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## Comprehensive Autopsy Program for Individuals with Multiple Sclerosis

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**SHORT ABSTRACT:**

Multiple sclerosis is an inflammatory demyelinating disease with no cure. Analysis of brain tissue provides important clues to understanding the pathogenesis of the disease. Here we discuss the methodology and downstream analysis of MS brain tissue collected through a unique rapid autopsy program in operation at the Cleveland Clinic.

**LONG ABSTRACT:**

We describe a rapid tissue donation program for individuals with multiple sclerosis (MS) that requires scientists and technicians to be on-call 24/7, 365 days a year. Participants consent to donate their brain and spinal cord. Most patients were followed by neurologists at the Cleveland Clinic Mellen Center for MS Treatment and Research. Their clinical courses and neurological disabilities are well-characterized. Soon after death the body is transported to the MS Imaging Center, where the brain is examined in situ by 3 T magnetic resonance imaging (MRI). The body is then transferred to the autopsy room, where the brain and spinal cord are removed. The brain is divided into two hemispheres. One hemisphere is immediately placed in a slicing box and alternate 1 cm-thick slices are either fixed in 4% paraformaldehyde for two days or rapidly frozen in dry ice and acetone. The short-fixed brain slices are stored in a cryopreservation solution and used for histological analyses and immunocytochemical detection of sensitive antigens. Frozen slices are stored at -80 °C and used for molecular, immunocytochemical, and in situ hybridization/RNA scope studies. The other hemisphere is placed in 4% paraformaldehyde for several months, placed in the slicing box, re-scanned in the 3 T magnetic resonance (MR) scanner and sliced into centimeter-thick slices. Postmortem in situ MR images (MRIs) are co-registered

with 1 cm-thick brain slices to facilitate MRI-pathology correlations. All brain slices are photographed and brain white-matter lesions are identified. The spinal cord is cut into 2 cm segments. Alternate segments are fixed in 4% paraformaldehyde or rapidly frozen. The rapid procurement of postmortem MS tissues allows pathological and molecular analyses of MS brains and spinal cords and pathological correlations of brain MRI abnormalities. The quality of these rapidly-processed postmortem tissues (usually within 6 years) is of great value to MS research and has resulted in many high-impact discoveries.

## **INTRODUCTION:**

One of the best ways to study a disease is to examine the diseased tissue itself. This presents challenges to those who study diseases of the central nervous system (CNS). Biopsies of diseased brain and spinal cord are extremely rare and usually involve atypical cases. Autopsy rates for individuals with CNS diseases have decreased dramatically in recent years, and when performed, they often do not provide rapid procurement of tissues. These challenges have resulted in the establishment of disease-centered brain banks, including several focused on collecting tissues from individuals with multiple sclerosis (MS). MS is an inflammatory-mediated disease of the CNS that destroys myelin, oligodendrocytes (myelin forming cells), neurons, and axons. The majority of MS patients have a bi-phasic disease course that starts with bouts of neurological disability with variable recovery that eventually evolves into a gradually-progressive disease that is likely neurodegenerative in nature<sup>1</sup>. For the majority of donated MS brains, postmortem intervals (PMI) between death and tissue processing exceed 24 h. While these tissues have provided valuable information regarding pathological changes in MS brains, they are not suited for more advanced molecular studies that can provide powerful insights into disease pathophysiology. This is particularly the case for gene profiling studies, which require intact RNA.

To overcome the limitations discussed above, we have developed a rapid tissue donation program that allows for MRI/pathological correlations. This protocol provides well-preserved tissues suitable for modern molecular studies and allows direct comparison of brain pathology and MRI abnormalities in MS brains. The Cleveland Clinic Multiple Sclerosis Tissue Donation Program has been in existence for over 20 years. This rapid tissue donation program procures brains and spinal cords from individuals with MS and other associated autoimmune neurological conditions. The program aims to obtain in situ MRIs within 6 h of death, followed by removal of brain and spinal cord for tissue processing.

## **Recruitment**

Donations are obtained through either ante-mortem consent obtained directly from patients (pre-consented) or from next of kin after death. Pre-consented patients are typically identified from the clinical population at the Mellen Center for Multiple Sclerosis Treatment and Research in Cleveland, Ohio. Although preference in recruitment in the rapid tissue donation program is given to patients who have been followed in longitudinal research studies, it is open to all patients seen at the center. Participants who enroll prior to death are given instructions for family members or care providers to contact the research team, either at the time of death or when death is thought to be imminent. The second method for individuals to enter the tissue donation program is at the time of death through consent by next of kin. The State of Ohio requires deaths

to be referred to a federally-mandated organ procurement organization named LifeBanc, which operates in 20 counties of northeastern Ohio. LifeBanc screens all deaths for a diagnosis of MS, which is an exclusion for organ donation. Arrangements were made for LifeBanc to notify investigators from the MS tissue donation program for all deaths with an associated diagnosis of MS occurring within a 75-mile radius from the Cleveland Clinic. Next of kin and hospital staff are then contacted by the Tissue Donation Program Staff and consent is obtained for donation of brain and spinal cord tissues. These two methods of recruitment—ante-mortem and post-mortem through LifeBanc—result in approximately 10–12 brain donations per year. Adjustments are made to the upper age limit of death to manage the number of referrals derived from LifeBanc.

### **Procurement of donations**

The program requires 24 h coverage, 365 days a year by a member of the tissue donation program for tissue procurement. A centralized tissue donation notification email/pager/mobile device text notification system is used by the clinical team covering tissue donations. LifeBanc is provided numbers to contact on-call personnel for the tissue donation program. Members are notified of death by hospital providers/next of kin (pre-consented) or by LifeBanc and other referral sources. First, a determination of time of death and feasibility to tissue donation is made. Deaths are then screened for conditions that potentially result in poor-quality tissue, including prolonged pre-mortem hypoxia, massive destructive brain tissue (e.g., large intracranial hemorrhage, extensive bi-hemispheric strokes, extensive tumor burden), prolonged ventilator support (>3 days), and prolonged use of vasoactive agents (>3 days) prior to death. When a medical examiner is involved in a death, the study neurologist can speak with the medical examiner to explore a way to receive timely tissues without compromising the medical examiner's responsibility. If viable tissue is felt to be present, then written consent is obtained (if not obtained pre-mortem) and preparations are made for body transportation. A previously-contracted decedent transportation service is then contacted for transport to the MRI facilities at Cleveland Clinic. Care is taken to ensure that the body remains at room temperature and is not placed into refrigeration, as lower body temperatures are associated with alterations in MRI signal characteristics.

### **Clinical history**

Clinical history includes details on diagnosis of MS, onset of symptoms, treatments used, results of clinical and para-clinical testing (evoked potentials, cerebrospinal fluid results, optical coherence tomography), multiple sclerosis functional composite, and expanded disability status scale (EDSS; actual or estimated), which are collected from the medical record (where available), and direct interview of next of kin. Pre-mortem MRI are also collected.

### **PROTOCOL:**

This protocol has been approved by the Cleveland Clinic Institutional Review Board and follows guidelines of the Cleveland Clinic human research ethics committee.

### **1. In situ MRI**

1.1. Take the donor body to the MRI suite and conduct a 2 h MRI imaging protocol at the MS Imaging Facility. Conduct MRI on a 3 T or 7 T imager.

NOTE: Priority is given to 3T as the majority of legacy data has been performed on 3 T, but when not available, imaging is conducted with 7T. Designated core sequences are performed for all cases (**Table 1**) and additional sequences that depend upon current research interests are performed if time permits (constrained by achieving tissue fixation less than 12 h after death). **Table 1** describes the core sequences.

## 2. Autopsy

NOTE: Following the in situ MRI, the body is transported to the morgue for brain and spinal cord extraction by a diener and tissue processing by lab members.

2.1. Perform the following steps prior to body arrival at the morgue. Two hours prior, prepare 3 L of 4% paraformaldehyde (PFA) and label containers and bags for tissue storage. Prepare 3 L of 8% PFA and dilute 1.5 L of 8% PFA to 4% PFA. Place the remaining 8% PFA in 4 °C for day 2.

2.2. Prior to traveling to the morgue, fill 2 traveling coolers to 50% capacity with dry ice (large blocks broken to fit and small pellets).

2.3. At the morgue, fill a stainless-steel container halfway with 2-methylbutane and dry ice and cover with a lid in preparation for snap freezing the tissue.

2.4. Weigh and photograph the brain once removed by the diener.

2.5. Place any attached dura in a container filled with PFA.

2.6. Separate the cerebellum and brainstem from the cerebrum and photograph the cerebrum.

2.7. Identify the optic nerves, chiasm, and tracts and separate using a probe and tweezers. Resect the structure with a scalpel.

NOTE: The distal segment of one side of the optic nerve is marked using Higgins ink for identification.

2.8. Separate the cerebral hemispheres longitudinally and photograph each hemisphere individually.

2.9. Ink the primary motor cortex (PMC) for the left hemisphere, re-photograph it, and place it in a 3.3 L container for long-fixation. Document the **start time** for brain fixation.

2.10. The PMC for the right hemisphere may be inked or excised.

177 2.10.1. If being excised, first remove the covering meninges.

178  
179 2.10.2. Re-photograph inked or excised PMC.

