Rodent EAE model for the study of axon integrity and remyelination

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Method Article

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Abstract

Introduction

Sha Mi, Bing Hu and Wutian Wu made equal contributions to this work. Myelin oligodendrocyte glycoprotein \(MOG)-induced murine experimental autoimmune encephalomyelitis \(EAE) is a widely accepted model for studying the clinical and pathological features of multiple sclerosis \(MS). The model has previously been used to demonstrate that fostering remyelination can be effective in moderating disease progression. Approaches to enhance myelination include the transplantation of OPCs, Schwann cells, olfactory ensheathing cells, or neural stem cells into the primary demyelinated lesions[1], or the promotion of oligodendrocyte precursor cell \(OPC) differentiation[2-5], such as by LINGO-1 antagonism[6,7]. Functional recovery from demyelination can be assessed via approaches for the histological preparation of tissues for light microscopy, magnetic resonance DTI imaging, and electron microscopy, as presented in this protocol.

Reagents

ANIMALS Mouse: 8-12 week-old wildtype \(WT) and LINGO-1 knockout \(KO) mice in >95% pure C57BL6J background. Rat: 9 week-old Sprague-Dawley rats \(220-280g). It is important to use age- and weight-matched animals in experimental groups so as to minimize variance in EAE disease onset and progression. \! CAUTION All experiment procedures should comply with national and institutional regulations for the care and use of laboratory animals. REAGENTS Recombinant rat MOG, used as an immunogen for EAE induction, corresponds to the N-terminal sequence of rat MOG \(amino acids 1–125). Store peptide stocks at -20 °C. Complete Freund’s adjuvant \(CFA, cat# 7001, Chondrex, Inc, WA). Store at 4 °C. CAUTION CFA contains heat-inactivated Mycobacterium tuberculosis \(strain H 37 RA; Difco Laboratories) that stimulates the innate immune response. Avoid inhalation and contact with skin and eyes. Three-way stopcock \(Luer connector) 5 ml glass syringe Pertussis toxin \(List Biological Labs, Campbell, CA) DMEM \(Invitrogen, CA) Gey’s hemolytic solution \(Invitrogen, CA) Heat-inactivated fetal calf serum \(FCS, Invitrogen, CA) Cell strainer with 100 mesh \(BD Falcon, MA) 3H-thymidine \(Amersham, NJ) Mouse interleukin 12 \(mIL-12, R&D System, MN) MOG 35-55 peptide \(Bachem) Mixture of ketamine/xylazine for anesthesia Anti-LINGO-1 antibody and IgG1 isotype antibody \(Biogen Idec, Inc.) 4% paraformaldehyde 0.1M phosphate buffer \(PB, pH7.4) 1M sodium hydroxide solution Pentobarbital sodium \(Dorminal 20%) Graded ethanol Chloroform Paraffin wax Gelatin \(for coating glass slides) Toluene Ethanol Methanol Acetic acid Lithium carbonate Mounting medium \(Permount, Fisher Scientific) 25% glutaraldehyde aqueous solution \(Calbiochem) 2% osmium tetroxide solution 1, 2-propylene oxide \(Merck) Methyl nadic anhydride – MNA \(TAAB) Dodecenyl succinic anhydride – DDSA \(TAAB) Taab 812 resin \(TAAB) 2, 4, 6-tri\(dimethylaminomethyl) phenol – DMP \(TAAB) Poly cut-ease \(Polysciences) Luxol fast blue \(0.1%) in methanol for myelin staining Toluidine blue \(0.5 %) in sodium tetraborate \(1%) for semithin section staining 1% neutral red solution \(Sigma) Sodium tetraborate Lead nitrate \(for preparing lead citrate

solution) ▯ Tri-sodium citrate \(for preparing lead citrate solution) ▯ Uranyl acetate ▯ Sodium hydroxide pellet CAUTION Chemicals such as pertussis toxin, ethanol, methanol, paraformaldehyde, sodium hydroxide, chloroform, toluene, acetic acid, permount, osmium tetroxide, 1, 2-propylene oxide, dodecenyl succinic anhydride, Taab 812 resin, phenol, uranyl acetate and sodium hydroxide are volatile, irritant, corrosive, toxic or/and harmful, wear gloves and mask, handle in a fume hood.

Equipment

EQUIPMENT ▯ Basic cell culture equipment ▯ Centrifuge with temperature control ▯ Cell incubator ▯ Vortex with an attachment capable of holding multiple eppendorf tubes ▯ 1 ml syringe with 26 gauge x 3/8” needle \(Becton Dickinson & Co.,) ▯ Eppendorf tubes ▯ Scale \(0.1 g sensitivity) ▯ Surgical microscope \(6X-40X) ▯ Alzet mini-osmotic pump with brain infusion kit \(Alza Corporation) ▯ Light microscope with digital camera ▯ Image J \(NIH free image processing software) ▯ Glasswares ▯ Hot plate with magnetic stirrer ▯ Surgical tools ▯ Perfusion kit ▯ Oven ▯ Dissecting light microscope ▯ EM tissue processor ▯ Glass knife ▯ Ultracut microtome ▯ Diamond knife \(Diatome) ▯ 200 mesh copper grid ▯ Electron microscope \(Philips, Model 208) ▯ 7 Tesla MRI scanner with maximum gradient of 370 mT/m \(70/16 PharmaScan, Bruker Biospoin GmbH, Germany) using a mouse brain resonator with an inner diameter of 23mm for transmit and receive ▯ Matlab program \(The Mathworks Inc., Natick MA)

