**Materials:**

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| **Name** | **Company**  | **Catalog Number** |
| **Animals**Female Mice 6-10 weeks of age | Envigo or Jackson Laboratories |  |

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| **Name** | **Company**  | **Catalog Number** |
| **Materials**1 ml syringes10 ml syringes15 ml conical tube#15 blades4-0 monofilament26G 5/830 guage needle, bent 90°Alpha-dri BeddingBacitracinBiosafety hoodCastroviejo needle holderClippersCoverslipsCryostatCotton tip applicators Dural scissorsEmbedding MoldsFriedman-Pearson micro ronguers, curved tipsGauze padsGel foamGlass micropipette tipsGraefe forcepsHeating padInverted MicroscopeJeweler’s forcepsLacrilubeMicropipette bevellerMicropipette pullerMicroscope SlidesMouse decapiconeMouse vertebral stabilizerNitrogen compressed air tankPicoinjector or nanoinjectorScalpel handleSponge spearsSpring scissors with fine bladesStaple applicatorStaplesStereotaxis frameStraight iris scissorsSurgical microscopeThoracotomy ScissorsWater heating pad | B-DB-DFisherF.S.T.Henry ScheinB-DHenry ScheinCincinnati LabsHenry ScheinFlow SciencesF.S.THenry ScheinHarvard ApparatusLeicaHenry ScheinF.S.TFisherF.S.THenry ScheinHenry ScheinWarner Instruments CorpF.S.THenry ScheinNikon InstrumentF.S.THenry ScheinSutter Instrument Sutter InstrumentFisherBraintree ScientificLouisville Injury System Scott-GrossF.S.TF.S.TF.S.TF.S.TF.S.TBenchmark digitalHenry Schein ZeissFSTHarvard Apparatus | 30962830960414-959-70C10015-00036-626, 4-0305115036-345ADT108-2912FS3060m12565-14025-11364-0712CM 3050S003-26315002-081260319B16221-14006-936031-551G120F-411053-10263-5521Nikon TIE Inverted11254-20039-886BV-10P-9712-550-17Handmade (equivalent)PV80010003-1218105-0315013-1212031-0912032-07012-33814002-14729715 |

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| **Name** | **Company**  | **Catalog Number** |
| **Reagents**1% Lysolecithin4% Paraformaldehyde0.9 % sodium chloride2,2,2 TribromoethanolAlexa Fluor 647 (mse)Alexa Fluor 488 (rbt)APC MseBSABuprenorphine (0.3mg/ml)Derma ChlorEriochrome CyanineEthidium Bromide (0.2mg/ml)EtOHFeClFluromountGentamicin (0.6mg/ml)GFAP ChkHCLHoeschtH2SO4KCLKetamine (100mg/ml)KH2PO4KimWipesMBP RbtNaClNa2HPO4NDSNH4OHOlig-2 RbtPerMountSucroseTexas Red conjugated (chk)Tissue Freezing MediumTritonTriz AcidXylazine (20mg/ml)Xylene  | SigmaSigmaHenry ScheinSigmaJackson ImmunoThermo FisherMilliporeSigmaReckitt BenckiserHenry ScheinSigmaInvitrogenSigmaSigmaSouthern Biotech Henry ScheinabcamSigmaSigmaSigmaSigmaHospiraSigmaFisherChemiconSigmaSigmaJackson ImmunoSigmaMilliporeFisherSigmaJackson Immuno Fisher, General DataSigmaSigmaLLOYD laboratories, Akorn IncFisher | L1381, 5mgP6148040-198T48402-25G715-605-151A21206OP80A8806055-4803275215585-011E7023F2877-500G0100-01006-913AB1674H1758-500 ml320501-500 mlP-3911P5379-500G06-666AAB980S7653S0751 – 1kg017-000-121221228-500 mlAB9610SP15-500S9378703-076-15515-183-39, TFM-GT8787 X-100T59414811 – 20 mlX3P-1 Gal |

**Procedures:**

All animal procedures were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, “Guide for the Care and Use of Laboratory Animals, Eighth Edition” (Committee for the update of the Guide for the Care and Use of Laboratory Animals, National Research Council, 2010), and with the approval of the University of Louisville Institutional Animal Care and Use Committee. Adult C57Bl/6 mice 8 - 10 weeks of age weighing 15-25 gm were used for this methods section (Envigo – Harlan; Indianapolis, IN). Rats weighing 180-220 gm are also commonly used. Because of the similarities in procedures, details of the mouse surgery are presented below.

