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### Publication Date

2014

Peer reviewed

# Improved pre-embedded immuno-electron microscopy procedures to preserve myelin integrity in mammalian central nervous tissue

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Preparation of specimens for pre-embedded immuno-electron microscopic analysis of CNS tissue is challenging. Conventional methods frequently result in significant damage to myelin integrity, ultimately confounding accurate analysis of myelin quality and assessment of changes in the number of lamellae. We have developed and optimized a protocol for maintaining good myelin morphology in CNS tissue while allowing for detect of antigens of interest with immunocytochemistry. We demonstrate that the presence of potassium ferrocyanide during lipid fixation is critical for obtaining optimal myelin ultrastructure. Further, our data illustrate the importance of rapid and efficient fixation for preservation of myelin integrity. Using this optimized technique, studies investigating potential efficacy of remyelination via administration of specific treatments can accurately distinguish between degenerating myelin after CNS injury/disease and new myelin (loose or compact) formed as a result of the intervention strategy.

**Keywords:** oligodendrocyte; ultrastructure; spinal cord injury; immunocytochemistry

## 1. Introduction

Pre-embedded immuno-electron microscopy (immuno-EM) of fixed biological specimens isolated from mammalian nervous tissue allows simultaneous detection of antigens of interest and morphological analysis of cellular ultrastructure at high resolution. However, acquisition of artifact-free images that accurately reveal myelin quality within the central white matter coupled with antigen detection is highly dependent on efficient antigen detection as well as prompt and uniform tissue fixation.

Due to its high lipid content, fixation of heavily myelinated tissue requires infiltration of strong lipid fixatives such as osmium tetroxide [1]. However, when performed in conjunction with immunohistochemical procedures, lipid fixation prior to antibody exposure (pre-embedded immuno-EM) can interfere with antigen accessibility. To circumvent the confounds of pre-embedding immuno-EM, many protocols use post-embedding techniques [2], but these procedures limit both antigen access and antibody penetration efficiency. Alternatively, when lipid fixation is followed by immunohistochemical procedures, use of detergents to permeabilize cellular membranes results in myelin degradation [3]. Thus, there is a paucity of successful pre-embedding immuno-EM techniques for the central white matter tissue where preservation of myelin integrity is critical.

The importance of obtaining adequate myelin ultrastructure becomes apparent in studies where the experimental treatment strategy is predicted to alter the number of lamellae and/or the quality of myelin wrappings (i.e. loose versus compact). Myelin is a defining feature of the vertebrate nervous system and variability in thickness of the myelin envelope may reflect conduction of neuronal signals [4], suggesting that preservation of myelin ultrastructure is an important feature of studies that address changes in neuronal conduction. As an example, the dysmyelinated mutant *shiverer* (*shi*) mouse has long been used in proof-of-principle experiments to test the remyelination capacity of potential therapeutics [5-8]. Critically, accurate investigation of treatment efficacy in studies using *shi* mice or similar animal models necessitates a clear distinction between endogenous axons containing few and often loose myelin sheaths, and any remyelination generated by the therapeutic intervention. However, when performed in conjunction with immunostaining to identify antigens, harsh immunohistochemical conditions can cause artifacts that significantly interfere with preservation of myelin integrity and may result in the false appearance of myelin degeneration. Hence, current immuno-EM procedures can limit accurate distinction between endogenously dysmyelinated axons and newly generated myelin (loose or compact), impeding accurate assessment of remyelination.

Additionally, a number of cell-based therapeutics for the treatment of neurological injuries and diseases have focused on remyelination of de/dysmyelinated axons as a mechanism for repair [8-14]. While increased myelin-basic protein (MBP) expression and/or oligodendrocytic differentiation of transplanted cell populations is often provided as evidence for generation of new myelin by the engrafted cells, analysis of remyelination at the level of EM has rarely been performed. In fact, of the studies cited above, only one utilized immuno-EM [8] in part due to the difficulties associated with this procedure.