180  
181 2.10.3. If PMC is excised, cut into 6 equally-sized sections.

182  
183 2.10.4. Ink the rostral aspect of each section.

184  
185 2.10.5. Place odd-numbered sections into PFA-filled containers for short-fixation.

186  
187 2.10.6. Snap-freeze even-numbered sections and place in sealed freezer bags into cooler #1.

188  
189 2.11. Cut the right hemisphere anterior to posterior into 1 cm-thick coronal sections.

190  
191 2.11.1. Document gross abnormalities (e.g., cutting artifact, hemorrhage, and lesions).

192  
193 2.11.2. Place odd-numbered sections into containers filled with PFA for short-fixation.

194  
195 2.11.3. Snap-freeze even-numbered sections and place in sealed freezer bags.

196  
197 2.11.4. Document **end of brain fixation time**.

198  
199 2.12. Separate the brainstem from the cerebellum and place in a PFA-filled container for short-  
200 fixation.

201  
202 2.12.1. Separate cerebellar hemispheres longitudinally.

203  
204 2.12.2. Cut each hemisphere into 4 equally thick sagittal sections.

205  
206 2.12.3. Photograph medial and lateral views.

207  
208 2.12.4. Place left cerebellar hemispheric slices into a container filled with PFA for short-fixation.

209  
210 2.12.5. Snap-freeze right cerebellar hemispheric slices and place in sealed freezer bags into cooler  
211 #1.

212  
213 2.13. Obtain the spinal cord with nerve roots from the diener.

214  
215 2.13.1. Remove spinal cord dura mater and store dura in a container with PFA.

216  
217 2.13.2. Separate left and right anterior and posterior nerve roots. Cut the left anterior and  
218 posterior nerve roots cut from the spinal cord and place in a PFA filled container for short-  
219 fixation.

220

2.13.3. Cut the right anterior and posterior nerve roots from the spinal cord, snap-freeze, place in sealed freezer bags, and then place in cooler #2.

2.13.4. Photograph the caudal-most 20 cm of the spinal cord. Document the location of the lumbar enlargement.

2.13.5. Cut 2 cm transverse sections of the cord proceeding from caudal to rostral.

2.13.6. Ink the rostral aspect of each cut section.

2.13.7. Place odd-numbered sections into containers filled with PFA for short-fixation.

2.13.8. Snap-freeze even-numbered sections, place into sealed freezer bags, and then place in cooler #2.

2.13.9. Document the start time for spinal cord fixation and any gross abnormalities.

2.13.10. Photograph the remaining rostral portion of the spinal cord.

2.13.11. Document the position of the cervical enlargement. Follow steps 2.11–2.13 for the remaining spinal cord.

2.4. Following the morgue place frozen tissue in labeled boxes in -80 °C freezers. Place fixed tissue in at 4 °C.

2.5. At 24 h post-autopsy (day 2) dilute the remaining 8% PFA to 4%.

2.6. Replace 4% PFA in fixation containers with freshly diluted 4% PFA.

2.7. At 60 h post-autopsy prepare solutions of 2.5% glutaraldehyde in 4% PFA from glutaraldehyde, PFA, dH<sub>2</sub>O and Sorenson's buffer (prepared by mixing in sequence: 0.2 M phosphate buffer pH 7.4, polyvinylpyrrolidone 1% w/v, sucrose 30% w/v, and ethylene glycol 30% v/v).

2.8. Remove used 4% PFA from short-fixation containers.

2.9. Rinse tissue in Sorenson's buffer and place it in cryoprotection solution (glycerol 20%, 0.4 M Sorenson's buffer 20%, and 0.02% sodium azide in dH<sub>2</sub>O).

2.10. Photograph short-fixed brain slices, cerebellum, brainstem and motor cortex (if applicable).

2.11. With a scalpel blade cut 2 mm thick transverse sections from each 2 cm short-fixed spinal cord section.

2.12. Place sections in 2 mL scintillation vials and fill with solution of 2.5% glutaraldehyde in 4% PFA.

2.13. Return the remaining section to the original 20 mL scintillation vial. Rinse section with Sorenson's buffer and replace with cryoprotection solution.

### 3. Pathology

NOTE: Short-fixed slices of the right hemisphere as well as the long-fixed left hemisphere (placed in 4% PFA for several months) are either cut into 30  $\mu$ m sections (referred to as free-floating) or embedded in paraffin and cut as 12–14  $\mu$ m sections (referred to as paraffin-embedded). These sections are processed usually with proteolipid protein (PLP) for detecting demyelinating lesions and major histocompatibility complex II (MHC-II) for immune activity using the diaminobenzidine (DAB) method. These protocols have been standardized and used in several publications<sup>2-12</sup>.

#### 3.1. Free-Floating (30 $\mu$ m) DAB-Avidin-Biotin Complex (ABC) Tissue Staining

3.1. Remove sections from cryostorage solution, transfer sections to a six-well plate, and wash them 3x for 5 min each in 2 mL of 1x phosphate buffered saline pH 7.0 (PBS). When transferring to the next well in the six-well plate use care not to tear the tissue. During each wash and incubation step, place the six-well plate on a shaker and allow tissue to gently shake.

NOTE: Tissue sections incubated in smaller volumes and in larger plates (i.e., 12- and 24-well plates) tend to exhibit surface and edge tearing.

3.2. Perform antigen retrieval by microwaving sections in a glass beaker containing roughly 30 mL of 10 mM citrate buffer (pH 6.0). Ensure tissues are not folded by manipulating with a paintbrush and microwave sections for 2–3 min or until citrate buffer comes begins to boil. Allow sections to cool to room temperature (~20 min).

3.3. Transfer sections back to a six-well plate and wash sections 3x for 5 min each in 2 mL of PBS/0.3% Triton X-100. Block endogenous peroxidases by incubating sections in 2 mL of 3% H<sub>2</sub>O<sub>2</sub>/0.3% Triton X-100/PBS for 30 min at room temperature (RT).

3.5. Wash sections 3x for 5 min each in 2 mL of PBS/0.3% Triton X-100. Block section in 2 mL of 3% normal goat serum/0.3% Triton X-100/1xPBS for 1 h at RT.

3.6. Incubate sections overnight for 5 days (depending on the antibody) in primary antibodies directed against microglia and myelin epitopes to detect inflammation (MHCII) and demyelination (PLP) (see the **Table of Materials**) at 4 °C.

NOTE: Ensure that sections are not folded when incubating in this step or subsequent steps as this will lead to areas within the sections being void of stain.



3.7. Wash sections 3x for 5 min each in 2 mL of 1x PBS. Then incubate sections in secondary biotinylated antibodies (see the **Table of Materials**) for 1 h at RT. Prepare Avidin-Biotin Complex (ABC) solution during incubation roughly 45 min prior to the next wash step to allow ABC complexes to form.

3.8. Wash sections 3x for 5 min each in 2 mL of 1x PBS. Then incubate sections in ABC for 1 h at RT.

3.9. Wash sections in 2 mL of 1x PBS three times for 5 min each. Incubate sections in filtered DAB (2 mL/well/section) containing H<sub>2</sub>O<sub>2</sub> (1:500 dilution of 30% H<sub>2</sub>O<sub>2</sub> in DAB) until color develops adequately (~3–8 min).

3.10. Wash sections 3x for 5 min each in 2 mL of 1x PBS. To enhance signal (optional), osmicate using 0.04% OsO<sub>4</sub> (~30 s).

3.11. Wash sections three times for 5 min each in 1x PBS. Transfer slides from the six-well plate to a small container full of 1x PBS and position them underneath a tissue slide as flat as possible.

3.12. Gently lift the slide out of the PBS while ensuring the tissue section is as flat as possible. Using two paint brushes, gently flatten and stretch the tissue out on the slide and dab away the excess water with paper towel. Mount tissue sections with glycerol (or an equivalent mounting media) and seal the coverslip with clear nail polish.

#### 4. Paraffin-embedded hemispheric sections: DAB staining

4.1. Melt paraffin off the slides in a 60 °C oven for 5–10 min.

4.2. De-paraffinize sections by incubating in xylene 3x for 5 min each.

4.3. Rehydrate tissue in graded ethanol at 100% (2x for 5 min each), 95% (2x for 5 min), 70% (2x for 5 min each), and 50% (1x for 5 min each). Cover slides in PBS.

4.4. Antigen retrieve by microwaving slides in a beaker of 10 mM citrate buffer (pH 6.0).

4.5. Wash slides in 1x PBS (3x for 5 min each) once cooled to room temperature. Block endogenous peroxidases by incubating tissue in 3% H<sub>2</sub>O<sub>2</sub>/1% Triton-X 100/PBS for 30 min.

4.6. Wash tissue in 1x PBS three times for 5 min each. Block tissue with 6% normal goat serum in PBS for 1 h.

4.7. Incubate sections in primary antibody (see the **Table of Materials**) in PBS at room temperature (RT) overnight (max 20 h).

4.8. Wash sections 3x for 5 min each in 1x PBS. Then incubate sections in corresponding secondary antibody (see the **Table of Materials**) in PBS for 1 h at RT.

