates a small "pocket" within the brain parenchyma that facilitates the flow of lysolecithin.

Note: the glass capillary should be able to easily poke through the brain parenchyma. If it does not, this would indicate that there are pieces of skull remaining on top.

d. Inject the lysolecithin by running the program set in the Micro4Pump. This step takes 10 min.

Note: Use visual references to control that the lysolecithin solution is being injected and not refluxed. Reflux is normally obvious after 200 nL of solution has been injected, and appears as blue liquid progressively accumulating around the glass injection site. If you observe repetitive reflux, please refer to troubleshooting 3.

e. Once the program is complete, wait for 2 more min.
f. Slowly raise the glass capillary until it is out of the brain.
g. Remove the mouse from the stereotaxic device by releasing the incisor and ear adapters and transfer to the warm pad.

Note: These steps describe the procedure to perform one unilateral injection into the corpus callosum. However, bilateral injection or multiple injection sites are also possible. We have performed up to 6 injections per mouse using the coordinates below. However, we have only confirmed the feasibility of the volumetric calculation for injection site 2. Sites 1 and 3 were used additionally when big amounts of demyelinated white matter were needed for –omics studies (see Penkert et al., 2021).
Injection site 1: X = −1/+1, Y =+1.10, Z = −2.30 → −2.25
Injection site 2: X = −1/+1, Y = −0.10, Z = −1.45 → −1.40
Injection site 3: X = −0.55/+0.55, Y = −1.22, Z = −1.45→ −1.40

Recovery and monitoring

Timing: 20 min – 3 days

These steps ensure correct recovery and monitoring of mice after surgery.

8. Inject 0.05 mg/kg BW of buprenorphine subcutaneously.
9. Apply saline solution onto the skull to ensure brain and skull moisture.
10. Pull the skin edges of the incision together and stitch using braided absorbable suture (suture 4/0 or 5/0 work, DSS 13) (Figure 3H).
11. Inject 2.5 mg/kg BW of Atipamezole, 0.5 mg/kg BW Flumazenil and 1.2 mg/kg BW Naloxon subcutaneously to terminate the anesthesia.
12. Clean away the Bepanthene from the eyes.

13. Keep the mouse in a warm, preferably dark space until full recovery from the anesthesia.
14. Monitor the mouse 24, 48 and 72 h after the injection by checking its weight. Inject 0.05 mg/kg of Buprenorphin subcutaneously 24 and 48 h after the injection as analgesia.

Perfusion and cryosectioning

Timing: 4 days (2 h hands on)

In these steps, we describe how to fix the brain for correct tissue preservation and how to cut the demyelinated tissue in a serial manner to allow for subsequent quantification.

15. Perfuse the mouse with fixative:
    a. Prepare 4% paraformaldehyde solution and 1× PBS.
    b. Anesthetize the mouse by injecting 150 µL of 10% ketamine and 2% xylazine intraperitoneally.
    c. Expose the heart, insert a venofix needle into the left ventricle and open the right atrium.
    d. Perfuse using a peristaltic pump with 5 mL of 1× PBS followed by 30 mL of 4% PFA at a rate of 2 mL/min.
    e. Remove the brain and keep overnight in 4% PFA at 4°C for 24 h. Wash the brain 3 times in 1× PBS and transfer to cryoprotecting sucrose solution for 2 days or until the brain sinks to the bottom of the tube.
16. To freeze the brain (Figure 4A);
    a. Cool down an aluminum surface on dry ice (e.g., a blade)
    b. Dry the excess sucrose around the brain and cut the cerebellum off (to provide a flat surface)
    c. Dip a 1 cm x 1 cm square of paper on water, then place on top of the cooled aluminum surface, on top of dry ice.
    d. Add a small drop of Tissue-Tek O.C.T. onto the paper, and then quickly place the brain on top of the paper so that the flat surface, i.e., the most caudal part of the brain, is on top of the paper.

Note: Avoid using Tissue-Tek O.C.T spread around the brain to facilitate cryosectioning.

17. To store the brains, cover the sample in pre-cooled aluminum foil and keep at −80°C.
18. Using a cryostat, cut serial 15-µm-thick coronal sections of the lesion and directly mount them on Superfrost glass slides. The lesion can be localized by the blueprint left by the Monastral blue dye used during injections. To assure the acquisition of the entire lesion, some sections before the beginning and some after the end of the lesion can be collected. We recommend sectioning from the dorsal side through to the ventral side of the brain (i.e., upside down) to prevent physical damage on the demyelinated lesion. Serial sectioning ensures a representative sampling of the whole lesion in different slides, which can subsequently be used for different stainings.