In a systematic comparative study, we have tested the efficacy of multiple procedures to preserve myelin integrity, and demonstrate that acquisition of optimal CNS myelin ultrastructure is highly dependent on the prompt immersion of tissue into osmium tetroxide in the presence of potassium ferrocyanide. Further, we show that when multipotent human

CNS stem cells with the capacity to differentiate into myelinating oligodendrocytes are transplanted into the spinal cord of immunodeficient NOD-*scid* mice 30 days following injury, our optimized immuno-EM procedures demonstrate the presence of electron-dense DAB reaction products of a human-specific antibody (identifying engrafted human cells), while still providing significant preservation of myelin integrity to allow association of the DAB reaction product with intact myelin. Collectively, our data suggest that using optimized procedures, standard immunohistochemical conditions can be used in combination with myelin fixation to detect antigens while preserving CNS myelin ultrastructure.

## 2. Experimental Procedures

All animal housing conditions, surgical procedures, and post-operative care was conducted according to the Institutional Animal Care and Use Committee (IACUC) guidelines at the University of California, Irvine.

Two animal models were used in this study. Uninjured female C57Bl/6 mice (9-10 weeks old, JAX Laboratories) were utilized in optimization of myelin visualization for EM analysis in the absence of immunohistochemistry. Contusion-injured female NOD-*scid* mice (9-10 weeks old at the time of injury, JAX Laboratories) receiving human cell transplants were used in immuno-EM experiments. A contusion injury paradigm (see below) was used to generate a de/dysmyelinated niche near the injury epicenter that could be distinguished from regions of intact myelin. Immunodeficient NOD-*scid* mice were used to reduce confounds of xenograft rejection and enhance human cell engraftment success.

### 2.1 Contusion Injuries and Cell Transplantation

Young adult NOD-*scid* mice (9-10 weeks old) were anesthetized using Avertin (0.5ml/20g tribromo-ethanol), and received a laminectomy at the thoracic vertebrae 9 (T9) using a surgical microscope followed by 50kd (1 dyne = 10 $\mu$ N) contusion injuries using the Infinite Horizon (IH) Imapctor (Precision Systems and Instrumentation, Lexington, KY). Post-operation procedures were identical to previous publications [8, 15].

Thirty days following spinal cord injuries, NOD-*scid* mice received human-derived neurospheres [16] which were dissociated into a multi-cell suspension (<70 $\mu$ m aggregates) and concentrated to a final density of 75,000 cells/ $\mu$ l of injection buffer and transplanted into the intact spinal cord paranchyma, as previously described [8, 15].

### 2.2 Perfusion and Tissue Dissection of Blocks for EM Analysis

Non-transplanted adult C57Bl/6 mice or transplanted NOD-*scid* mice at 16 weeks post-transplant were anesthetized and transcardially perfused with 0.1M phosphate buffered saline (PBS) at pH 7.2, at 37°C, with a flow rate of 10ml/min for 5 minutes, to ensure blood clearing. Animals were then perfused using 4% paraformaldehyde/1% glutaraldehyde or 4% paraformaldehyde/0.5% glutaraldehyde (Table 1) in PBS (fixative cocktail) at 37°C at a rate of 10ml/min for 15 minutes to ensure optimal tissue fixation at the time of perfusion. Physiological temperature was used to avoid stress to the cellular membrane. A flow rate of 10ml/min was used to limit rupturing of micro-capillaries while simultaneously maximizing solution delivery. Immediately after perfusion, spinal cord tissue at the vertebral levels T6-T12 was dissected and placed in 6-well tissue culture plates containing fixative cocktail for a maximum of 30mins prior to block cutting. This process ensured steady and constant tissue hydration.

Fixed spinal cord tissue (T6-T12) was placed on a PEG-covered slide box containing the fixative cocktail and a ruler, and 1mm spinal cord pieces were cut using a sharp razor blade under a dissecting microscope while immersed in fixative, then submerged into microfuge tubes containing 1ml fixative cocktail, and placed at 4°C for 1-1.5hrs of post-fixation (Table 1).