4.9. Prepare ABC solution roughly 45 min prior to the next wash step during incubation.

4.10. Wash sections 3x for 5 min each in 1x PBS and then incubate in ABC for 1 h at RT.

4.11. Wash section 3x for 5 min each in 1x PBS.

4.12. Incubate sections in filtered (0.45 µm filter pore size) DAB containing H<sub>2</sub>O<sub>2</sub> (1:500 dilution of 30% H<sub>2</sub>O<sub>2</sub> in DAB) until color develops adequately (~3-8 min).

4.13. Wash sections (3x 5 min) in 1x PBS. To enhance signal, osmicate using 0.04% osmium tetroxide (OsO<sub>4</sub>; approximately 30 s).

4.14. Wash sections (3x 5min) in 1x PBS.

4.15. Dehydrate tissue in a graded series of ethanol 50% (1x 5min), 70% (1x 5min), 95% (2x 5min), 100% (2x 5 min), and 100% xylene (1x 5min). Allow xylenes to evaporate.

4.16. Mount sections with toluene-based rapid dry mounting media. Formulations that contain anti-oxidants are recommended to prevent stain bleaching. Remove excess mounting media from slide edges with a razor for subsequent storage.

## 5. MRI/pathology correlations

NOTE: For correlating MRI with pathology, we first perform ex vivo MRI of the long-fixed intact brain hemisphere (step 2.9 above) in an adjustable box with MRI-visible markers indicating slicing slots. We then slice the brain and photograph the 1 cm slices to enable co-registration of in situ MRIs to the individual brain slices. We can then perform MRI-guided analyses, where regions of interest (ROIs) are identified on MRI to direct tissue analysis. We can also perform histopathology-guided analyses, where ROIs are identified on tissue (e.g., white matter lesions, white matter without demyelination, etc.) and then characterized using MRI measures at the site (**Table 1**).

### 5.2. Identification of MRI-based ROIs

NOTE: In previous studies, we have identified ROIs in white matter<sup>11-15</sup> and gray matter<sup>16-18</sup>. The example below is for white-matter analysis.

5.2.1. Segment T2 hyperintense lesions on in situ MRIs (from step 1.1), initially processed by an automatic algorithm, and then corrected manually by experienced users.

5.2.2. Segment T1 hypointense lesions within T2 lesions as voxels with a signal intensity less than or equal to 80% of the signal intensity of the surrounding normal-appearing brain tissue.

5.2.3. Segment hypointense areas in magnetization transfer ratio (MTR) maps with an 80% threshold.

5.2.4. Create three classifications using the above segmentations: (a) T2-only lesions which are abnormal on T2-weighted/FLAIR scans but normal on T1-weighted or MTR scans, (b) T2T1MTR lesions, which are abnormal on all T2-weighted/FLAIR, T1-weighted, and MTR scans; and (c) normal-appearing white matter (NAWM), which are normal on all T2-weighted/FLAIR, T1-weighted, and MTR scans.

NOTE: Our selection of slices is based on the existence of all three regions-of-interest types (T2-only, T2T1MTR, and NAWM) on the same slices.

5.2.5. Calculate normalized intensities for each ROI to minimize variability arising from different brains and different brain locations.

### 5.3. Co-registration of in situ MRI to brain slices

5.3.1. Scan the fixed brain hemisphere in a custom slicing box with four rows of MRI-sensitive markers localizing the slots where a knife can be inserted to slice the brain. Perform a T1-weighted 3D MPAGE acquisition with 1 mm isotropic resolution covering the fixed brain and all of the markers.

NOTE: This is called post-fixation MRI and is only used as an intermediate step for co-registration of the in situ MRIs to the brain slices.

5.3.2. Immediately after scanning, slice the brain hemisphere in slots located 1 cm apart, resulting in approximately 15 slices.

5.3.3. Photograph the brain slices on both the anterior and posterior sides.

5.3.4. Co-register the in situ MRI and photographs of brain slices with the following steps.

5.3.4.1. For segmentation of post-fixation and in situ MPAGE, pre-process both post-fixation and in situ MPAGE MRIs for intensity non-uniformity<sup>19</sup>.

5.3.4.1.1. Segment the brain and four rows of markers from preprocessed post-fixation MPAGE.

5.3.4.1.2. Segment the hemisphere corresponding to the post-fixation MRI<sup>20, 21</sup> from the pre-processed in situ MPAGE.

5.3.4.2. Co-register the brain-extracted in situ and post-fixation MRIs through a series of linear registration processes up to 12 degrees-of-freedom (affine registration) using FSL FLIRT<sup>22</sup>. The scaling and shearing components account for the effect of fixation shrinkage.

5.3.4.3. Find the slicing plane's normal direction by minimizing the sum of maximum projected intensities using the segmented markers. These re-orientation angles are incorporated in the transformation matrix obtained from the previous step.

5.3.4.4. Visually match MRI images to photographs of fixed posterior brain slices using an in-house image viewer that allows for changing the depth and orientation of the normal vectors. Small modifications are needed because the brain slicing is imperfect.

5.3.4.5. Apply AFFINE image transformation<sup>13</sup> for each slice to transform the in situ MRIs to the same slicing locations as the photographs of the brain slices.

### **REPRESENTATIVE RESULTS:**

As mentioned above, almost half of the cerebral hemisphere is frozen and available for molecular studies using DNA, RNA, or protein. Historically, studies using postmortem brain tissues have been shown to be affected by pre-mortem conditions, age, sex, tissue pH, mRNA integrity (RIN), postmortem interval (PMI), diagnostic certainty, comorbid substance use, and prior medication treatment status<sup>23</sup>. Based on studies using brain tissues, DNA and protein appears to be affected by lesser extent as compared to RNA. Based on our experience, RNA isolation and downstream analysis have, however, been found to be most affected by the pre-mortem conditions and postmortem interval of the brain tissue. We therefore discuss some of the conditions to be followed for conducting RNA based analysis using postmortem MS tissues.

For all our studies, after the brain is collected at autopsy, it is sliced (1 cm thick) and then either fixed in 4% paraformaldehyde for morphological studies or rapidly frozen for biochemical analysis. All tissue blocks are characterized for demyelination by immunostaining using PLP as described above. A representative analysis scheme is shown in **Figure 1**. Tissue sections are examined for the presence of white-matter lesions (**Figure 1A**). Selected regions are stained for immune activity (**Figure 1B**) and demyelination (**Figure 1C**). The frozen tissue is mounted on the cryostat (**Figure 1D**) and frozen 30  $\mu$ m sections are cut. This is followed by collection of 3–4 subsequent sections, separation from the adjoining tissues, and storage for DNA, RNA, or protein isolation. Using this protocol, we have successfully isolated DNA<sup>24, 25</sup>, RNA<sup>5-9</sup> as well as proteins<sup>26</sup>. While major findings from some of the studies analyzing RNA from MS brains are discussed, here are some of the issues related to analysis of RNA postmortem MS brains.

[INSERT FIGURE 1 HERE]

**Axonal transection in the lesions of MS<sup>10</sup>.** An initial scientific focus of this program was on the characterization of cellular components of demyelinated white-matter lesions. Among the antigens localized was non-phosphorylated neurofilaments (NFs). Most NFs are phosphorylated in myelinated axons. Upon demyelination, axons are dephosphorylated. We detected the

expected expression of non-phosphorylated NFs in demyelinated axons. In acute MS lesions, many of these demyelinated axons ended as axonal retraction bulbs (**Figure 2A**), which reflect the proximal ends of transected axons. Transected axons exceed 11,000 mm<sup>3</sup> in the acute lesions compared to adjacent normal regions<sup>10</sup>. These observations helped catalyze a paradigm shift in MS research that moved the field toward characterizing neurodegeneration as the major cause of permanent neurological disability in individuals with MS.

[INSERT FIGURE 2 HERE]

**Remyelination in chronic MS brains**<sup>3</sup>. Remyelination can be robust during early stages of MS. Many chronic MS lesions, however, are not remyelinated. We investigated whether the presence of oligodendrocyte progenitor cells (OPCs) or the generation of new oligodendrocytes limits remyelination of chronically demyelinated white-matter lesions. While OPC density was often decreased, they were present in all chronically-demyelinated lesions<sup>3</sup>. Newly-generated oligodendrocytes were also present in many chronic MS lesions. Oligodendrocyte processes associated with, but did not myelinate demyelinated axons (**Figure 3**). These studies indicate that OPCs and their ability to produce new oligodendrocytes are not limiting remyelination of chronic white-matter lesions. We hypothesized that the chronically-demyelinated axons, which often appeared dystrophic, were not receptive to remyelination by newly-produced oligodendrocytes.

[INSERT FIGURE 3 HERE]

**Mitochondrial dysfunction in MS**<sup>6</sup>. We performed an unbiased search for neuronal gene changes in rapidly-frozen motor cortex obtained from chronic MS patients (**Figure 4A**). An unbiased search of this data set identified significant reductions in 23 nuclear-encoded mitochondrial mRNAs in MS (Figure 4B). Credentialing studies using immunocytochemistry and in situ hybridization indicated that these genes were highly enriched in cortical projection neurons (Figure 4C) and that mitochondria isolated from projection axons display reduced glycolysis (Figure 4D). This paper catalyzed a focus on mitochondrial dysfunction and reduced ATP production as a major contributor to axonal degeneration in MS.