Note: the Monastral blue dye is mostly concentrated at the center of the lesion. Thus, the blue color will be most prominent at the core of the lesion, while the rest of the lesion should be stained in lighter blue.

Note: in the beginning, it is useful to collect several sections before and after the lesion, to ensure the acquisition of the entire lesion. If the correct coordinates were reached during the injection, the lesion should be found between approximately Y: +0.1 mm and −1 mm from Bregma, and there should be approximately 60–70 15-µm-thick sections with lesion, depending on the time point after injection.

Note: Sometimes, it might happen that some sections will be lost during cutting. If this problem is constant and persistent, refer to troubleshooting 4.

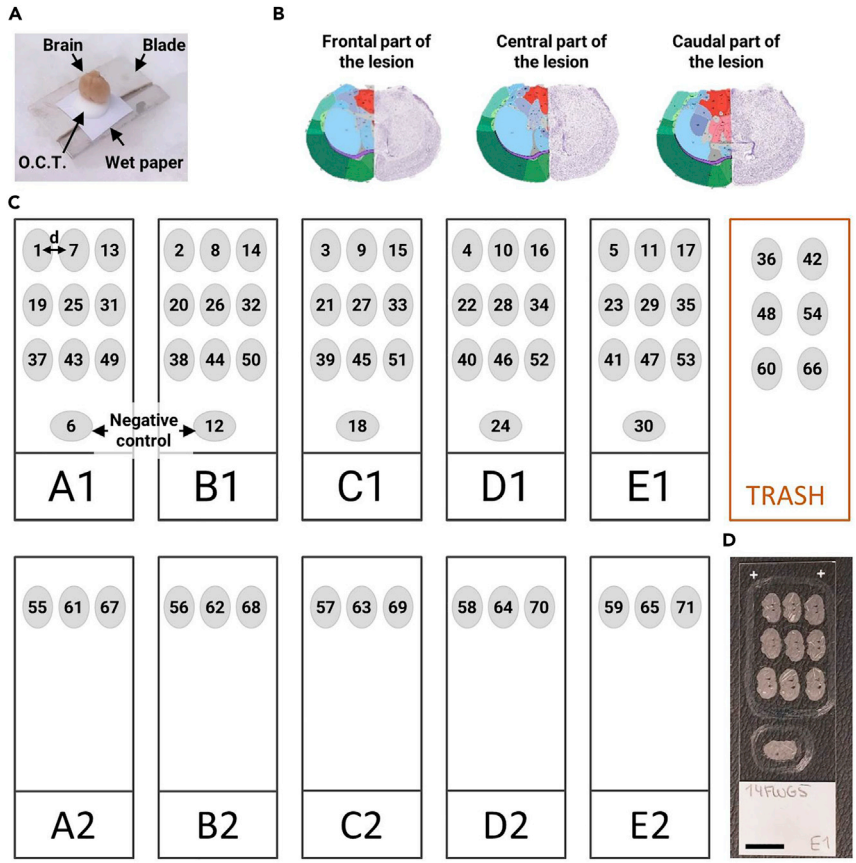

Figure 4. Freezing and sectioning the lesioned brains

(A) to freeze the brain quickly, place a wet paper on top of a metallic blade on top of dry ice. Then, apply a drop of Tissue Tek O.C.T and place the brain with its caudal end on the paper. Use little O.C.T. to prevent having O.C.T around the brain.

(B) Schematic of how the neuroanatomy of the brain at the expected localization of the demyelinated lesion looks like at the frontal, central and caudal part of the lesion. The brain is displayed upside-down because we recommend sectioning corona sections starting from the most dorsal part of the brain and cutting through to the most ventral part of the brain to prevent tissue destruction at the lesion site.

(C) Cryosectioning scheme to ensure serial sectioning of the demyelinated lesion with a known distance between sections of the same slide. Here, 5 series are produced (A to E) and 2 slides are needed for each series (1 and 2 for each series). The first section with lesion tissue should be on slide A, the next on slide B, … until section 5 goes on slide E. Section 6 should go on the very end of slide A1, to serve as a negative primary control for the immunostaining. From section 7, the procedure should be repeated, with the next section for negative control staining going to slide B1. This way, each section on one same slide is separated by a known distance ("d") of 6 sections, i.e., 90 μm if 15-μm sections are being used. Once all the positions for the negative control are occupied, one section should be trashed after each round, to be able to keep the constant distance.