Procedure

Induction of active EAE in mouse 1| Dilute the MOG peptide in 0.01M phosphate buffered saline \(PBS, pH 7.4) to a final concentration of 2 mg/ml. Mix equal volumes of MOG/PBS and complete Freund’s adjuvant \(CFA, 2mg/ml) thoroughly between two 5-ml glass syringes using a three-way stopcock with a Luer connector to form a thick peptide/CFA emulsion. Centrifuge the emulsion gently and load slowly into 1-ml syringe using an 18 gauge needle ▲ CRITICAL STEP Due to the viscosity of the MOG/CFA emulsion, use a 18 gauge needle to minimize introduction of air bubbles. Replace the18 gauge needle with a 27 gauge needle for subcutaneous injection of 200 µl of MOG/CFA emulsion into two sites at flank, 100 µl per site. On the same day, Inject 50 ng \(100 µl) of Bordetella pertussis toxin intraperitoneally. After MOG induction, weigh each animal and determine the EAE scores for motor functions daily. ▲ CRITICAL STEP Special attention on food, water and urination should be paid once mice have clinical symptoms of EAE. EAE scores are used as a clinical metric for demyelination. Disease severity is scored on a clinical scale of 0 to 6 with the following criteria: 0, no detectable sign of EAE; 1, weakness of the tail; 2, definite tail paralysis and hind limb weakness; 3, partial paralysis of hind limbs; 4, complete paralysis of hind limbs; 5, complete paralysis of hind limbs with incontinence and partial or complete paralysis of forelimbs; 6, dead. **Induction of active EAE in mouse by transfer of encephalogenic T-cells** 2|Euthanize the MOG-immunized mice 10 days later. Remove and pool inguinal and axillary lymph nodes and spleen in DMEM. Pass the tissues through a sterile cell strainer with 100 µm mesh attached to a sterile 1-ml syringe to form single cell suspension. Centrifuge the cell suspension at 1000 rpm for 10 min at 4 °C, discard the supernatant and resuspend the pellet in DMEM. Lyse the red

blood cells with Gey's hemolytic solution and incubate for 10 min at 4 °C. Wash the cell with DMEM and count the number of live cells. Centrifuge the cells at 1000 rpm for 10 min at 4 °C and resuspend at 5 x 10^6 cells/ml in DMEM containing 10% FCS, 20 μg/ml MOG 35-55 peptide and 20 ng/ml murine IL-12. Keep the cells at 37 °C, 10% CO_2 for three days. Wash once with PBS before harvest the cells. Inject mouse intraperitoneally with 2.5-3 x 10^7 cells in 200 μl PBS and 50 ng pertussis toxin in 100 μl PBS. After encepahologenic T-cell transfer, assess each animal assessed daily, as described above, using the active induction of EAE protocol. **Induction of rat EAE model** 3| Procedures for induction of EAE rat model are similar to that in mice above and the previous studies[8,9]. Briefly, mix 50 μl of CFA with 50 μl of recombinant rat MOG \(50 μg in PBS, corresponding to the N-terminal sequence of rat MOG) in the eppendorf tube. Anesthetize the rats with ketamine \(80 mg/kg)/xylazine \(8 mg/kg) mixture, with 6-8 rats anesthesized at a time before emulsion injections. Inject the 100 μl cocktail into rat by intradermal injection at the base of the tail. ▲ CRITICAL STEP Generally, excess emulsion is prepared due to loss from surface adhesion. The injection should be performed slowly, requiring about 30-60s before removing the needle. After MOG induction, determine the EAE scores for each rat using the following system: grade 0.5, distal paresis of the tail; grade 1, complete tail paralysis; grade 1.5, paresis of the tail and mild hind leg paresis; grade 2.0, unilateral severe hind leg paresis; grade 2.5, bilateral severe hind limb paresis; grade 3.0, complete bilateral hind limb paralysis; grade 3.5, complete bilateral hind limb paralysis and paresis of one front limb; grade 4, complete paralysis \(tetraplegia), moribund state, or death. Generally the induced EAE rats are required to be killed when their body weight loss exceeds 1/3 of initial body weight or severe clinical signs occur \(EAE score ≧3.5). It is difficult to study animals that followed a progressive EAE course for more than 60 days because these animals developed considerable loss of body weight and other complications. The EAE animals survived for 35 days in our study. ▲ CRITICAL STEP Special attention on food, water and urination should be paid once rats have clinical symptoms of EAE. Clinical signs of EAE usually begin between 9 and 20 days post-immunization. **Treatment with Anti-LINGO-1 antibody in rat EAE model** 4| Treat the randomly assigned MOG-induced animals by intrathecal delivery of antagonist antibody to LINGO-1 or isotype control antibody \(IgG) three days after MOG induction and before the onset of EAE symptoms. Each 200 μl- mini-pump held 2.6 mg anti-LINGO-1 antibody or control IgG and delivered 185 μg/day for two weeks, with the minipump replaced every two weeks. The mini-osmotic pump is placed and fixed on the vertebra column following the procedures below. Anathetize the rats and expose the vertebrae at lumbar level \(Fig 1a,b). Drill a 1 mm hole on the fourth lumbar vertebra \(Fig 1c,d). Insert the tip of the cannula connected with the pump into the hole for about 2 mm depth on the L4 vertebra and fix with instant glue \(Fig 1e). At the end of the study, check the position of the pump's tip to ensure that it is in the subarachnoid space \(Fig 1f). Finally, check the residual volume in the pump as to ensure the success of deliver. Deliver the drug systemically after the onset of EAE clinical symptoms by intraperitoneal injections of 8 mg/kg antibody twice per week \(either anti-LINGO-1 antibody or isotype control antibody), commencing when an EAE score of 1 is reached. Sacrifice the rats after two weeks of treatment. **Histology** **Animal perfusion** ˙TIMING approximately 2hrs 5 | Prepare fixative \(4% paraformaldehyde in 0.1M phosphate buffer, pH7.4) by stirring and heating at 60°C for ~1.5 hrs on a magnetic hotplate. Filter the fixative before use. Use fixative after cooling to 37 °C or below. Deeply anesthetize the animal with an overdose of pentobarbital \(150