[INSERT FIGURE 4 HERE]

**Pathogenesis of cognitive dysfunction in MS**<sup>8</sup>. Forty to 60% of MS patients have cognitive decline and reduced executive function. We identified the hippocampus, which is a functional site of memory/learning, as a common site for demyelination in MS. We next compared neuronal gene expression in myelinated and demyelinated hippocampi and found significant reductions in neuronal mRNAs encoding proteins involved in memory/learning. We extended these data by demonstrating that select microRNAs are increased in demyelinated hippocampus and that these microRNAs can decrease the expression of glutamate receptors. We have reproduced and extended these observations in rodent models. We next compared neuronal gene expression in myelinated and demyelinated hippocampi and found significant reductions in neuronal mRNA encoding proteins involved in memory/learning.

[INSERT FIGURE 5 HERE]

**Pathological correlates of MRI changes<sup>12</sup>.** While MRI is a valued indicator of MS diagnosis and response to treatment, and is also a predictor of MS disease progression, the pathological correlates of MRI changes are poorly understood. Our postmortem MRI studies have focused on two MRI ROIs. Cerebral white-matter ROIs that were only T2 hyperintense (T2 only) and ROIs that had a combination of T1 hypointensity, T2 hyperintensity, and reduced magnetization transfer ratio (MTR) (T2T1MTR). Approximately 45% of cerebral white-matter T2-only ROIs were myelinated, confirming their non-specific nature. In contrast, 83% of the T2T1MTR ROIs were chronically demyelinated and appeared as black holes. T1 and MTR values are semi-quantitative and their values varied extensively in the T2T1MTR ROIs. If loss of myelin is the only contributor to these MRI changes, then the values should be constant. Swollen demyelinated axons correlated with both T1 and MTR values.

[INSERT FIGURE 6 HERE]

**Neurodegeneration independent of demyelination<sup>11</sup>.** Historically, neurodegeneration in MS has been thought to result from demyelination. Brain imaging studies, however, have raised the possibility that neurodegeneration and demyelination can be independent events. We recently identified a subpopulation of MS patients that have demyelination of the spinal cord and cerebral cortex, but not of the cerebral white matter. We coined this MS subtype as myelocortical MS (MCMS). MCMS cases provided a platform to investigate the relationship between cerebral white-matter demyelination and cortical neuronal loss. Compared to control cortices, cortical neuronal loss was significantly greater in MCMS cortices than in typical MS cortices. Control brain tissue was obtained from the Pathology Department at the Cleveland Clinic. This study provides the first pathological evidence for neurodegeneration in the absence of demyelination.

[INSERT FIGURE 7 HERE]

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Sample collection for mRNA analysis.** (A) Autopsy tissue is selected for analysis. Areas of tissue are selected and a portion of tissue is excised. All sections are stained with (B) MHC-II (Major histocompatibility complex (MHC) class II HLA-DR CR3/43) antibody to detect inflammatory activity and with (C) proteolipid protein (PLP) to determine myelin status using published protocols. Based on the myelin status, the block is scored by a scalpel (D). Sections (60  $\mu$ m) are cut (E) and the areas that have been previously scored are removed, separated in tubes, and labeled (F). PLP and MHC-II stains are repeated after every 5 sections to ensure proper collection of tissue. Normal appearing white matter (NAWM) is noted and white matter lesions (WML) are outlined in red.

**Figure 2: Axonal transection during inflammatory demyelination.** Axonal transection occurs during inflammatory demyelination (A, arrowheads) and induces formation of terminal axonal ovoids (A, arrows). When quantified (B), transected axons are abundant in MS lesions and appear

to correlate with inflammatory activity of the lesion. Panel A reproduced from Trapp et al.<sup>10</sup> with permission. Red: proteolipid protein, green: Anti nonphosphorylated neurofilament.

**Figure 3: Processes of pre-myelinating oligodendrocytes associated with axons.** Confocal micrographs of MS lesions stained with PLP antibodies (red in Panels A, B) and neurofilament antibodies (green in Panels A, B) are shown. A pre-myelinating oligodendrocyte (red in Panel A) in the subventricular zone (SVZ) extended processes into the region of demyelinated axons (green in Panel A) in a chronic MS lesion. Many of these processes (arrows in Panel A) spiraled around axons, as shown at higher magnification (Panel B). Scale bars represent 20  $\mu\text{m}$  (A) and 5  $\mu\text{m}$  (B). Reproduced from Chang et al.<sup>3</sup> with permission.

**Figure 4: Microarray data and downstream validation techniques performed in MS motor cortex.** (A) Hierarchical clustering of significantly altered transcripts from control (C1–C6) and SPMS (MS1–MS6) motor cortex samples, separately supporting disease-related gene expression patterns. Among the decreased transcripts in MS motor cortex, twenty-six belonged to the electron transport chain (B). Mitochondrial complex I (NDUFA6) mRNA was decreased in neurons ( $n = 55\text{--}130$ ) in MS motor cortex (CII) compared to control (CI), whereas PLP mRNA densities were similar between control (CIII) and MS (CIV) cerebral cortex. Activity of electron transport complexes I and III were decreased in mitochondrial-enriched fractions from motor cortex of MS patients ( $n = 3$ ) (D). Reprinted from Dutta et al.<sup>6</sup> with permission. Error bars represent SEM; Scale bar in CI–IV are 25  $\mu\text{m}$ . \*  $p < 0.05$  Student's  $t$ -test.

**Figure 5: Tissue collection, histological analysis, and gene expression studies in MS hippocampus.** Brain slices containing hippocampus are selected during autopsy (A) and the hippocampus and adjoining region is removed (red box) for further analysis. Immunostaining for PLP showed preservation of myelin in all control (B) and 40% of MS hippocampi (C). Extensive demyelination was detected in ~60% of MS hippocampi (D). When compared to control hippocampi (E, G, I), significant neuronal loss was not detected in CA1, CA3, or CA4 regions of demyelinated MS hippocampi (F, H, J) as shown by HuR immunohistochemistry. Double-labeling immunofluorescence for myelin (myelin basic protein (MBP), green) and axons (SMI32, red) showed loss of myelin (L) with relative preservation of axons (N) in MS demyelinated hippocampus compared to control hippocampus (MBP, K; SMI32, M). Dual clustering of mRNA expression levels arranged samples into discrete clusters based on myelin status (myelinated and demyelinated) and location (hippocampus vs. motor cortex) (O). High mRNA levels are indicated by red and blue denotes low expression levels. Panels C–O Adapted from Dutta et al.<sup>8</sup> with permission. B–D: 2 mm, E–J: 100  $\mu\text{m}$ , K–N: 50  $\mu\text{m}$ .

**Figure 6: Magnetization transfer ratios (MTR) and T1 contrast ratios linearly correlate with the percentage of  $\text{Na}^+/\text{K}^+$  ATPase-positive axons in chronic MS lesions.** Chronically-demyelinated lesions stained for  $\text{Na}^+/\text{K}^+$  ATPase (green) varied from nearly 100% (A) to zero (B) in neurofilament (red). Many axons without  $\text{Na}^+/\text{K}^+$  ATPase had increased diameters (B). A comparison of the percentage of  $\text{Na}^+/\text{K}^+$  ATPase-positive axons in chronically-demyelinated MS lesions correlated with quantitative postmortem MTR ( $p < 0.0001$ , C) and T1 contrast ratios ( $p < 0.0006$ , D). Each data point is from a single lesion and each unique color-symbol combination denotes one of the

brains studied. Scales bars = 5  $\mu\text{m}$ . Reproduced from Young et al.<sup>12</sup> with permission.

**Figure 7: Neuronal loss in the absence of cerebral white-matter demyelination.** A cresyl violet - stained coronal hemispheric section from an individual classed as having typical MS (A). Neuronal densities were compared in cortical layers III, V, and VI in each of the five labelled areas. Neurons with an area greater than 60  $\mu\text{m}^2$  (yellow) are shown in a representative image from the superior temporal cortex (B). Labelling for PLP and the distribution of demyelinated lesions (white-matter demyelination is highlighted in blue; subpial demyelination is highlighted in pink) in hemispheric sections from individuals with typical MS (C) and myelocortical MS (D) are shown. A significant correlation between reduced cortical neuronal density and increased cerebral white-matter lesion volume was found in typical MS, but not in myelocortical MS (E); dashed lines indicate 95% confidence interval (CI). IFG = inferior frontal gyrus. STG = superior temporal gyrus. INi = inferior insula. INs = superior insula. CG = cingulate gyrus. Reproduced from Trapp et al.<sup>11</sup> with permission.

#### Table 1: Postmortem imaging protocol.

#### DISCUSSION:

We describe a protocol that has been used to rapidly procure and process tissue from over 150 individuals with MS. An important feature of this protocol is that the scientists that utilize the tissue are also in charge of establishing the protocol and performing the tissue collection. This provides flexibility in meeting the scientific needs of individual research projects. Several aspects of this protocol enhance its utility. The patients are usually well-characterized prior to death, as many of them have been followed by neurologists at our center. A critical step is the processing of tissue donations soon after death, which increases the quality of the frozen tissue compared to some other brain banks. This enables molecular studies that are of great value in describing changes in transcriptional and translational gene products, which are essential for substantiation of histological and immunocytochemical observations. Leveraging of morphological/immunocytochemical and molecular data across multiple cases enhances the reliability of conclusions. This is best illustrated by our description of mitochondrial gene changes in cerebral cortex and neuronal gene changes in demyelinated hippocampi. Novel gene profiling protocols are being developed at a rapid rate and the frozen tissue in our bank should provide high-quality RNA for tissue and single-cell analysis.