(D) Picture of a slide with brain sections. It should be ensured that the demyelinated lesion lays perfectly flat on the glass slide. The negative control section is circled separately from all other sections for subsequent immunostaining. Scale bar: 1 cm.

CRUCIAL: It is essential that the cutting paradigm enables the recognition of the order of sections, as well as the exact distance between one section and the next. For example, at 15-μm-thick sections and 6 series, each section in one series is separated by 90 μm. We suggest following the scheme presented in Figure 4.

19. Air-dry the sections for 16–20 h (overnight) at room temperature. Then, transfer and keep the sections at −20°C.

Staining and imaging

Timing: 3 days

In this part, we describe how to stain myelin and microglia/macrophages so that the demyelinated area and the area of IBA1$^+$ cell accumulation can be visualized. All steps are performed at RT unless stated otherwise. From step 20i on, the solutions containing secondary antibodies and the slides should be protected from light to prevent bleaching of the fluorophores.

20. Stain the sections with anti-IBA1 antibodies and DAPI and Fluoromyelin fluorescent dyes to visualize the microglia/macrophages, demyelinated area, and nuclei:
    a. Transfer the glass slides to be stained (1 series of each lesion is sufficient) to 37°C for 30 min.
    b. Rinse the slides with 1× PBS, 3 × 5 min.
    c. Permeabilize the tissue for 10 min using 1× PBS with 0.3% Triton X.
    d. Incubate the sections for 1 h with a blocking solution.

    Note: encircle the sections with a hydrophobic PAP Pen to ensure that the liquid stays on top of the sections.

    e. Dilute IBA1 at a concentration of 1:2000 in staining solution.
    f. Incubate the sections with the primary antibodies for 16 h at 4°C.
    g. Continue incubation with the same solution for another hour at RT.
    h. Wash the sections with 1× PBS, 3 × 5 min.
    i. Dilute the anti-rabbit Alexa 555 secondary antibody at a concentration of 1:1000 in staining solution.
    j. Incubate the sections with secondary antibodies for 1 h at RT.
    k. Wash the sections with 1× PBS, 3× 5 min.
    l. Dilute Fluoromyelin (1:400) and DAPI (1:1000) in 1× PBS.
    m. Incubate sections with Fluoromyelin and DAPI for 15 min.
    n. Wash the sections with 1× PBS, 3× 10 min.
    o. Dip slides in ddH_2O, remove all excess water using a tissue and keep the slides protected from light until all water is evaporated and the sections are dry (approx. 10 min).
    p. Cover sections using a glass coverslip (thickness #1.5) using Mowiol as a mounting medium, dry for 16–20 h (overnight) at RT and keep at 4°C until imaging.

    Note: immunostaining with an anti-MBP antibody to stain for myelin is also possible.

21. Acquire fluorescence images of the stained sections for three channels (DAPI, Fluoromyelin, AF555) using standard settings for multi-channel acquisition (both an epifluorescence microscope or a laser-scanning confocal microscope are suitable) (Figures 5A and 5B):
    a. Select an objective with a field of view large enough to fit the lesion region (e.g., 10×). If the field of view of the objective is smaller than the whole lesion, then acquire and merge tile-scan images, so that the entire demyelinated lesion is visible in one single image.
    b. Set the excitation intensity and detection gain for each channel, so that the fluorophores are properly excited but not saturated.
    c. Use the same pixel size, as well as the same excitation and detection settings for all acquisitions.
    d. The demyelinated lesion is shown as the area of corpus callosum with FluoroMyelin-negative signal and prominent IBA1+ cell accumulation (Figure 5A). Image all the demyelinated areas in order, so that the order of the different lesions can be tracked afterwards (Figure 5B).
    e. Save the images with consecutive numbering in their names, as individual gray-scale .tiff files, or, if possible, inside an image container file (recommended).