**Glass Micropipette Preparation:**

1. Prepare micropipette needles by pulling a glass capillary tube with an inner diameter (ID) = 0.53 mm to 2.0 mm, using a Sutter Instrument model P-97 micropipette puller (or equivalent). Size of the glass capillary depends on the volume of demyelinating solution. For this procedure glass capillaries with an ID = 0.69 mm, OD = 1.2 mm, with a length = 10 cm (warner Instruments, 64-0790) are commonly used.

Note: Capillaries contain a filament that helps draw up and expel liquids.

1. Pull glass capillaries to very fine tips using a high temperature setting (600 °C -800 °C) with small force (20 psi) to produce a long tapered tip for deep tissue injection. If using the Sutter Instrument use program 21-1 (Heat – 600, Pull - 29, Velocity - 37, Time 150).
2. Once pulled trim micropipette needles to an ID of 20-30 µm for mice and 30-35 µm for rats using #5 forceps with the aid of a micrometer. Each hash mark on a micrometer represents 5 micrometers.

Note: The inner diameter is based on the viscosity of solution, cell diameter (if cellular therapies are used), and size of the animal being injected. The larger the ID the less resistance for fluids.

1. Once an ID has been achieved bevel pipettes using a Sutter Instrument model BV-10 micropipette beveller (or equivalent). Tips should be beveled at 35-45° to facilitate tissue penetration. After beveling, flush pipette needles with 100% Ethanol to remove any debris.
2. Always prepare 5-10 pipettes for each operative day to replace broken or clogged needles.
3. Sterilize glass pipettes by laying them on adhesive tape inside a petri dish exposing them to UV light for one hour.

**Preparing micropipette for Injection:**

1. Reconstitute lysolecithin to a 1% solution with sodium chloride (NaCl; pH 7.4) or prepare 0.2mg/ml ethidium bromide solution (Kuypers, 2013). Store Lysolecithin in small aliquots (75 µl) at -20 °C. Thaw aliquots to room temperature before use. Keep ethidium bromide at room temperature.

Note: Glial toxins will vary depending on experimental conditions of demyelinated lesions.

1. Handle the pre-pulled glass pipette with extreme caution to avoid damaging the fine tip. Label micropipette needles with hash marks to represent 0.25 µl between each mark.
2. Attach the pipette needle directly to the arm of the stereotactic device.
3. Load the pipette needle by connecting a 60 ml syringe to the inlet of the vacuum compressed air tip with plastic tubing. Make sure seal is tight so that no air escapes.
4. Once connected lower your pipette needle into your demyelinating solution.
5. Gently pull back on the syringe plunger to slowly fill the pipette needle with glial toxin until the required amount is achieved.

Note: Avoid introducing air bubbles into the pipette needle. If air bubbles are present pipette preparation must be repeated. Pipettes will be able to inject 4-8 animals before needing to be refilled.

1. Remove the 50 ml syringe from the vacuum inlet and reconnect the pneumatic pico-pump (PV800) to the stereotaxic frame.

**Pre – Surgical Animal Preparation:**

1. Anesthetize mice with an intraperitoneal injection (IP) of ketamine (50mg/kg) and xylazine (5mg/kg) or 2.0% avertin (2,2,2-tibromoethenol in 1.25% 2-methyl-2-butanol in sodium chloride solution) at 400mg/kg I.P. using a decapi-cone or by grabbing the scruff of the animal at the shoulder blades. Make sure the animal is safely secured before inserting the needle. Insert the needle with the bevel up and gently pull back on the syringe to test for proper placement.

Note: When the needle is properly placed you will get negative pressure. If blood, feces or urine are pulled into the syringe then remove the needle without injecting and discard the syringe and repeat the procedure again. Avertin is not an FDA approved drug and needs IACUC approval before use.

1. Place the animal in a secured location until they are fully anesthetized. The animal will be under anesthesia for approximately 45 minutes - 1 hour.
2. Firmly pinch the toe of the animal to assure that adequate sedation has been achieved. A properly anesthetized animal will not respond to toe pinch. Anesthetic depth should be monitored every 5-10 minutes throughout the surgical procedure.
3. Shave a 2 inch square along the dorsal spinal column using clippers. Make sure the clipper blade lays flat against the animal’s skin to prevent tissue damage.
4. Make sure all clipped hair has been removed from the surgical area.
5. Clean the surgical area with 100% chlorohexidine solution applied to a 2X2 gauze pad. Then repeat using a 10% chlorhexidine solution. Carefully apply a small amount of lacrilube to the eyes to prevent drying during the surgical procedure.
6. Administer 1 ml of pre-warmed subcutaneous fluids (SQ) to prevent dehydration.