Another valued aspect of our protocol is the short-fixed brain slices. These tissues are cut into 30  $\mu\text{m}$ -thick, free-floating sections. These sections are ideal for using confocal microscopy to analyze two or more antigens in three dimensions. Good examples include the interaction of pre-myelinating oligodendrocyte processes with dystrophic axons in chronic MS lesions, as well as the identification of single axonal connections to transected axonal retraction bulbs. This is in contrast to the routine use of 7  $\mu\text{m}$ -thick paraffin sections, where 3D images are not feasible. Paraffin-embedded tissues have great value for some questions, especially the quantification of neuronal densities in hemispheric 7  $\mu\text{m}$ -thick sections. Our tissue processing protocols, therefore, are diverse and provide flexibility to assure fixed and rapidly-frozen tissues.



Another unique feature of our protocol is the postmortem in situ brain MRI. Brain MRIs are an irreplaceable biomarker of MS disease. It is essential, therefore, to establish the pathological correlates of abnormal MRI signals. Our studies established that both T2 only and T2T1MTR ROIs are often myelinated. This finding supports the need for more specific imaging modalities that reliably distinguish between myelinated and demyelinated cerebral white matter. MRI appears to be sensitive for detection of myelin, but our studies demonstrate that even a combination of T1/T2/MTR is not specific for identifying myelination. Our postmortem protocol provides an ideal platform for testing the ability of new imaging modalities to distinguish between myelinated and demyelinated cerebral white matter. MRI also provides the ideal vehicle for translation of basic science results into clinical practice, given the use of MRI in our translational research and its widespread clinical use in living patients.

While cutting short- and long-fixed as well as frozen slices offers an advantage for processing tissue in multiple modes for different studies, there are some limitations with this method. Evaluating the entirety of a structure may be limited as portions of it may be processed differently on adjacent slices. The large volume of the tissue bank, however, offers the ability to investigate a structure of interest in multiple subjects to improve sampling. Another general limitation to studies utilizing postmortem tissues is that they are cross-sectional. Conclusions regarding timing and progression of changes need to be interpreted within this context. There may be a selection bias to patients that donate their tissues, which may limit generalization of data to all patients with MS. Since most donors die from complications of advanced MS, it may not be appropriate to extrapolate findings from these patients to those in earlier stages of MS. Nonetheless, we have received tissues from younger patients who died from non-MS-related conditions (i.e., acute myocardial infarction, drug overdose, suicide). The scope of our protocol does not include sampling of other organs (e.g., gastrointestinal and bone marrow) which have been implicated in MS. We believe that the strengths of the program greatly outweigh its limitations.

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#### **DISCLOSURES:**

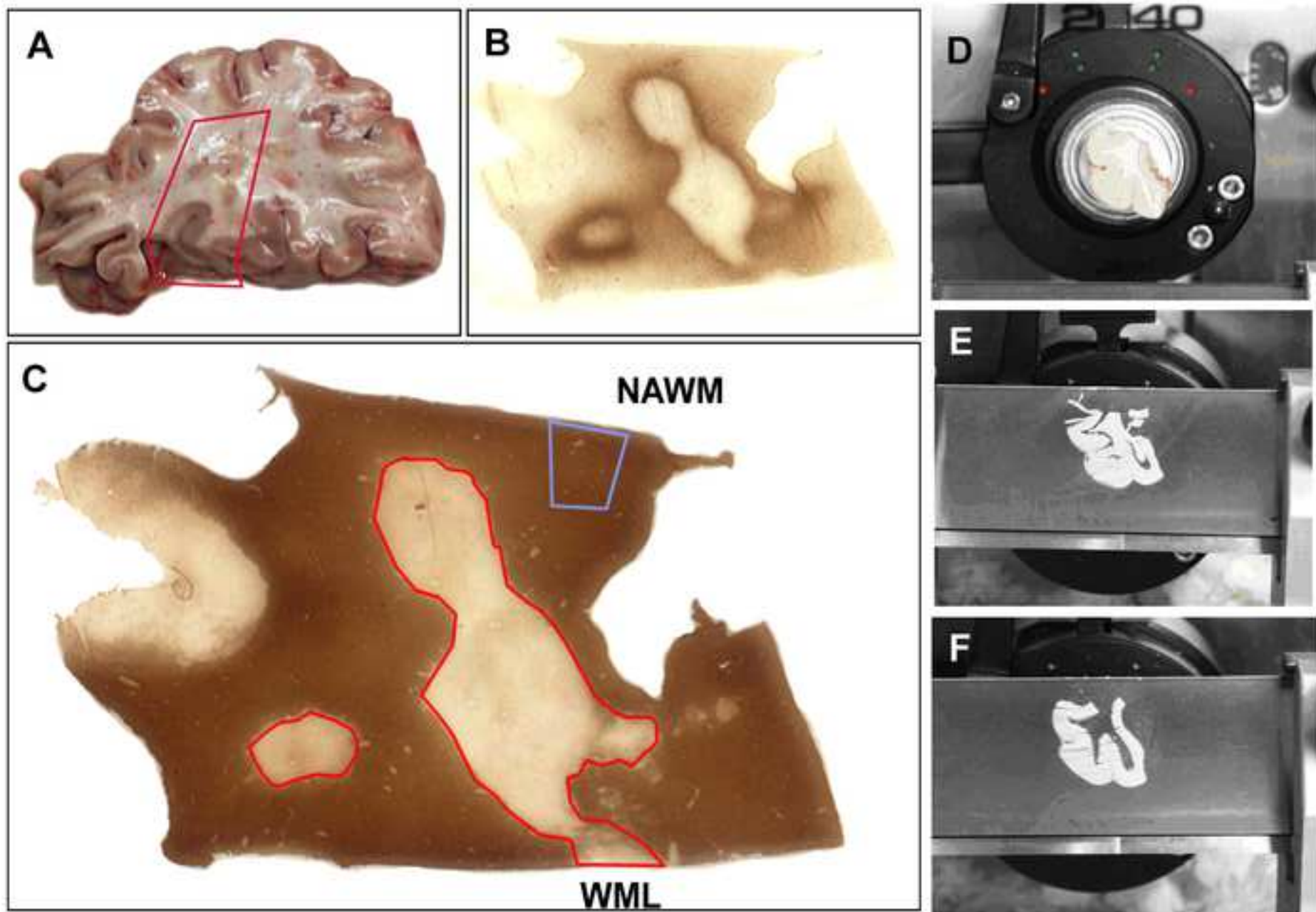
The authors declare no conflicts of interest.

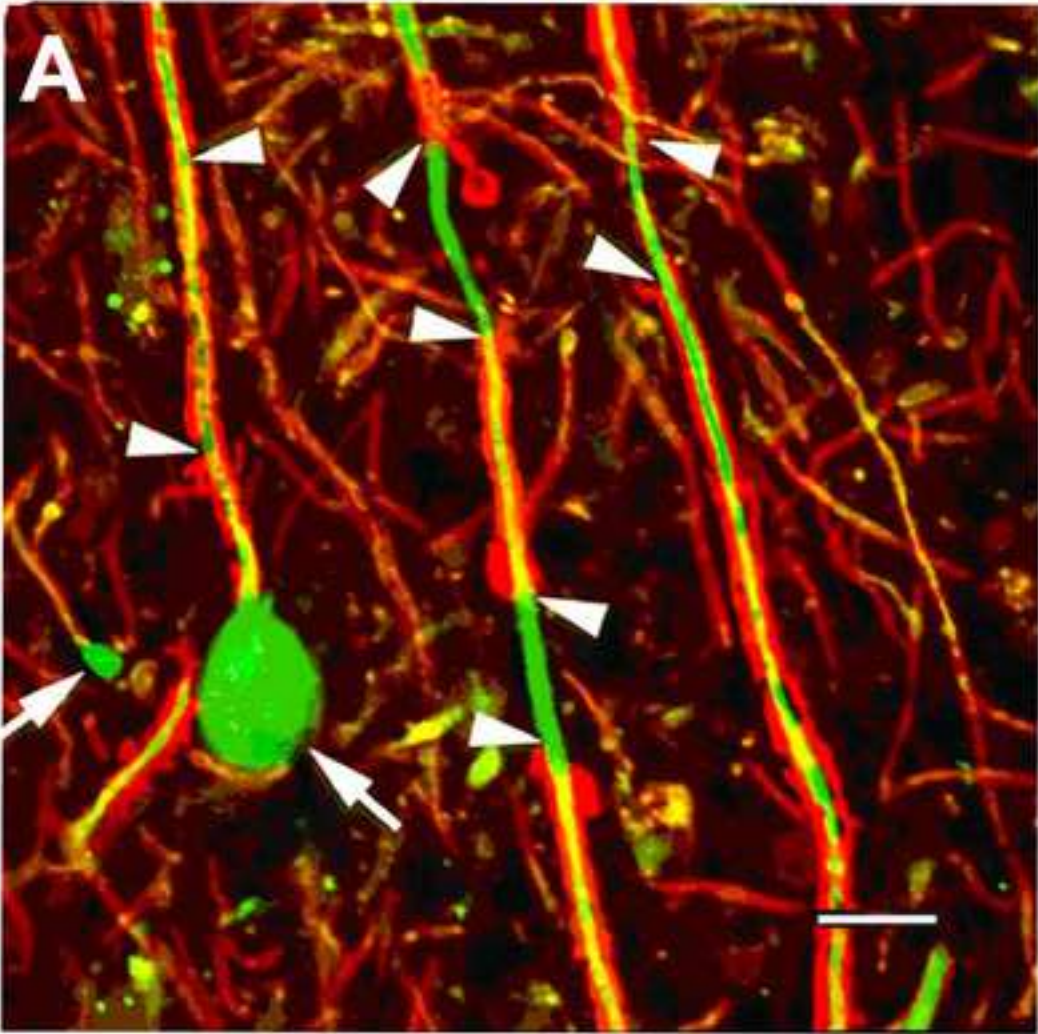
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752  
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**B**    **Transected Axons in MS Lesions**