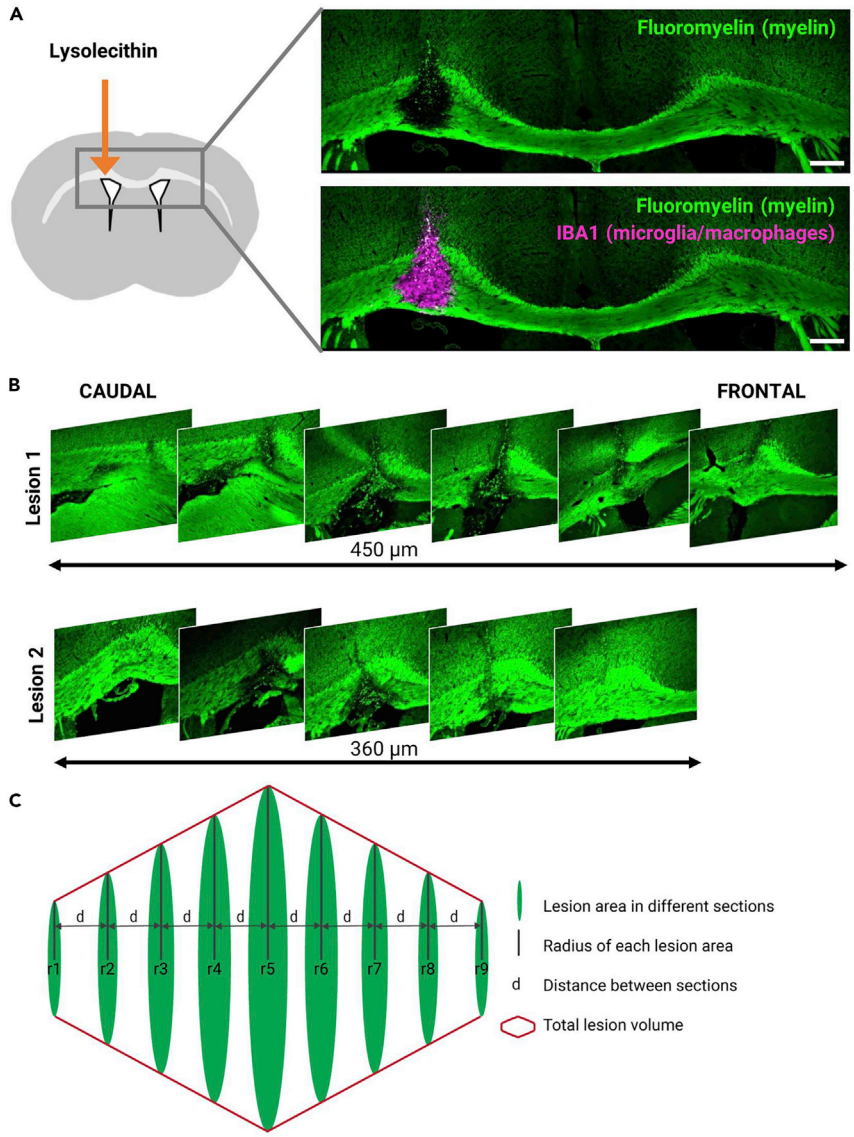

Figure 5. Staining and imaging of the demyelinated lesion

(A) Cartoon demonstrating the site of lysolecithin injection. The gray square delineates the area shown in the microscopy images on the right, which are representative images of the demyelinated lesion at 14 days post injection (dpi), showing in green the myelin labeling (Fluoromyelin) and in magenta the microglia/macrophages (IBA1). The injection of lysolecithin creates a small focal lesion at the site of injection. Scale bar: 200 µm.

(B) Representative images of two demyelinated lesions at 14 dpi. All the pictures of these lesions are shown, from the most frontal part of the lesion to the most caudal part. Considering that each section is separated by 90 µm, the total distance between the first and the last section in lesion 1 is 450 µm, while in lesion 2 it is 360 µm. C. Cartoon representing the components required for the volume calculation. Each lesion area is measured using our custom-made ImageJ macro. The radii of the areas are calculated, while the distance between sections is dictated by the serial cutting paradigm. The output of the iPython script will be the total lesion volume.

Area quantification and volume calculation

Timing: 30 min

Finally, these steps describe how to first analyze the areas of demyelination or the area of clustered IBA1^{+} cells using an ImageJ macro and how to compute the volume of the lesion using our iPython script.