**Performing the Surgical Procedure:**

To create the desired demyelinating lesion, sterotaxis is used to align the depth of the injection in the ventral-dorsal plane as well as the location of the injection in the medial-lateral plane. To generate demyelinating lesions of the Ventral Lateral Funiculus (VLF) the following parameters are used:

VLF lesions in rats, inject at 1.6 and 1.3 mm depths and 0.7 mm lateral to midline.

VLF lesions in mice, inject at 1.1 and 0.7 mm depths and 0.45 mm lateral of midline.

\*Two unilateral injections spaced 1.75 mm apart were given at a depth of 1.1 and 0.7 mm and 0.45 mm lateral of midline for this demonstration.

**Additional Comments**: Aseptic technique should be used for all steps during this procedure (gloves, bonet, mask). Surgical tools should be sterilized before coming in contact with the animal. Each surgical procedure may take 15-20 minutes. It is recommended to have at least 1-2 people assisting for each surgical procedure. If anesthetic boosting is required during the surgical procedure inject ketamine (50mg/kg) IP or Avertin (20-40mg/kg). This surgical procedure may be modified for rats.

1. Move the animal to the surgical table dorsal side up. Surgical tape (optional) may be used to secure the animal to the operating station.
2. Under a surgical microscope with adequate lighting use small surgical scissors to make a sagittal incision in the skin from T6-T12.
3. Cut away the connective tissue overlaying the musculature of the dorsal column.
4. Using extra fine spring scissors separate the muscles from the lamina, exposing the lateral aspects of the facets bilaterally. Adequate exposure is important to allow the vertebral column to be grasped when lifted onto the stabilizer using the facets.

Note: The spine stabilizer used was developed by the Louisville Injury System Apparatus (LISA) However other devices may work well provided the spine is securely immobilized.

1. Using forceps or closed spring scissors, feel for the hard surface of T11 (T11 will protrude slightly higher than the other vertebrae).
2. Once T11 has been located, use spring scissors to make shallow lateral cuts (2-3 mm deep) in the connective tissue between T8 - T9 and T9 - T10 repeat between T10 - T11.

Note: If bleeding occurs hold sponge spears or cotton tipped applicators to area for 15-30 seconds or until bleeding subsides. Gel-foam may be applied to the area as aid for coagulation.

1. Using Graefe forceps pull the T9 and T10 vertebrae up and off of the column. Removal of the vertebrae will slightly expose the spinal cord so that a laminectomy may be performed.
2. Place the animal in the Louisville Injury System Apparatus.
3. Use extra fine Graefe forceps to grasp the lateral facets and place the toothed blades of the stabilizer on either side of the facets, thereby securing the vertebral column in the stabilizer by tightening both sides of the stabilizer.

Note: The vertebral column must be aligned parallel with the base of the stabilizer.

1. Very gently use #2 laminectomy forceps to remove the lamina from the vertebral column. Make sure the area does not contain any sharp edges which may interfere with pipette placement.
2. Clean the interlaminar soft tissue with a cotton tipped applicator. Open the dura using the tip of a bent (90°) 30 gauge needle. Once the dura has been penetrated expand the opening with laminectomy forceps. Remove any cerebrospinal fluid that may have leaked out when the dura was penetrated.

Note: The dural opening should only be large enough to allow the micropipette tip to enter the spinal cord.

1. Transfer the animal in the spine stabilizer to the injection system. Place the animal perpendicular to the tip of the micropipette. Align the tip to the midline of the dural opening. Slowly lower the pipette tip until it is just 1-2 mm above the cord.
2. Using the graded measurements of the Y-axis move the pipette needle 0.4 mm lateral of midline.
3. Slowly move the pipette needle in a downward direction until it just barley touches the spinal cord to obtain a surface reading of x-axis. From this reading, subtract 1.1 mm. Use a quick downward motion to pierce the spinal cord surface and slowly lower the pipette tip until the new measurement is reached.
4. Using the pico-pump deliver half of the total volume per injection site of glial toxin into the spinal cord. Wait 2 minutes to allow the demyelinating agent to diffuse.

Note: Lesions depend not only on the target but also on the volume and concentration of the solution, each of which has a specific pattern of diffusion.

1. Using your x-axis reading, add 0.5 mm. Raise the tip to this new measurement. Inject a second bolus of glial toxin into the spinal cord. Wait 2 minutes.

Note: If lesions are required at a different location, refer to a mouse brain atlas for specific parameters.

1. Remove the pipette needle from the spinal cord.
2. Calculate the z-axis to move 1.75 mm caudal to the first injection site.

Note: This only applies if a second injection site is needed.