Activity of Lesion	Transected Axons/mm <sup>3</sup> (Mean + sem)
Active	11236 + 2775*
Chronic Active	
Edge	3138 + 688*
Core	875 + 246*
Non-lesion White Matter	15 + 3*
Control White Matter	0.7 + 0.7



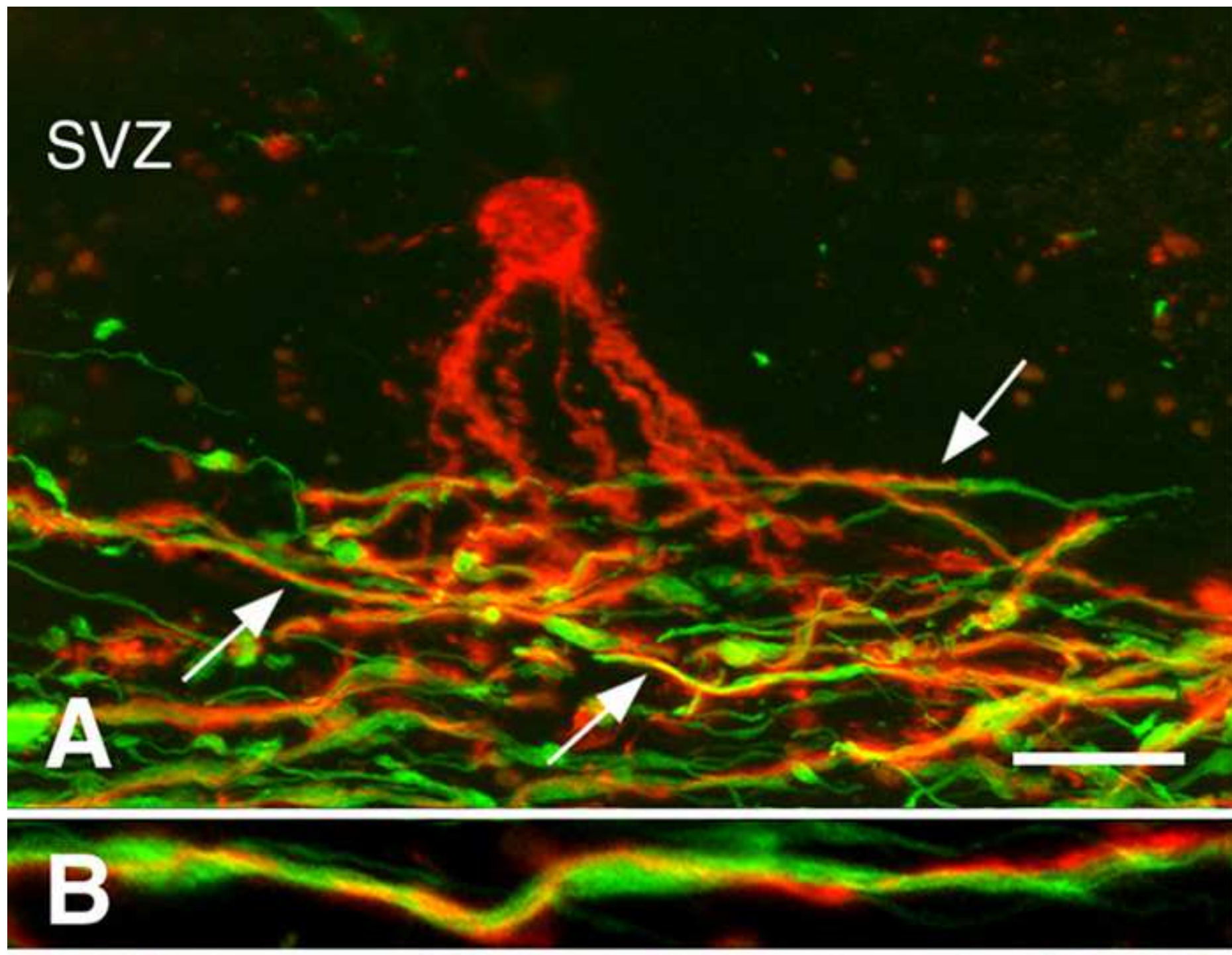
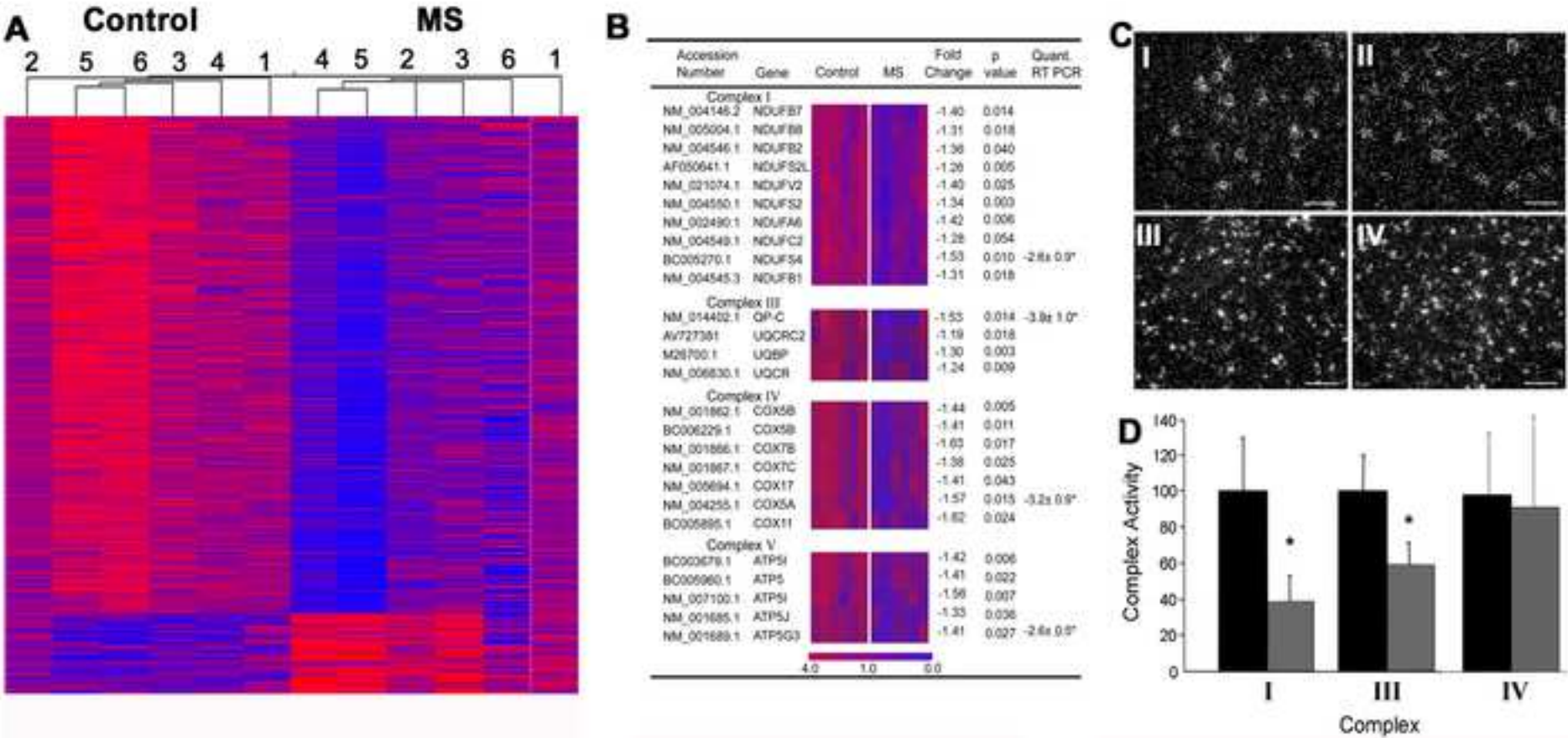