22. Quantify the demyelinated area and the area of clustered IBA1+ cells in each section using the ImageJ/FIJI macro "Macro_Analyse_Lesion_Area.ijm" (find it in the File S2 of this paper or GitHub and Zenodo).
    a. Run ImageJ/FIJI.
    b. Open the macro in FIJI by dragging and dropping the .ijm file on ImageJ/FIJI or File→ Open… and selecting the macro file. Then click on Run.
    c. The option "Analyse single image container file" is selected by default. This means that the macro will process only single files (images or image containers). If this option is not checked, then the macro asks for an input folder and will process all images (or image container) files located in this folder.
    d. The option "Save ROI Selections" is marked by default. Uncheck this if you do not wish to save the area selections.
    e. Then give the basic name of the output folder where the result text files and the regions of interest (ROIs) will be saved. This new folder will be located in the same folder as the input files. The name of the output folder will also contain the date and time the macro has run (e.g., _Results_20211020_1535).
    f. Press OK and then select the folder containing the image files (or the image container file) to be analyzed.
    g. Every selected image will be opened, together with a pop-up message that states "Please select the region of lesion and press OK". With the free-hand selection tool delineate the area of Fluoromyelin negative staining or the area of IBA1+ clustered cells, depending on whether you aim to analyze the demyelinated or the IBA1+ volume, respectively. When done click OK.
    h. Step 22g will be repeated until all images are processed.
    i. In the saving folder selected in step 22e, you will find a .txt file that contains the results of your analysis. This file is a tab-separated file with two columns (name of image and area measurement) and as many lines as the analyzed images.

    Note: The macro expects input images with a single z-level, a single time-frame and three channels. However, it can accept as an input any kind of hyper-stack. In such cases, a notification will appear in the Log Window of ImageJ notifying the user during the process of the image.

    Note: The area measurement will have the same unit as the image pixel size (i.e. if the image is not calibrated, then the area's unit will be squared pixels).

    Note: If you observe many sections with a necrotic hole where the demyelinated tissue should be, refer to troubleshooting 5.

23. Copy the area measurements for each section in an excel file. This excel file will be composed of three columns: the first column listing animal names corresponding to each section (same name for all the sections of one lesion), the second column containing the areas A of each section and the third column specifying the radii r of the corresponding sections calculated using the formula for the area of a circle $r = \sqrt{A/\pi}$. See the example template in the File S1.
24. To calculate lesion volumes, run iPython 2.7 (Pérez and Granger, 2007) and input the prepared excel file and the known distance between consecutive sections in μm into the openly available code (GitHub and Zenodo). This script calculates the volume in between sections based on the truncated cone volume formula, sums up the single volumes between all sections per animal and outputs one file containing the animal names and their corresponding lesion volume (Figure 5C).

EXPECTED OUTCOMES

Given that 1 μL of 1% lysolecithin is injected into the corpus callosum, and the analysis is performed as describe, the experimenter should expect to obtain demyelination volumes between 0.2 to

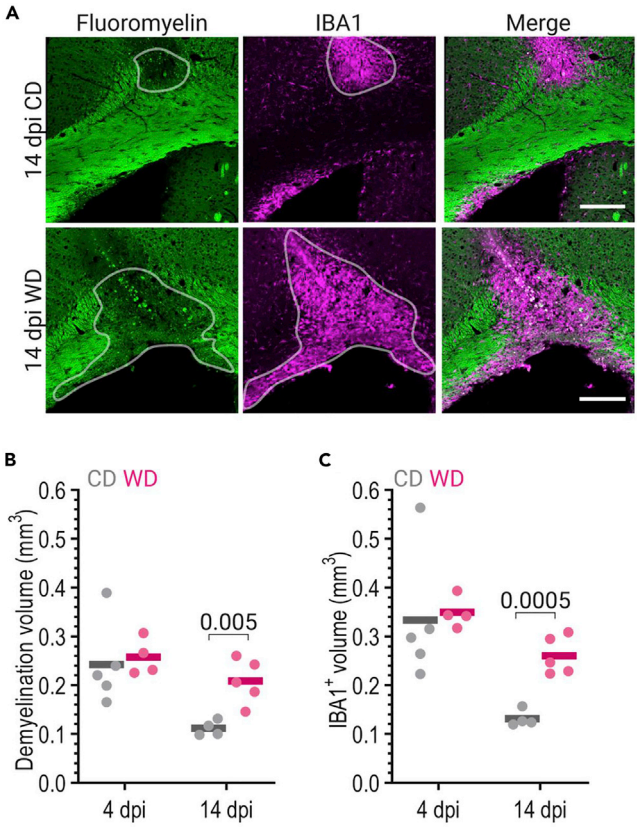

Figure 6. Western diet impairs microglia-mediated myelin-debris clearance and myelin repair after demyelination

(A) Images of demyelinated lesions in the corpus callosum in control diet (CD) and western diet (WD) mice at 14 days post injection (dpi). Double immunostaining with Fluoromyelin (myelin) and IBA1 (microglia and macrophages). The area of demyelination and of IBA1$^+$ cell accumulation is delineated with a semi-transparent white line. Scale bar: 200 μm.