mg/kg). Perfuse the rat with 50 ml of 0.9% normal saline solution and 250-300 ml of 4% paraformaldehyde solution by intracardiac infusion. Examine the infusion cannula and pump as indicated above and harvest the spinal cord. Post-fix the tissues in the same fixative at 4°C for at least 4 hrs. ▲ CRITICAL STEP The paraformaldehyde should be fully dissolved and the solution cleared by adding a few drops of 1M sodium hydroxide solution. **Tissue processing for light microscopy** ˙TIMING approximately 4 days 6 |After post fixation, dehydrate the tissue in 70% ethanol at room temperature overnight. Remove the 70% ethanol, dehydrate the tissue in graded ethanol \(80%, 95% and 100%) at room temperature for total 5 hrs. Clear the tissue with chloroform overnight at room temperature. Embed tissues in melted paraffin wax at 56-58°C for 3 hrs, with 3 changes of wax and maintain embedded blocks at 4°C until sectioning. Cut 10µm sections with a microtome and collect on a drop of distilled water on a gelatin coated glass slide, then dry overnight at 37°C. De-wax sections with toluene and graded ethanol \(100%, and 95%). Stain sections in filtered 0.1% Luxol Fast Blue solution at 37°C overnight, then wash in 70% ethanol followed by tap water. Differentiate stained sections in 0.05% lithium carbonate solution and wash in tap water. After counterstaining with 1% neutral red solution for 5 min, dehydrate the sections in graded ethanol, clear in toluene and mount with mounting medium. ▲ CRITICAL STEP Check sections under microscope until grey and white matters are distinguished. **Tissue fixation and processing for electron microscopy** ˙TIMING approximately 5 days 7|Freshly prepare EM fixative \(2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M PB, pH7.4) before use. Fix tissues at 4°C for 4 hrs, then wash 3 times with 0.1M PB. Post-fix the tissues in freshly prepared 1% osmium tetroxide at 4°C overnight, then wash 3 times with 0.1M PB. After post fixation, the following steps are done in an EM processor with agitation at room temperature. Dehydrate the tissues in graded ethanol \(30%, 50%, 70%, 80%, 90%, 95% ethanol) for 5 min each followed by three changes in absolute ethanol for 10 min each. After two changes in 1, 2-propylene oxide \(PO) for 15 min each, immerse the tissues in a 1:1 PO:Epon mixture for 1 hr, then overnight in pure Epon. Finally the tissues are embedded in pure Epon and keep at 60°C for 3 days. Prepare Epon by mixing MNA, DDSA, Taab 812 resin, DMP and Poly cut-ease \(12.5 ml, 15 ml, 22.5 ml, 0.9 ml and 0.75 ml respectively). Store at -20°C and use at room temperature. Semi-thin section cutting and staining for light microscopy ˙TIMING approximately 5 days 8 |Cut 1µm semi-thin sections from the Epon embedded tissue using a glass knife with an ultracut microtome, and collect on a drop of distilled water on a gelatin coated glass slide. Dry on a hot plate at 80-100°C for 2 hrs. Prepare 0.5% toluidine blue in 1% sodium tetraborate solution \(semi-thin section staining dye). Stain on a hot plate at 80-100°C for 2 min, then wash with distilled water and dry on a hot plate. Dehydrate the sections in graded ethanol and clear the sections in toluene. Mount with mounting medium. ▲ CRITICAL STEP The temperature of the hot plate should not be set below 80°C as sections cannot fully expand at low temperature. Drying for 2 hrs is needed to prevent detachment of sections from the glass slide. **Ultra-thin section cutting and staining for electron microscopy**: ˙TIMING approximately 4hrs 9|Cut 90 nm sections from the Epon embedded tissue using a diamond knife with an ultracut microtome and collect on a 200 mesh copper grid. Prepare and filter lead citrate \(approximately 3%) and 8% uranyl acetate before use10. Stain the grids with lead citrate droplets for 20 min in a Petri dish. Wash the grids 3 times with distilled water. Then stain the sections on droplets of 8% uranyl acetate inside a Petri dish at room temperature for 20 min, wash with distilled water for 3 times. Sections are now

ready for electron microscopic analysis. ▲ CRITICAL STEP Lead citrate forms precipitates with ambient carbon dioxide, which can adhere onto the sections. This is avoided by placing sodium hydroxide pellets in the Petri dish to absorb the carbon dioxide. **Quantification of demyelination area and axon preservation** 10 |To estimate the areas of demyelination on transverse Luxol fast blue stained spinal cord sections \(fourth lumbar segment, L4). Blue areas indicate intact myelin while pale areas indicate demyelination \(Supplementary Fig. 4a online). Trace the total cross-sectional area \(yellow trace, Supplementary Fig. 4b online) and the demyelination area \(red trace, Supplementary Fig. 4b online of each section) with Image J software. Express the demyelination as a percentage of the total demyelination area over total spinal cord area, using random sections from L4 of each animal. 11 |To quantify axons, a line-sampling method is adopted similar to that described previously7. In the toluidine blue stained semithin sections of L4 spinal segment, sample axons which are intercepted by nine fixed sampling lines \(Red lines, Supplementary Fig. 5 online). Select nine tissue strips extending from the gray matter to the pial surface under the 100X oil-immersion objective of a light microscope. Count all myelinated axons whose axoplasm was intercepted by the sampling line. Pool the axonal profiles from the tissue strips of each rat. Due to the resolution limits of light microscopy, unmyelinated and myelinated fibers of cross-sectional areas smaller than 0.1 μm2 are not included in the counts. Magnetic Resonance Imaging All MRI experiments are performed on a 7 Tesla MRI scanner with maximum gradient of 370 mT/m \(70/16 PharmaScan, Bruker Biospoin GmbH, Germany) using a mouse brain resonator with an inner diameter of 23mm for transmit and receive. MRI Data Acquisition ˙TIMING approximately 2.5h 12 |After fixation, immerse the spinal cord into a plastic tube filled with 4% paraformaldehyde. Suspend the spinal cord in formalin solution before and during MRI scan Acquire the diffusion weighted \(DW) images with a diffusion-weighted spin echo imaging sequence. Use a rotationally invariant icosahedral encoding scheme with 6 encoding directions to acquire the DW images11,12. Set the he imaging parameters as follows: TR/TE = 1600/29.0 ms, δ/Δ=3/20 ms, slice thickness = 2 mm \(interslice gap = 0.2 mm), FOV = 27 mm, data matrix = 256 x 256 \(zero filled to 512 x 512), image resolution = 105 x 105 μm2 and two b-values as 0 and 1000 s/mm2. Repeat the sequence 3 times for signal averaging, resulting in an acquisition time of 145 min. **MRI Data Analysis** ˙TIMING approximately 2.5hrs 13 |Extract and diagonalize the diffusion tensors \(DTs) using an in-house Matlab program \(The Mathworks Inc., Natick MA) interfaced to a software implementing the constrained nonlinear least squares methods \(CNLS)[13] for the estimation of three diffusion eigenvalues, i.e., λ_1, λ_2 and $\lambda_{/3}$ \(Dr. CG Koay, STBB/LIMB/NICHD, NIH, USA). Measure the mean diffusivity See figure in Figures section., axial diffusivity See figure in Figures section., radial diffusivity See figure in Figures section. and fractional anisotropy See figure in Figures section. in the ventral, dorsal, left lateral and right lateral column of the second and third segment of the spinal cord. ROIs are drawn based on λ//, λ⊥ and T2-weighted images to avoid covering gray matter.