Following post-fixation, 1mm tissue blocks obtained from C57Bl/6 mice were followed up by EM embedding procedures described below (not immuno-EM), while NOD-*scid* mice underwent immunohistochemical procedures.

### 2.3 Immunohistochemistry

Immunohistochemical procedures were only performed on NOD-*scid* mice. After post-fixation, 1mm spinal cord blocks were sectioned on a Vibratome at 40 $\mu$ m and collected in 96-well plates containing TBS. All subsequent steps were performed at room temperature unless otherwise indicated. Vibratomed sections were incubated in TBS containing BSA and Triton X-100 (TBS-B) for 30mins, followed by a 2hr incubation in primary antibody (SC121; StemCells, Inc.). Excess primary was washed twice, for 5mins each, in TBS containing Triton X-100 (TBS-A), followed by a 15min incubation in TBS-B. Sections were then incubated in secondary antibody for 1hr, washed in TBS-A for 10mins, followed by TBS-B for 15mins. Sections were then incubated in Avidin-Biotin Complex (ABC) for 45mins, washed twice with TBS, and finally developed with DAB (5mins).

## 2.4 Embedding Procedures and Ultrathin Microscopy

After either development of vibratomed sections with DAB (NOD-*scid*) or incubation of 1mm blocks in fixative cocktail (C57Bl/6), specimens were washed in TBS for 10mins. As outlined in Table 1, conditions 1-5 were used for fixation of each tissue block from C57Bl/6 mice. Condition 5 was also used for fixation of vibratomed sections obtained from NOD-*scid/shi* mice. Prior to dehydration, sections/blocks were washed for 10mins in DI water. Sections were dehydrated through a series of graded ethanols for 2x5mins at 50%, 75%, and 3x10mins at 95% and 100%. Lastly, tissue was exposed to Propylene Oxide 3 times for 20mins each. Fresh Epon Resin containing Eponate 12 Resin, Dodecyl Succinic Anhydride, double distilled (DDSA), Nadicmethyl Anhydride (NMA), and 2,4,6-Tridimethylaminomethyl Phenol (DMP-30) (All from Ted Pella, Inc.) was prepared in advance and placed in a vacuum to eliminate bubble formation. Then, a 1:1 mixture of Resin:Propylene Oxide was prepared, tissue sections/blocks were submerged into the mixture and placed in a vacuum overnight.

The next day, fresh Epon resin was prepared and placed in a vacuum to eliminate bubble formation. Sections/blocks were then removed from the 1:1 mixture and placed in fresh resin for 6-8hrs in a vacuum. At the end of incubation, fresh resin was once again prepared and tissue was removed from the microfuge tubes. Blocks were embedded in flat bottom BEEM embedding capsules (Electron Microscopy Sciences) containing fresh Epon while vibratomed sections were embedded in one drop of fresh Epon on an Aclar embedding film of 7.8mm thickness (Electron Microscopy Sciences) that was placed on a glass slide (Fisher Scientific). A second Aclar film was then overlaid onto the sections and a small screw thred vial of 6.2g (Fisher Scientific) was placed on top of the films to flatten the tissue. Blocks and sections were then cured at 55°C overnight. Thin (90nm) sections were cut on a Leica Ultra-Microtome, transferred to a 150-mesh copper grid, stained with a saturated solution of uranyl acetate in methanol, rinsed, re-stained with sodium hydroxide containing lead citrate, and again rinsed. Ultrathin sections were viewed on a Philips C10 transmission electron microscope.

## 3. Results

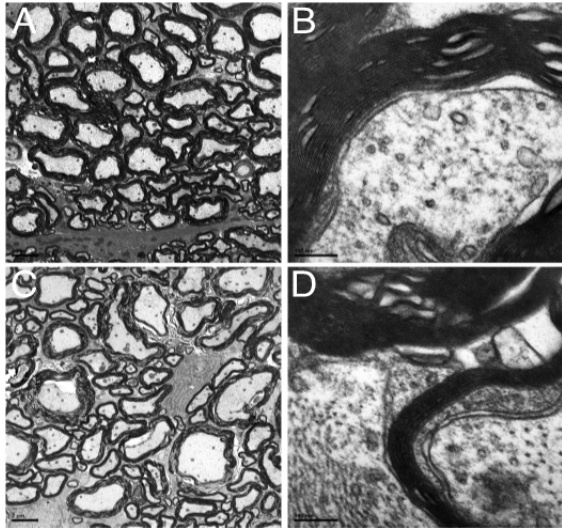
3.1 Use of osmium tetroxide, either before or after protein fixation, is insufficient for preservation of myelin integrity.