(B) Quantification of demyelination (Fluoromyelin-negative volume) in demyelinated lesions at 4 and 14 dpi. While at 4 dpi the volumes of both groups are similar, at 14 dpi the demyelinated lesions of WD mice are larger. Lines represent the mean, unpaired two-tailed Welch's t-test.

(C) Quantification of the extent of inflammation (IBA1+ volume) in demyelinated lesions at 4 and 14 dpi. Inflammation is similar at 4 dpi but increases in demyelinated lesions of WD-fed mice at 14 dpi. Lines represent the mean, unpaired two-tailed Welch's t-test. Reference: Bosch-Queralt et al. (2021), reused with permission.

0.3 mm^3 in an adult WT mouse at 4 days post injection (dpi). At 14 dpi, the demyelination volumes should range between 0.05 and 0.15 mm^3 in a WT mouse, and larger volumes can indicate inefficient myelin repair (Figure 6). It is usual to obtain larger volumes for the IBA1$^+$ volume than for the demyelination volume for the same section, because the demyelinated area is a focal region with clearly defined edges, while the microglia/macrophage reaction to the demyelination spreads slightly further than the focal demyelinated area and its edges are rather diffused. See references Bosch-Queralt et al. (2021), Cantuti-Castelvetri et al. (2018), Gouna et al. (2021), Penkert et al. (2021).

Typically, at 4 dpi, the demyelinated lesion should expand throughout the whole width of the corpus callosum, from its tip to the ventricle wall (similar to the Western diet (WD) 14 dpi picture at Figure 6). If the demyelinated area is restricted to the very tip of the corpus callosum at 4 dpi and does not spread all the way to the ventricle wall, this indicates an unsuccessful injection, possibly due to reflux of the lysolecithin-Monastral blue solution. See troubleshooting 3 for tips on how to solve this problem.

With this protocol, we have obtained consistent results with multiple experimenters and different mouse lines, and have been able to identify differences in the extent of remyelination in several models.

LIMITATIONS

To achieve trustworthy results and keep variability to a minimum, it is necessary that all steps are performed meticulously. This includes (1) making sure that the full volume of lysolecithin ends up in the desired coordinates, (2) ensuring good mouse perfusion and brain fixation, (3) collecting cryosections in a serial manner that keeps the distance between sections known and constant and (4) analyzing all demyelinated lesions using pre-defined, objective and consistent rules.

Thus, we highly encourage practicing this protocol beforehand, in order to fully establish all details of the protocol and discover potential problems in advance. In order to achieve reliable consistent results, it is important to pay attention to the four points described above by (1) ensuring that the lysolecithin solution is not being refluxed out of the brain neither during nor right after the injection, (2) perfusing the mice with freshly prepared, filtered, 4% PFA at a slow rate for 10–15 min, (3) following our cryosectioning scheme, which allows for a certain number of lost sections, and (4) performing all analysis in a blinded manner.

TROUBLESHOOTING

Problem 1

When you start injecting mineral oil (step 3-i-i), it might happen that the glass capillary falls down from the nanoliter injector.

Potential solution

This is usually caused by an incorrect fitting of the glass capillary in the nanoliter injector. If this happens, you need to unmount the capillary from the injector, dry all the O-rings and collet from any oil that might have spilled over, and use a new clean capillary to repeat step 3.

When re-mounting the fresh capillary to the nanoliter injector, make sure that all O-rings are in the correct order, that you push the capillary up to the injector while tightening the collet, and that the collet is tightened to finger strength.

Problem 2

When trying to fill the glass capillary with lysolecithin-Monastral blue solution (step 3-i-iii), the solution is not correctly withdrawn into the glass capillary.