Anticipated Results

ANTICIPATED RESULTS IN ADDITION TO THAT SHOWN IN THE PAPER Illustration of Non-destructive MRI Analysis Different parameter maps obtained from high-resolution DTI images show clearly distinguished

demyelinated regions \(as shown in Fig.2\(a)-\(d)). Fig. 2\(a) shows the axial diffusivity \($\lambda_{//}$) map which probes water diffusion along the long axis of the axons, whereas Fig. 2\(b), showing the radial diffusivity \($\lambda_{\perp}$), indicates the water diffusion perpendicular to the long axis14. The fractional anisotropy \(FA) map in Fig. 2\(c) illustrate whether the water diffusion has directional dependence or not. In the case of severe demyelination \(as shown in the dorsal horn Fig. 2), where myelin sheaths are spared, there would be less restriction to water diffusion perpendicular to the nerve and thus the $\lambda_{\perp}$ will be increased. Therefore, Fig. 2\(b) shows brighter pixels in the dorsal horn than in other regions of the spinal cord.

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Figures

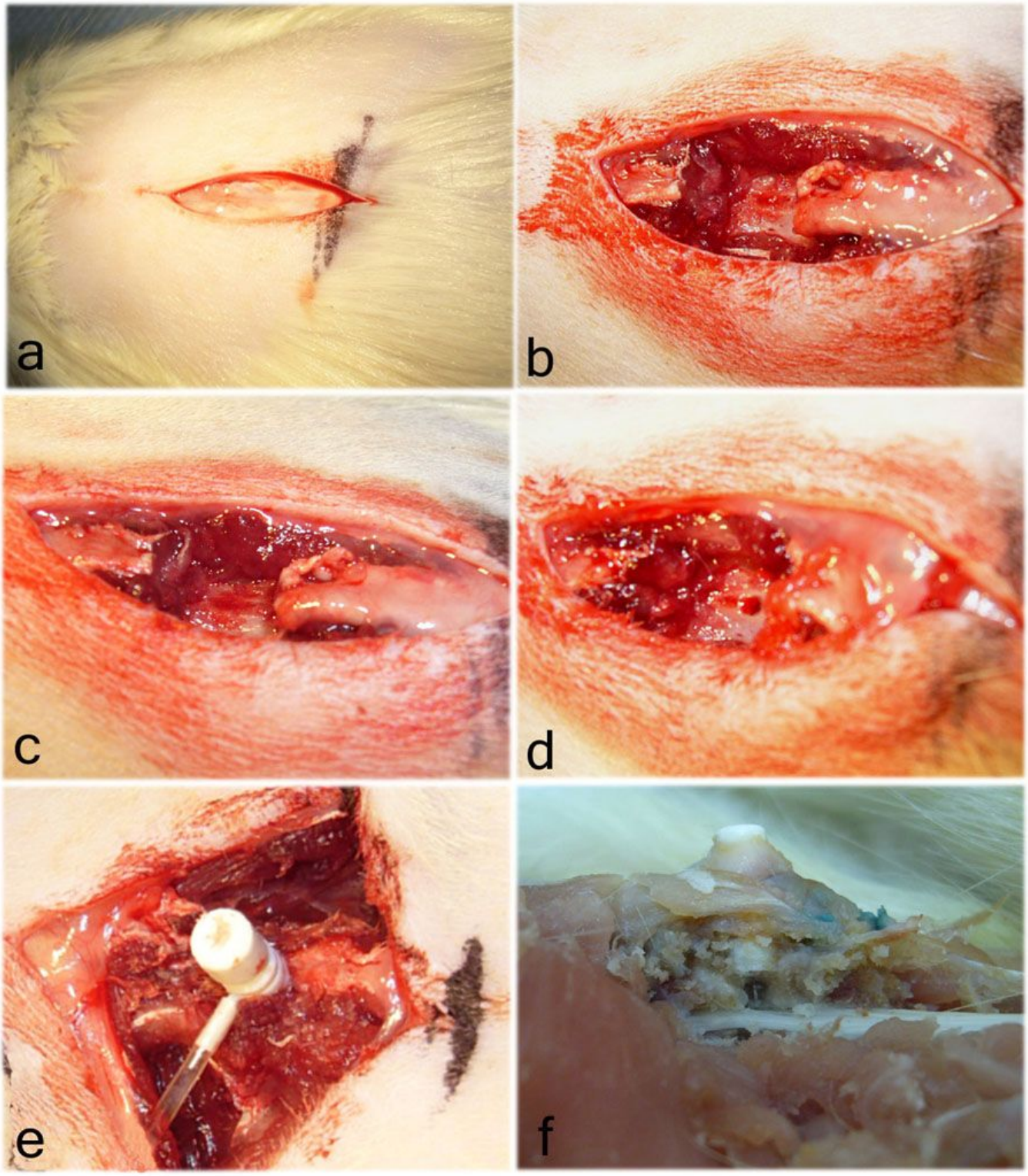

Figure 1

Surgical procedures for antibody delivery. (a) Anesthetize the rats, incise skin and expose the vertebrae at lumbar level. (b, c) Remove the spinous process of the 4th lumbar vertebra and the surrounding muscles and connective tissues. (d) Drill a 1 mm hole through the lamina of the fourth lumbar vertebra (arrow). (e) Insert the tip of the cannula connected with the pump into the hole for about 2 mm depth and fix with

instant glue. (f) Check the position of the pump’s tip to ensure that it is in the subarachnoid space at the end of the study (arrow points to the tip).