Prior to testing the optimal protocol for performing immuno-EM, we first processed 1mm tissue blocks obtained from C57Bl/6 mice using standard EM protocols, followed by testing of methodologies that were predicted to be more effective when used in more harsh conditions of immuno-EM. Following post-fixation in fixative cocktail, 1mm blocks of spinal cord were dissected and processed using a variety of conditions (Table 1).

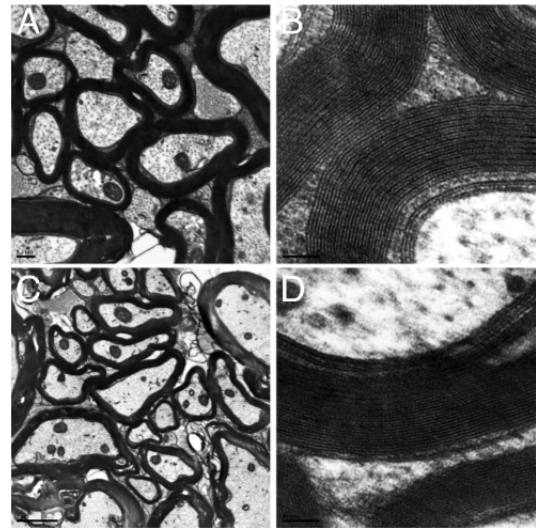
**Table 1** List of conditions that were tested for optimization of an EM/immuno-EM protocol that provides adequate myelin integrity. Cells highlighted in dark gray indicate protein fixation and cells highlighted in light gray indicate lipid fixation. Abbreviations: Para=Paraformaldehyde; Glut=Glutaraldehyde; Na Cac=Sodium Cacodylate; Os=Osmium; KFeCN=Potassium Ferrocyanide

Condition	Setting	Perfusion	Post-Fixation	Lipid or Protein Fixation	Wash	Lipid or Protein Fixation
1	Reagent	4% para/1% glut	4% para/1% glut	1% Os	0.2M Na Cac	2% glut
	Time	~15mins	1hr	1hr	3x15mins	1.5hrs
	Temperature	37°C	4°C	4°C	4°C	4°C
2	Reagent	4% para/1% glut	4% para/1% glut	2% glut	0.2M Na Cac	1% Os
	Time	~15mins	1hr	1.5hrs	3x15mins	1hr
	Temperature	37°C	4°C	4°C	4°C	4°C
3	Reagent	4% para/1% glut	4% para/1% glut	1% Os/1.5% KFeCN	0.2M Na Cac	2% glut
	Time	~15mins	1hr	1hr	3x15mins	1.5hrs
	Temperature	37°C	4°C	4°C	4°C	4°C
4	Reagent	4% para/1% glut	4% para/1% glut	2% glut	0.2M Na Cac	1% Os/1.5% KFeCN
	Time	~15mins	1hr	1.5hrs	3x15mins	1hr
	Temperature	37°C	4°C	4°C	4°C	4°C
5	Reagent	4% para/0.5% glut	4% para/1% glut	-	0.2M Na Cac	1% Os/1.5% KFeCN
	Time	~15mins	2hrs	-	3x15mins	1.5hrs
	Temperature	37°C	4°C	-	4°C	4°C

In condition 1 and consistent with the classical EM protocols, blocks were incubated in 1% osmium tetroxide, followed by protein cross-linking using 2% glutaraldehyde. While tissue was isolated from the intact spinal cord of C57Bl/6 mice, where myelin sheaths are predicted to be normal, lipid fixation using 1% osmium tetroxide resulted in loose myelin morphology around most of the axons (Fig. 1A-B), suggesting the presence of artifact associated with the procedures. Interestingly, in condition 2 where the order of lipid versus protein fixation was reversed, damage to myelin integrity was further exacerbated with most axons demonstrating the presence of loosely wrapped myelin sheaths (Fig. 2A-B).