Potential solution

Normally, this is due to a clog caused by small particles in the solution. To prevent this problem, make sure the Monastral blue is correctly filtered and that the lysolecithin is fully dissolved, which is achieved by either temperature of 40°C–50°C or short sonication. Next, make sure the opening of the glass capillary is wide enough. The capillaries should be pulled to have a width of approximately 1 μm and one should break 1–2 mm off the tip by pressing with fine forceps. Finally, while trying to pipette the solution up into the capillary, use a slow withdrawal rate of the Micro4Pump (1000 nL/min) and repeatedly move the tip of the glass capillary inside the solution and carefully against the bottom and walls of the tube to prevent any particles from clogging the tube. If the problem persists, break 0.5–1 mm off the tip of the glass capillary and try again.

Problem 3

Reflux of the lysolecithin-Monastral blue solution might occur during the injection (step 7d). It is visible as a continuous accumulation of light blue solution around the capillary, which stops if the injection is paused.

Potential solution

The lysolecithin-Monastral blue solution might be refluxed when injected if the injection speed is too fast, there are clogs in the solution, or not enough space for the solution to disperse into the brain. To prevent this issue, pay attention to the following:

Ensure that the coordinates are correct and that the Bregma and Lambda coordinates are aligned in the Z plane.

Make sure the lysolecithin-Monastral blue solution is flowing regularly before injecting the capillary into the brain.

Ensure there is enough space in the brain for the solution to flow into by lowering the capillary to a deeper Z position before reaching the position of injection (see step 7c).

Use speeds of 100 nL/min to 150 nL/min.

Once reflux occurs, it is very difficult to stop it. If possible, use the contralateral side to perform the injection. Otherwise, raise the capillary again, dry out the refluxed lysolecithin on the brain, re-check your coordinates and inject at a slightly deeper Z coordinate (−1.50 to −1.45).

Problem 4

Too many cryosections are lost during cutting (step 18).

Potential solution

If many sections are lost because of curling of the section, folded section on the glass, or problems with the cryostat such as changing temperatures, please try the following:

Ensure that the cryostat you are using is able to keep the temperature of both the object and the chamber in a very stable value.

Follow our cutting scheme (Figure 4C). This places one section for negative primary control at a different place on the section. If, while cutting one round (from the first to the last series), a section is lost, then you can skip the section that goes in the negative control so that the distance between sections in the actual slides is kept constant.

Skip the position of the lost section and take a note that a section was lost during cutting for one particular slide. Once the areas of the surrounding sections are analyzed, take the average of the area above and the area below the lost section to calculate the Radius in the excel containing the areas so that the section is included in your volume calculation.

If the section is attached to the glass slide and the demyelinated lesion is folded, use PB solution to slightly moisten the section and carefully flatten it using a soft small brush.

Problem 5

Tissue necrosis is observed at the core of the demyelinated lesion (step 22).

Potential solution

We often observe a necrotic hole in the core of the demyelinated lesions. We believe this is either a consequence of (1) insufficient tissue fixation that causes the tissue to be lost during cryosectioning or (2) a consequence of the oedema that follows our injection. If the hole is caused by reason 1, you should be able to observe that the tissue is lost during cryosectioning. To avoid this, please improve mouse perfusion, brain freezing and the cryosectioning technique. For brain freezing, it is essential that the brain is frozen fast, within 1–2 min. For cryosectioning, we highly recommend cutting the coronal sections starting from the most dorsal and cutting through to the most ventral part of the brain at a constant slow speed.

If the necrotic hole is already present in the brain before cryosectioning, proceed with the protocol, considering the necrotic hole as a part of the demyelinated lesion and thus including the necrotic

tissue in the demyelinated area selection. For this, simply assume the normal shape of the corpus callosum while selecting the demyelinated area. Such an approach can introduce some variability into the area and volume calculations, thus; we highly recommend performing all analyses in a blinded manner.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Mar Bosch Queralt (mar.boschqueralt@medizin.uni-leipzig.de or mar.bosch-queralt@dzne.de).

Materials availability

This study did not generate new unique reagents or mouse lines.

Data and code availability

The ImageJ macro required to sequentially analyze lesion areas from a series of pictures can be found at GitHub and Zenodo.

The code generated during this study necessary to compute the lesion volume from a series of lesion areas is available at GitHub and Zenodo.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101141.

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AUTHOR CONTRIBUTIONS

M.B-Q and M.S. conceived the project and designed experiments. M.B-Q, V.T., and A.D. carried out experiments. L.V. and I.A. developed and provided tools, and M.B-Q wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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