Figure 2

Different parameter maps obtained from high-resolution DTI images of the third lumbar segment of the spinal cord (SC) of an EAE-induced rat with severe demyelination in dorsal column. (a) showing the axial diffusivity (λ~//~) map which probes water diffusion along the long axis of the axons. (b) showing the radial diffusivity (λ~┴~), indicates the water diffusion perpendicular to the long axis. (c) showing the fractional anisotropy (FA) map illustrating whether the water diffusion has directional dependence or not. (a-d) In the case of severe demyelination where myelin sheaths are spared, there would be less restriction to water diffusion perpendicular to the nerve and thus the λ~┴~ will be increased. (e) illustrates the ROI placement for ventral (V), dorsal (D), left lateral (LL) and right lateral (RL) column of the spinal cord. (f) T2-weighted image. (g-h) shows two λ~┴~ maps of the second lumbar segment. (i) Tissue section stained for myelin using Toluidine Blue showing the dymyelination in the dorsal column of the lumbar spinal cord.

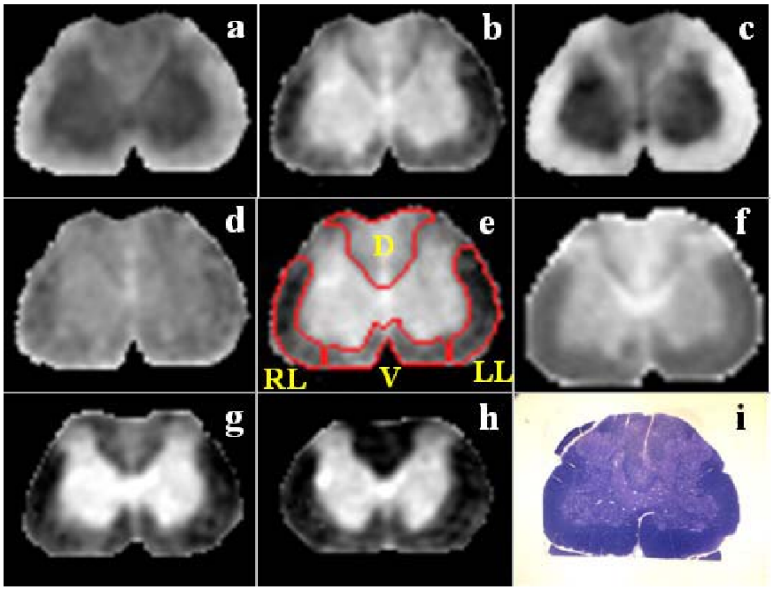

Figure 3

Figure 2 as a pdf

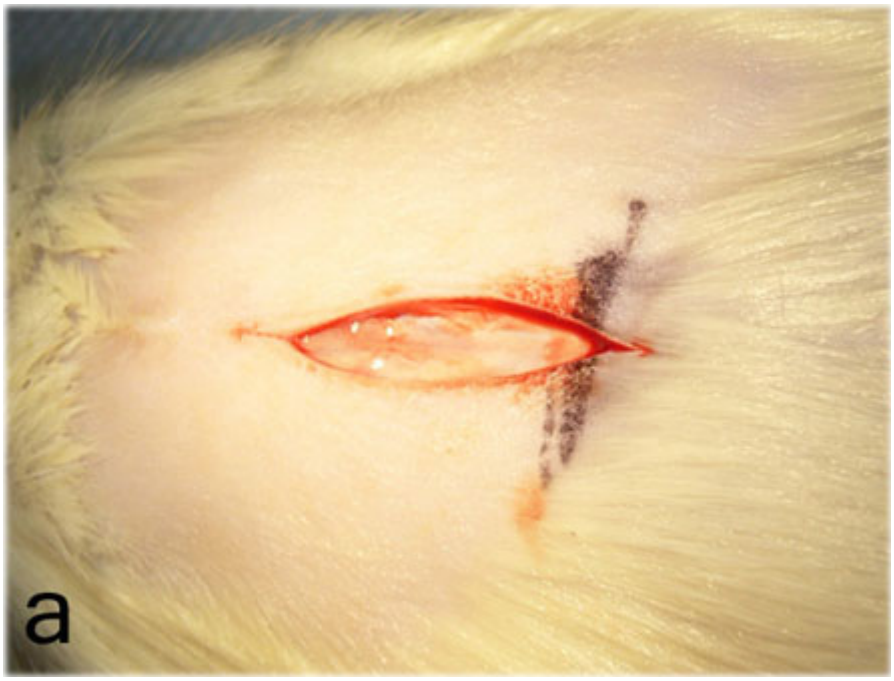

Figure 4

Figure 1 Surgical procedures for antibody delivery. (a) Anathetize the rats, incise skin and expose the vertebrae at lumbar level. (b, c) Remove the spinous process of the 4th lumbar vertebra and the surrounding muscles and connective tissues. (d) Drill a 1 mm hole through the lamina of the fourth lumbar vertebra (arrow). (e) Insert the tip of the cannula connected with the pump into the hole for about 2 mm depth and fix with instant glue. (f) Check the position of the pump's tip to ensure that it is in the subarachnoid space at the end of the study (arrow points to the tip).