**Fig. 1** Use of osmium tetroxide, either before or after protein fixation, is insufficient for preservation of myelin integrity. (A-B) Tissue fixation using condition 1 demonstrates the presence of many axons with loose myelin morphology around most axons, suggesting significant technical artifact. (C-D) Tissue fixation using condition 2 illustrates inadequate myelin fixation. Myelin wrappings around most axons are loose, suggesting that the procedures used significantly damage myelin integrity.



**Fig. 2** Use of osmium tetroxide in combination with potassium ferrocyanide, either before or after protein fixation, results in fine myelin integrity. (A) Tissue fixation using Condition 3 demonstrates the presence of compact myelin sheaths around all the axons in central white matter. (B) High power image clearly illustrates the number of myelin lamellae. Major dense lines can be clearly identified. (C-D) Tissue fixation using Condition 4 illustrates adequate myelin fixation and fine myelin ultrastructure.

### 3.2 Use of osmium tetroxide in combination with potassium ferrocyanide, either before or after protein fixation, results in fine myelin integrity.

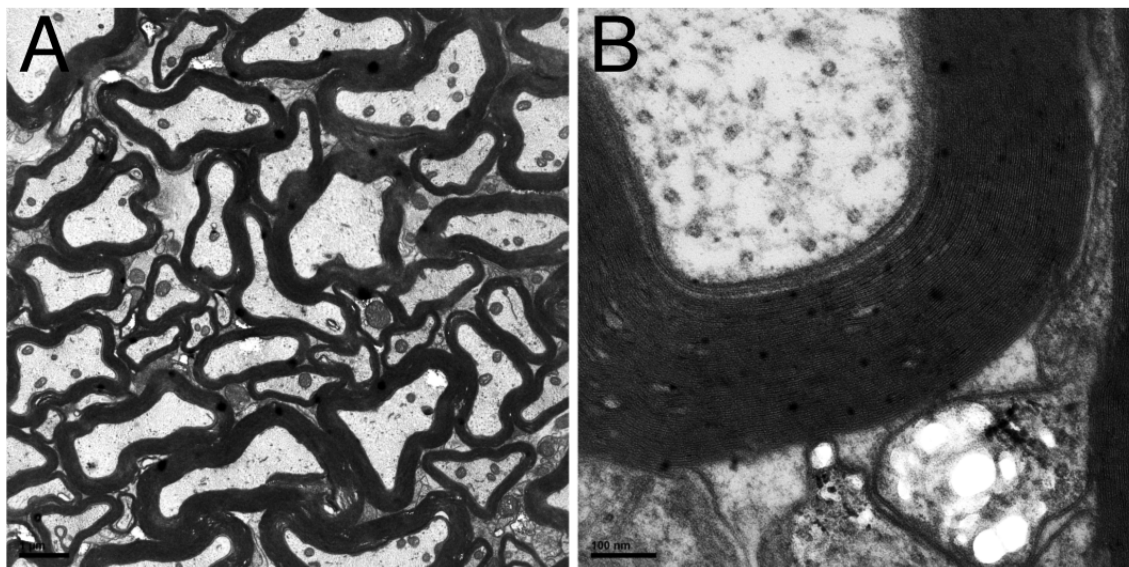
In condition 3, we combined osmium tetroxide fixation with potassium ferrocyanide to further enhance myelin morphology. This protocol was adapted from the presently best known EM methodology for fixation of peripheral myelin [17], and was followed by immersion of blocks into 2% glutaraldehyde (Table 1). In agreement with Coggeshall, our results demonstrated the presence of compact myelin with fine ultrastructure around all the axons of the spinal cord (Fig. 2A). The number of myelin sheath spiral wrappings around each axon, and the major dense lines could be clearly identified (Fig. 2B). These data confirm that potassium ferrocyanide is critical for preservation of CNS myelin integrity.