1. Repeat steps 14 – 17.
2. Loosen the pins of the facet blades and remove the animal from the LISA apparatus.
3. Using 4-0 or 5-0 monofilament tie a single suture through the muscle and adipose tissue overlaying the spinal column.
4. Close the skin incision by applying wound clips using a staple applicator. Apply bacitracin to the incision area. Administer 1 ml of pre-warmed SQ fluids, and gentamicin (5mg/kg) SQ.

Place the animal in a recovery cage that has been placed halfway onto a warm water circulating heating pad.

Note: Only place the cage half way on the heating pad so the animal may escape the warmed area if over-heating occurs.

1. Administer analgesics as directed by the institute’s animal care and use program. Animals are fully ambulatory and capable of self-feeding and drinking as soon as they recover from anesthesia.
2. Repeat the procedure on any remaining animals.

Note: Between each use the micropipette tip should be submerged in liquid to prevent clogging. The same pipette needle may be used throughout 4-8 surgeries before the needle dulls and has to be replaced. Separate needles should be used when multiple demyelinating agents are being considered. All used micropipette needles should be discarded at the end of surgery. It is not recommended to reuse needles.

**Post-op Animal Care:**

1. Keep animals on a water-heating pad (cage half on half off) overnight in a recovery room. Cages should contain alpha-dri bedding with food pellets laid directly on the alpha-dri for ease of access.
2. Return animals to normal housing condition 24 hours post-surgery.
3. Administer pain meds Buprenorphine (0.1 mg/kg) for 48 hours post-surgery, or other IACUC approved pain med.
4. Administer antibiotics Gentamicin (5mg/kg) SQ once daily for 7-10 days, or other IACUC approved antibiotic. Fluids may also be given as need for dehydration.
5. Keep daily health logs to record each animal’s health status for the remainder of the study.

**Tissue Processing and Analysis:**

Lesions will evolve over the next 3-4 weeks. 48 hours after injection OPC’s emerge in lesion, 3 Days post injection demyelinating Oligodendrocytes will appear, 14 days post injection Oligodendrocytes will be recruited, and 14-21 days after injection active remyelination will take place. Ethidium bromide lesions will not remyelinate. Animals were euthanized at 1, 2, and 4 weeks post injection for tissue processing.

1. Anesthetize the animal with ketamine (100mg/kg) xylazine (10mg/kg) IP or Avertin (800mg/kg) IP.
2. Firmly pinch the toe of the animal to assure that adequate sedation has been achieved.
3. Perform a thoracotomy and perfuse the animal with 30 ml of PBS (ice-cold) over 2-3 minutes followed by 30 ml of 4% paraformaldehyde (ice-cold) in PBS for 2-3 minutes.
4. Dissect out the spinal cord using #2 laminectomy forceps and small spring scissors. Use the forceps to remove the vertebrae and the scissors to cut roots along the spinal cord.
5. Post-fix the cords in 4% paraformaldehyde in PBS at 4 °C overnight (hard fixation) or 1-2 hours (light fixation). Wash cords in PBS by inversion in a 15 ml conical tube. Decant the PBS and add 30 % sucrose. Store cords at 4 °C for at least 72 hours or until they can be blocked in embedding molds containing tissue freezing media (TFM).
6. Cut 2-3 cm of tissue centering the injection site and embed into embedding molds. Place the embedding molds over dry ice until the TFM is fully frozen. Molds can be stored at -20 for short term or -80 for long term storage.

Note: Multiple cords can be placed in the same embedding molds. Method will depend on histological needs.

1. Section the embedding molds on a cryostat at 20-30 µm per section. Size of sections is based on the anatomical size of the structure being represented (ie. OPC’s, myelin sheaths, axons, etc). Transfer the section to a microscope slide and gently place your index finger on the back of the slide containing the tissue section until the section is embedded on the slide, freezing-media will look clear. Once all tissue sections have been cut store them at -20 °C until further processing is required.