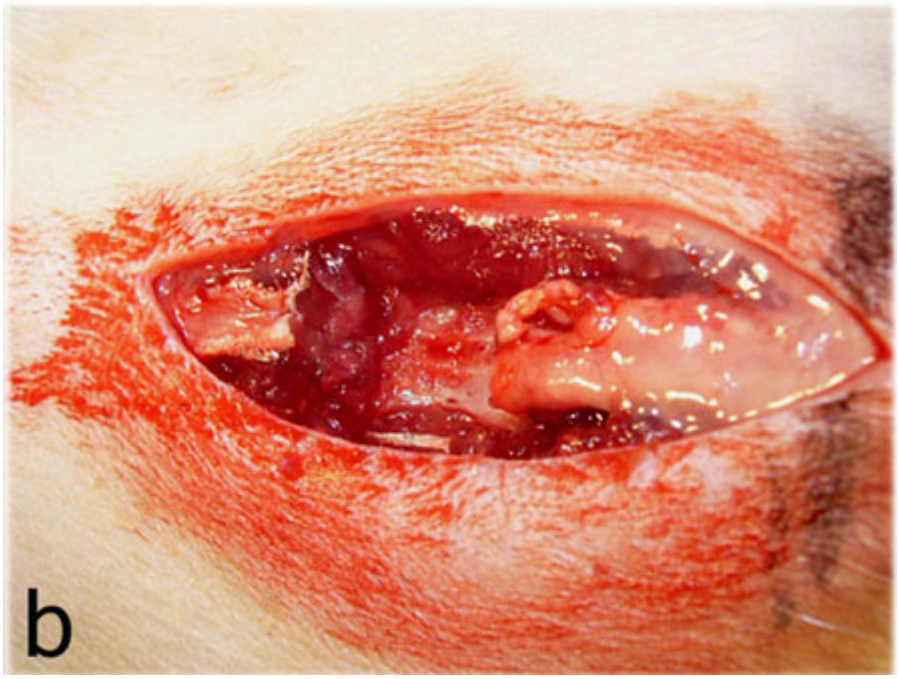

Figure 5

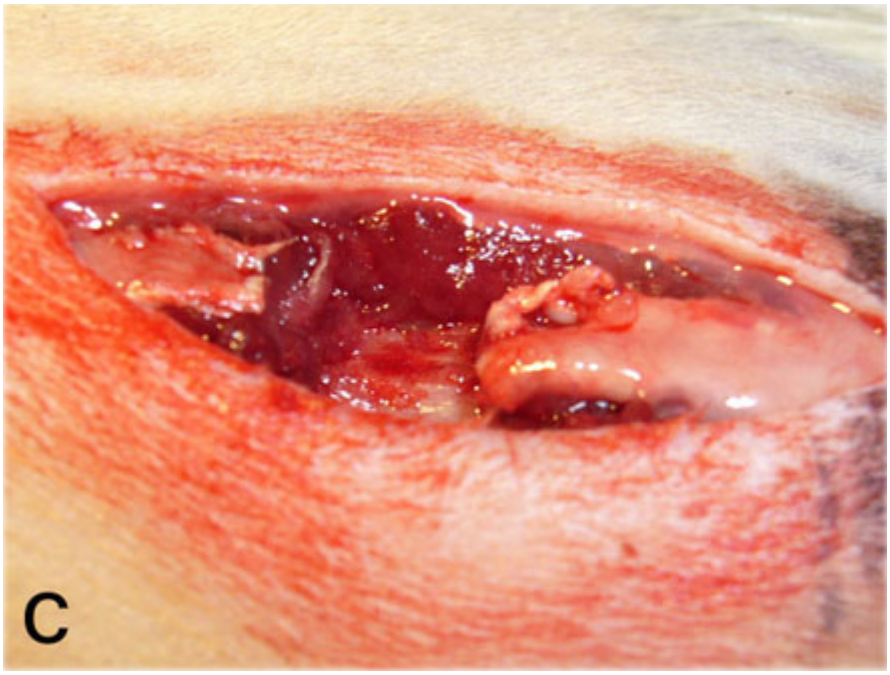

Figure 6

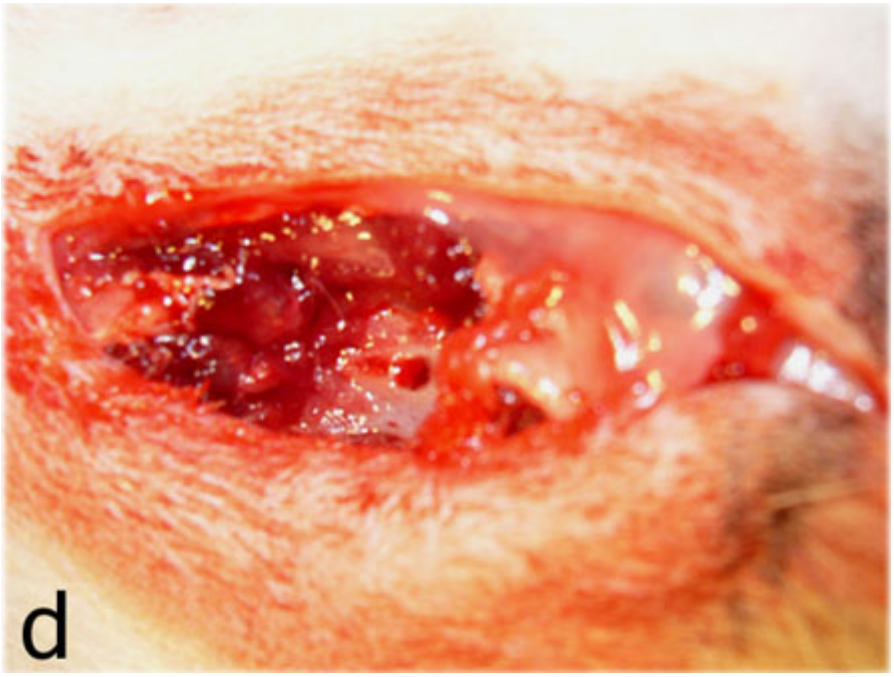

Figure 7

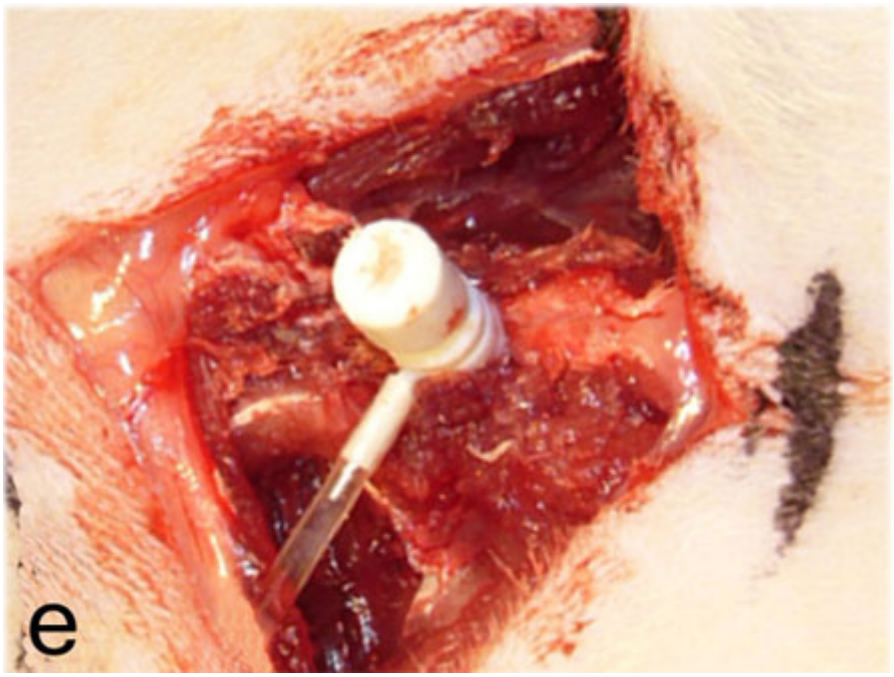

Figure 8

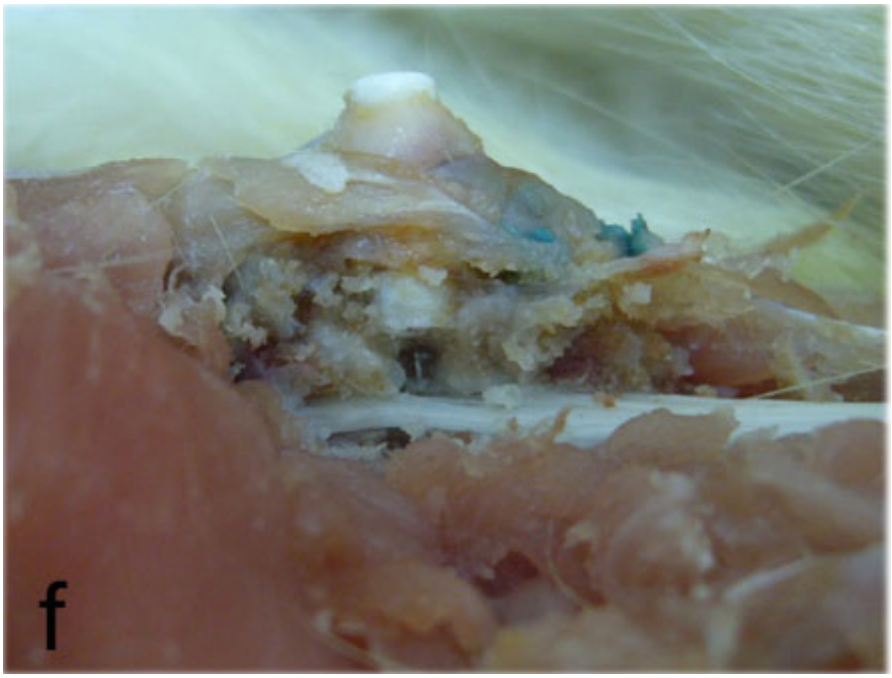

Figure 9

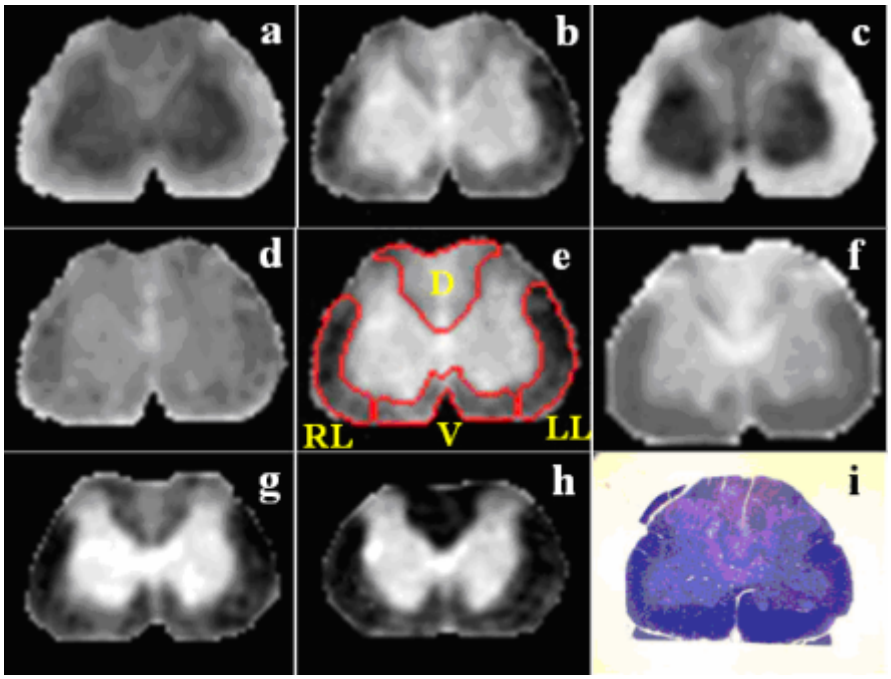

Figure 10

Figure 2 Different parameter maps obtained from high-resolution DTI images of the third lumbar segment of the spinal cord (SC) of an EAE-induced rat with severe demyelination in dorsal column. (a) showing the axial diffusivity (λ~//~) map which probes water diffusion along the long axis of the axons. (b) showing the radial