Fixation of lipids, which are the building blocks of myelin sheaths, prior to infusion of aldehydes or other protein fixatives, presents the challenge of insufficient protein fixation and limits antibody penetration past the cellular membrane. Thus, in condition 4, we reversed the order of protein and myelin fixation and addressed the question of whether rapid aldehyde use is sufficient for protein fixation. Based on previous data (Fig. 1C-D), we predicted that delay in lipid fixation compromises the ultrastructure of myelin sheaths. However, we found that the addition of potassium ferrocyanide is sufficient to significantly preserve myelin integrity of many spinal axons (Fig. 2C). High power images demonstrated fine myelin ultrastructure and the presence of compact myelin sheaths (Fig. 2D).

### 3.3 Use of mild aldehydes in protein post-fixation does not alter ultrastructural morphology and establishes grounds for immuno-EM procedures.

Prior to testing the optimal procedure for the harsh conditions of immuno-EM, we sought to investigate the effect of a protein fixative that was milder in comparison to glutaraldehyde. Due to the potential difficulties of antibody binding following the use of strong aldehydes, such as glutaraldehyde, we hypothesized that when used in combination with paraformaldehyde, reduction of glutaraldehyde concentration would result in sufficient protein fixation without the confounds of strong aldehydes. Thus, in Condition 5, we post-fixed tissue blocks in 4%para/1% glutaraldehyde, followed by osmium/potassium ferrocyanide. Our results demonstrated adequate protein ultrastructure as shown by the

clear presence of ribosomes and mitochondria in the tissue, as well as fine ultrastructural morphology of myelin sheaths (Fig. 3A). High power images demonstrated presence of compact myelin and adequate protein ultrastructure that was not compromised significantly by the reduction in glutaraldehyde concentration (Fig. 3B). These data established the grounds for preparation of specimens for immuno-EM analysis.



**Fig. 3** Use of mild aldehydes (0.5%-1% Glutaraldehyde) for protein fixation does not alter myelin ultrastructural morphology. (A) Tissue fixation using condition 5 demonstrates the presence of compact myelin sheaths around all axons in central white matter. (B) High power image clearly illustrates the number of myelin lamellae. Some embedding artifact can be identified but does not interfere with preservation of myelin.