Figure 4

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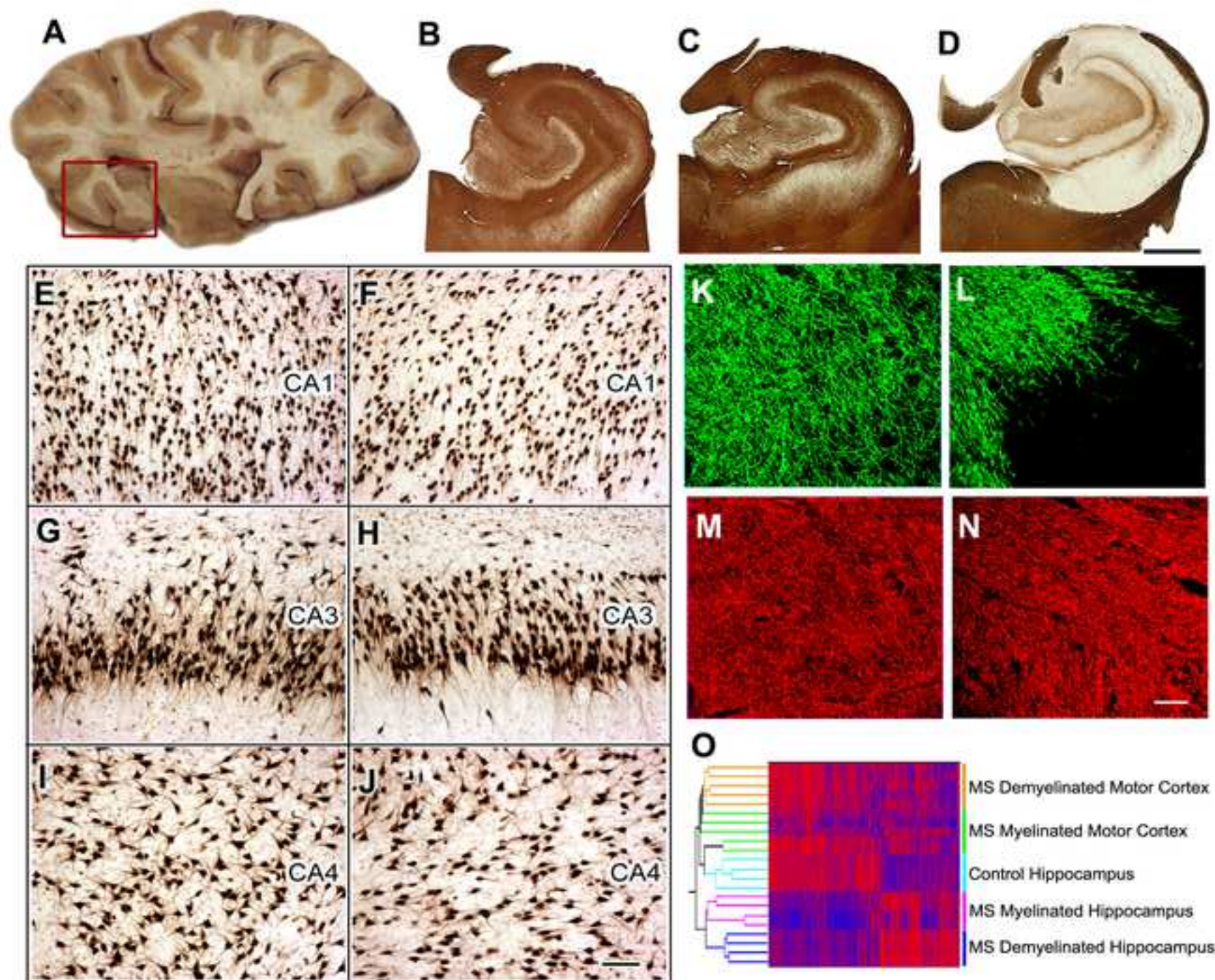
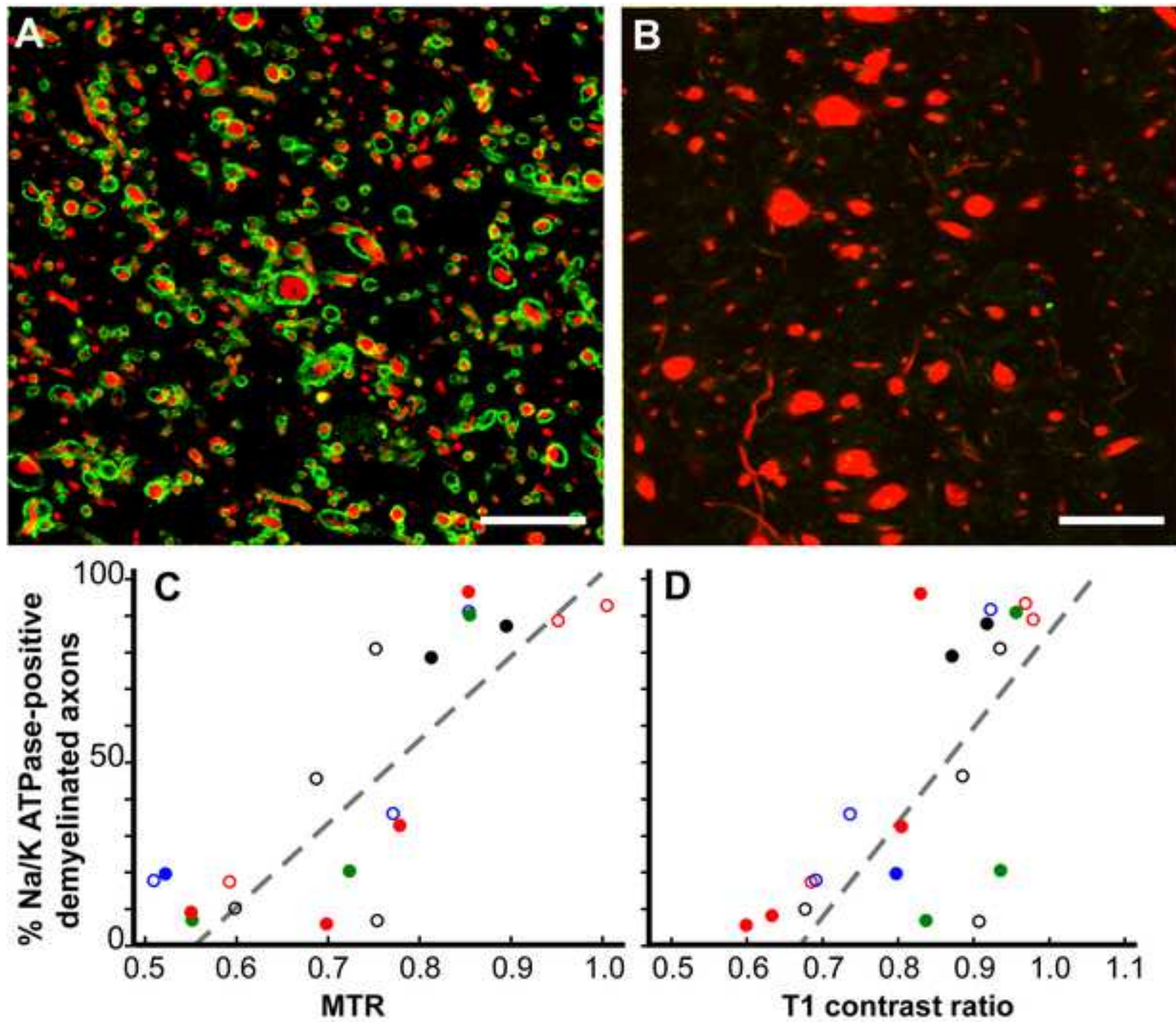
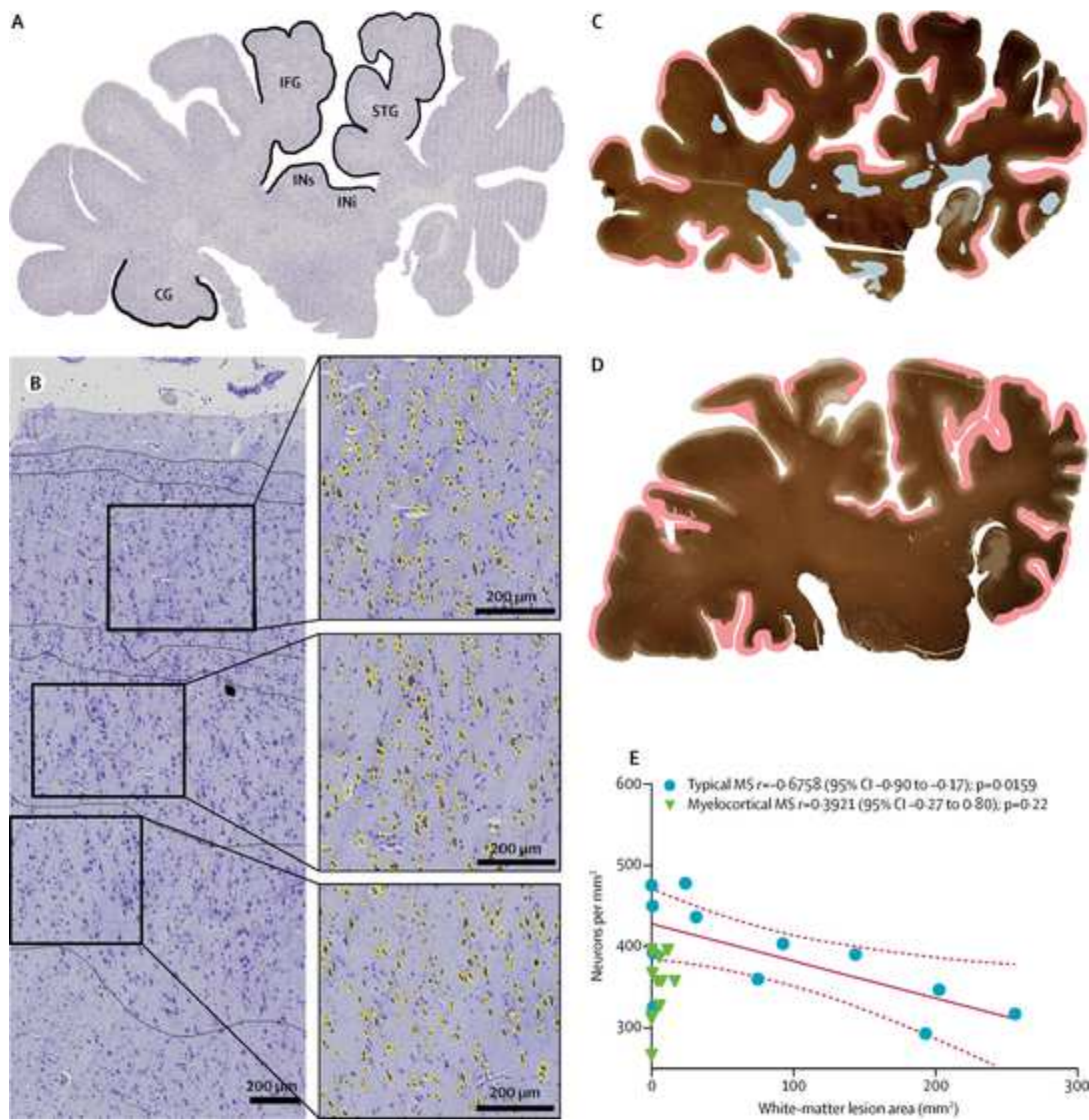