diffusivity (λ~┴~), indicates the water diffusion perpendicular to the long axis. (c) showing the fractional anisotropy (FA) map illustrating whether the water diffusion has directional dependence or not. (a-d) In the case of severe demyelination where myelin sheaths are spared, there would be less restriction to water diffusion perpendicular to the nerve and thus the λ~┴~ will be increased. (e) illustrates the ROI placement for ventral (V), dorsal (D), left lateral (LL) and right lateral (RL) column of the spinal cord. (f) T2-weighted image. (g-h) shows two λ~┴~ maps of the second lumbar segment. (i) Tissue section stained for myelin using Toluidine Blue showing the dymyelination in the dorsal column of the lumbar spinal cord.
($\lambda_{//} = \lambda_1$)

Figure 11

e2
($\lambda_{\perp} = (\lambda_2 + \lambda_3)/2$)

Figure 12

e3
$$\left(FA = \sqrt{3/2 \cdot \left[(\lambda_1 - \bar{\lambda})^2 + (\lambda_2 - \bar{\lambda})^2 + (\lambda_3 - \bar{\lambda})^2\right] / (\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}\right)$$

Figure 13

e4

$$(\mathrm{MD},\ \bar{\lambda} = (\lambda_1 + \lambda_2 + \lambda_3)/3)$$

Figure 14

e1

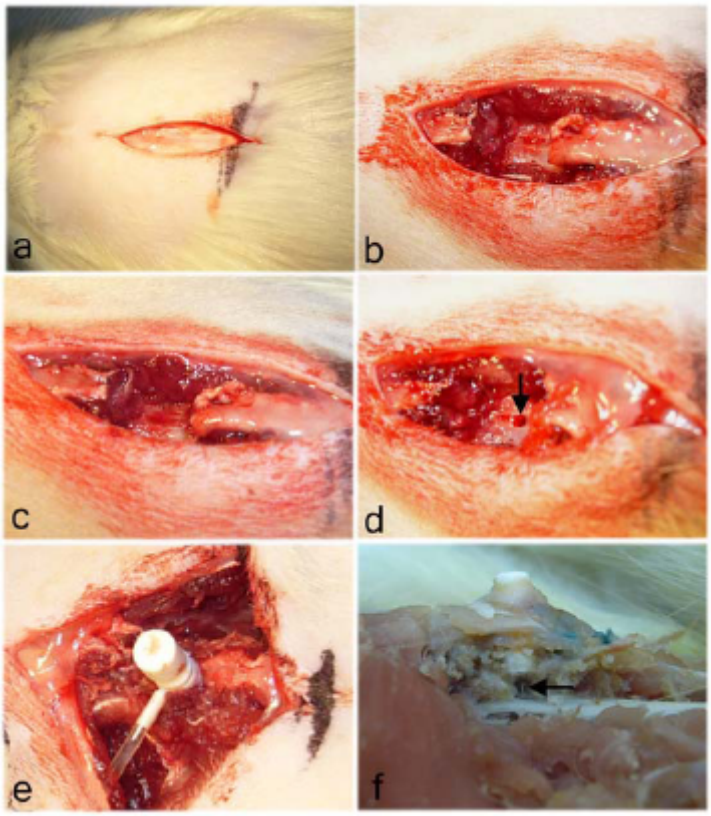

Figure 15

Figure 1

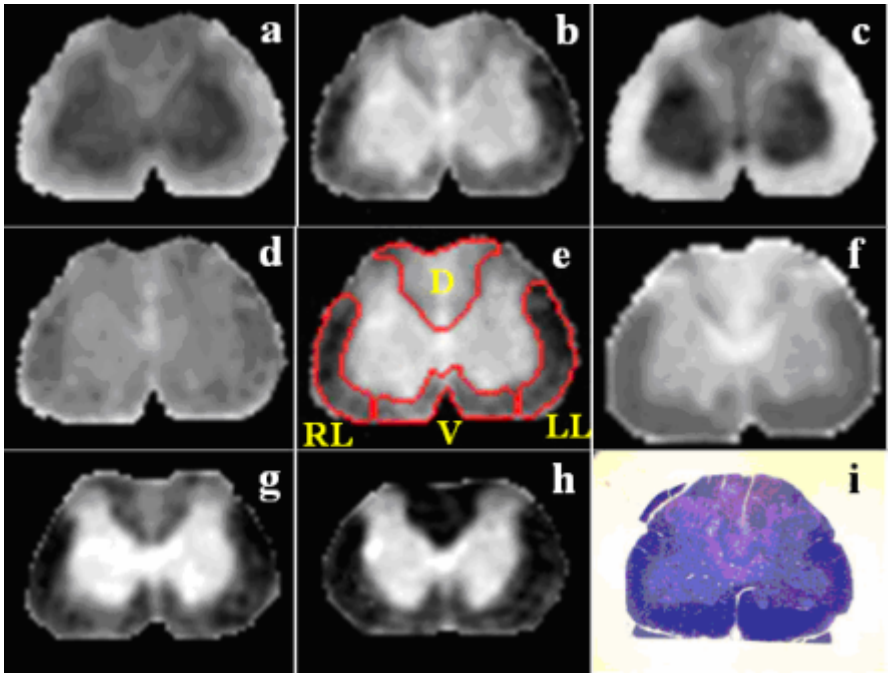

Figure 16

Figure 2