**White Matter Staining (Eriochrome Cyanine Stain):**

To detect the amount of white matter sparing after glial toxin injection. Prepare the following solutions:

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| **Solution A**Need:100% FeCl₃ = 10g FeCl₃ to 10ml dH₂O3% HCl = 3.0ml HCl in 97.0ml dH₂O**To make Solution A:** 10% FeCl₃ = 10ml 100% FeCl₃ + 90ml 3% HCl**Solution B**Need:-0.5% H₂SO₄ = 1.5ml H₂SO₄ + 298.5ml dH₂O-Eriochrome Cyanine**To make Solution B:** 0.2% ECRC = 0.6g ECRC in 300ml 0.5% H₂SO₄. Boil for 5 min. No need to filter. | **Solution C** (differentiation solution)Need:-0.5% aq. NH₄OH = 3.0ml 29.1% NH₄OH in300ml dH₂O\*Must be made fresh for each use; cannotreuse.**Solution D** (EC staining solution) -Can re-use multiple times after use.-Replace one time a year.Need:-12.0ml Solution A + 240.0ml Solution B- Bring total volume of 300ml with dH₂O.  |

EC Staining Procedure:

1. Remove frozen slides from -20 °C and let thaw at RT for one hour. A slide warmer may also be used, set to 37 °C for 20 minutes.
2. Clear and hydrate sections by submerging in wells in the following order:
	1. Xylene (2 x 30 min) gently agitate to remove excess gum.
	2. 100% EtOH (3 min)
	3. 95% EtOH (3 min)
	4. 70% EtOH (3 min)
	5. 50% EtOH (3 min)
	6. dH₂O (2 min)
3. Stain sections in EC staining solution for 10 minutes.
4. Dip Sections in 2 wells of tap H₂O to remove excess stain.
5. Differentiate 30 sec (gently dip slides up and down, 2-3 times) then wash in 2 wells of tap H₂O to stop differentiation. Verify stain under microscope before proceeding. Gray matter should be light blue, white matter should be deep blue. Additional differentiation may be needed (15-30 seconds, wash and verify).
6. Air dry in hood overnight. Next day: place slides in xylene for 10 minutes. Wipe off excess xylene with kimwipe and let dry in hood 20 minutes.
7. Coverslip with PerMount and let dry overnight in hood.

**Immunohistochemistry (Fluorescence):**

Immunohistochemistry is used to visualize axons and myelin. If using fresh tissue you will want to

follow this same protocol using PBS instead of TBS. Prepare the following solutions:

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| **Solution A** (Blocking Buffer, 0.5% BSA)Need:-5ml NDS (100%)-0.75 ml Triton (20%)-0.25g BSA-44ml TBS\*Combine in a 50 ml conical tube**Solution B** (10X PBS - 1 Liter)Need:-80g NaCl-2g KCL-14.4g Na₂PO₄-2.4g KH₂PO₄\*Dissolve solutes in 1000 mls dH20\*use as 1X working solution**Solution C** (0.1 M TBS – 1 Liter)Need:-15.76g Triz acid-8.76 NaCl\*Dissolve solutes in 1000 mls dH20\* For 0.1% Triton add 1ml of 100% triton. | **Solution D** (Primary Antibodies)Need:-GFAP (chk) 1:1000-Olig-2 (rbt) 1:100-APC (mse) 1:100-MBP (mse, rbt)\*Dilute antibodies in 0.1 M TBS + 0.3% Trition + 0.5 % BSA + 5% NDS**Solution E** (Secondary Antibodies)Need:-Hoescht (Blue) 1:1000-467 (mse) 1:200-488 (rbt) 1:200-Texas Red (chk) 1:200\*Dilute antibodies in 0.1 M TBS + 0.3% Trition + 0.5 % BSA + 5% NDS |

Day 1:

1. Warm slides on a slide warmer at 37 °C for 20 minutes
2. Remove media around the tissue
3. Pap pen around each section
4. Wash in 0.1 M TBS for 5 min (3 times)
5. Shake off excess TBS and dry with a kimwipe
6. Block with 10% NDS in 0.1 M TBS + 0.3% Triton + 0.5% BSA solution. Place in a humidor for 1 hour at RT.

Note: serum is based on secondary antibody.

1. Rinse 10 min in 0.1 M TBS
2. Shake off and dry with a kimwipe
3. Apply primary antibodies in 0.1 M TBS + 0.3% Trition + 0.5 % BSA + 5% NDS. Leave overnight on shaker at 4 °C.

Note: should have one slide as isotype control to see unspecific staining.

Day 2:

1. Wash slide in 0.1M TBS, 0.1 M TBS + 0.1% Triton, and 0.1 M TBS (10 minutes each).
2. Apply secondary antibody in 0.1 M TBS + 0.3%Trition + 0.5% BSA + 5% NDS. Incubate in dark at RT for 1 hour.

Note: Add hoescht at this step as a nuclear stain to check for live and dead cells.

1. Cover Slides since secondary antibodies may be sensitive to light.
2. Wash in 0.1 M TBS, 0.1 M TBS + 0.1% Triton, and 0.1 M TBS (10 minutes each)
3. Carefully dry slide with kimwipe and vacuum suction around tissue sections.
4. Coverslip with fluromount.