Figure 6





Sequence Duration	Sequence Description
0:09	Localizer
9:14	3D Magnetization prepared rapid gradient echo (MPRAGE)
5:14	3D Fluid attenuated inversion recovery (FLAIR)
2:35	2D T2 weighted
5:12	3D gradient-recalled echo with magnetization transfer pre-pulse , (MT-ON)
5:12	3D gradient-recalled echo without magnetization transfer pre-pulse , (MT-OFF)
0:27	Diffusion Tensor Imaging (DTI) field mapping
10:27	Diffusion Tensor Imaging (DTI) multi-shell
1:18	Diffusion Tensor Imaging (DTI) multi-shell
39:48:00	SUBTOTAL: CORE

## **Sequence use**

Localization for subsequent sequences

Structural Imaging Volumetric estimation of brain structures

Lesion identification Lesion segmentation Volumetric lesion assessment

Lesion identification Lesion segmentation Volumetric lesion assessment

Purported measure of myelin content in normal appearing and lesional tissue

Measure of water diffusion in brain tissue thought to reflect brain tissue integrity.



Name of Material/ Equipment	Company	Catalog Number	RRID	Comments/Description
<u>Antibodies</u>				
Biotinylated goat anti-mouse IgG	Vector Laboratories	BA-9200	AB_2336171	1:500 dilution for hemispheric; 1:1000 for 30µm free-floating
Biotinylated goat anti-rabbit IgG	Vector Laboratories	BA-1000		1:500 dilution
Biotinylated goat anti-rat IgG	Vector Laboratories	BA-9400	AB_2336208	1:500 dilution for hemispheric; 1:1000 for 30µm free-floating
Glial fibrillary acid protein (GFAP)	Dako	Z0334	AB_10013382	1:700 dilution for hemispheric
HuR, mouse IgG, 3A2 clone	Santa Cruz	SC-5261	AB_627770	1:500 for 30µm free floating
Major histocompatibility complex (MHC) class II HLA-DR CR3/43	Dako	Mo746	AB_2313661	1:250 dilution for hemispheric; 1:500 for 30µm free floating
Non-phosphorylated neurofilament (SMI32)	Biolegend	801701	AB_2564642	1:5000 dilution for hemispheric; 1:2500 for 30µm free-floating
Phosphorylated neurofilament (SMI31)	Biolegend	801601	AB_2564641	1:5000 dilution for hemispheric; 1:2500 for 30µm free-floating
Proteolipid protein (PLP)	Gift from Wendy Macklin			1:250 dilution for IHC; alternative anti-PLP antibodies commercially available.

<u>Reagents</u>				
125 mm filter paper	Whatman	1452-125		For filtering PFA
50% Glutaraldehyde	Electron Microscopy Sciences	16320		Electron microscopy grade
Cytoseal	ThermoScientific	8310-16		
Ethylene glycol	Fisher Chemical	BP230-4		
Glycerol	Sigma-Aldrich	G7893		400mL/2L Cryoprotection solution
Millex-HV Syringe Filter Unit, 0.45 µm, PVDF, 33 mm, gamma sterilized	Millipore-Sigma	SLHV033RB		

Paraformaldehyde	Electron Microscopy Sciences	19200		Prills form
Polyvinylpyrrolidone (PVP-40)	Fisher Chemical	BP220-212		
Sodium azide	Fisher Chemical	S227I		2g/2L Sorenson's buffer
Sodium phosphate dibasic	Sigma-Aldrich	S0876		98.8g/2L Sorenson's buffer
Sodium phosphate mono basic monohydrate	Sigma-Aldrich	S9638		14.352g/2L Sorenson's buffer
Sucrose	Sigma-Aldrich	PVP40-500G		
VectaStain ABC Kit	Vector Laboratories	PK-6100	AB_2336819	1:1000 dilution of A and B
Waterproof drawing black ink	Higgins	44201		
Xylene	Fisher Chemical	X3S		Histological grade

#### Equipment

3T MRI Magnetom Prisma	Siemens Healthineers
7T MRI Agilent 830AS	Siemens Healthineers

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Main document changes and comments

**Page 0:** Please focus the title on the highlighted portions of the protocol so that the title best represents the video. The current title does not represent the portions you have highlighted.

**Ranjan Dutta:** We will keep the current title. The video portion (highlighted) have been expanded accordingly to fit the title

**Ranjan Dutta:** Based on the contribution towards the revision, we have changed the order of the authors.

**Page 1:** I have moved the long descriptive sections from the protocol to introduction to conform with JoVE's style.

**Ranjan Dutta:** OK

**Page 2:** I have edited the protocol to meet JoVE's style requirements and also eliminated tradenames as per JoVE's policy against commercial names. Please ensure that all instruments and items used are listed in the table of materials.

**Ranjan Dutta:** New 'materials' section is uploaded

**Page 2:** I recommend extending the highlight to show a bit more of your steps from section 2 and/or 5.3. If you extend highlighting please ensure that continuity and completeness is maintained and that the steps will be filmable on the day of filming. Ensure that the highlight is under 2.75 pages including line spacing.

**Ranjan Dutta:** Additional section is highlighted to match the title of the article

**Page 4:** Add ink to the table of materials

**Ranjan Dutta:** Added

**Page 5:** Please update.

**Ranjan Dutta:** OK

**Page 5:** Add to the table of materials.

**Ranjan Dutta:** Added

**Page 5:** On a vibratome? Add it to the table of materials.

**Ranjan Dutta:** Changed

**Page 5:** Add to the table of materials.

**Ranjan Dutta:** Added

**Page 5:** Glutaraldehyde OR PFA? or a mix of both?

**Ranjan Dutta:** It's a mix of both

**Page 6:** Cryoprotection solution? Add to the table of materials.

**Ranjan Dutta:** Added

**Page 7:** Which antibodies? Add them to the table of materials along with RRIDs and working concentrations.

**Ranjan Dutta: Added**

**Page 8:** Please mention the step number where this was last handled? OR What is the source of the slices?

**Ranjan Dutta:** Step 2.9 Above

**Page 8:** Please mention MRI sequences to be acquired (e.g. flair etc.)

**Ranjan Dutta:** Listed in Table 1

**Page 8:** MRIs of which tissues? Those processed in the above steps?

**Ranjan Dutta:** In situ MRIs of whole head (from 1.1)

**Page 9:** Add the scanner to the table of materials.

**Ranjan Dutta: Added**

**Page 10:** What kind of transformations? Is this done in software? Please cite references where this is described in detail.

**Ranjan Dutta:** AFFINE image transformation according to Fisher et al., 2007 (Reference 13)

**Page 12:** Add to the table of materials

**Ranjan Dutta: Added**

**Page 12:** Add to the table of materials

**Ranjan Dutta: Added**

**Page 13:** Add scale bar to panel B.

**Ranjan Dutta: Added**

**Page 13:** Mention statistical test used and sample size.

**Ranjan Dutta: Added**