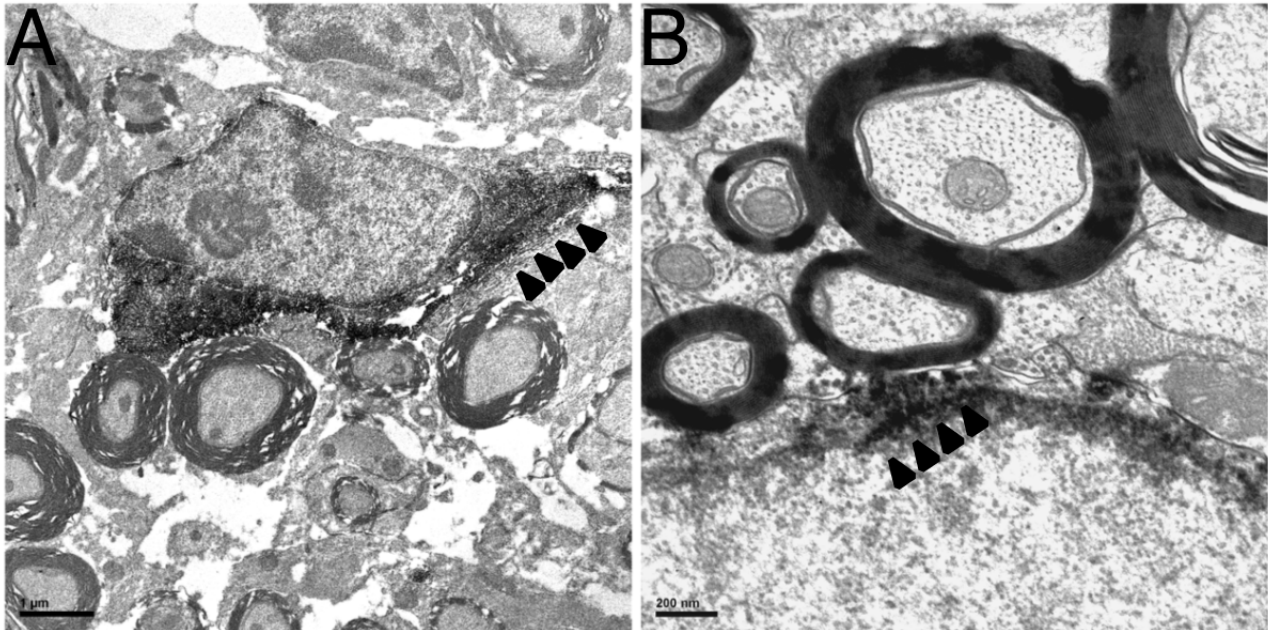
Interestingly, studies suggest that the cellular membrane quickly becomes permeable to ions upon fixation with osmium tetroxide, thus destroying osmotic activity, and resulting in tissue volume changes [18]. Conversely, aldehydes that are prepared in solutions of salts whose osmolarity is about 60% of cellular osmolarity in equilibrium do not destroy osmotic activity and do not alter tissue volume [19]. Further, after aldehyde fixation, even following the removal of aldehyde fixatives, the cells continue to respond in an osmotically active manner, thus minimizing tissue shrinkage/swelling. Here, our parameters for preparation of solutions were selected specifically to match cellular osmolarity. Notably and in support of previous findings above, using condition 5 (Fig. 3) where osmium tetroxide infusion was the final fixation step, parenchymal preservation is significantly higher than condition 1 where osmium tetroxide fixation was done prior to aldehyde infusion (Fig. 1A).

### 3.4 Conditions established in the present study optimize myelin integrity even in combination with harsh immunohistochemical procedures.

In order to investigate the efficacy of our established protocol in the more harsh conditions of immuno-EM, we transplanted human neural stem cells with the capacity to differentiate into myelinating oligodendrocytes into spinal cord-injured NOD-*scid* mice as a potential therapeutic strategy to restore functional recovery. We hypothesized that transplantation of human neural stem cells results in locomotor recovery via remyelination of host axons. Thus, in order to preserve myelin integrity and accurately investigate remyelination by the engrafted cells, we utilized the optimized conditions discussed above, in combination with harsh immunohistochemical conditions using the pan-human cytoplasmic marker SC121 (STEM).

Observations of ultrathin sections demonstrated the presence of electron-dense peroxidase reaction products indicative of SC121-positive human cells, suggesting that our procedures provided adequate protein fixation as well as antibody penetration. High power images demonstrated that low concentrations of glutaraldehyde were sufficient to obtain fine ultrastructure in protein-containing organelles. Critically, the harsh conditions that utilize strong detergents for cellular permeabilization to allow antibody penetration, could also damage the integrity of myelin sheaths. However, we have shown that rapid lipid fixation circumvents this issue: Following immunohistochemical procedures, vibratomed sections were promptly submerged in osmium tetroxide in combination with potassium ferrocyanide (Fig. 4B). In fact, we have found that increasing the delay in osmium tetroxide infusion (i.e. overnight incubation in sodium cacodylate buffer) results in increasing damage to myelin integrity (Fig. 4A), suggesting that efficient myelin fixation largely depends upon prompt technical procedures. Analysis of ultrathin sections demonstrated that using these procedures, myelin sheath integrity is highly preserved. Critically, dense DAB reaction products indicative of human-specific cytoplasmic antibody was found near axons containing many wraps of well-preserved myelin sheaths (Fig. 4B).

Thus, not only do these procedures provide adequate antibody penetration, but they also allow for accurate analysis of myelinated axons without the confounds of artifact that may result in false appearance of myelin loss/degeneration.



**Fig. 4** Optimized EM procedures preserve myelin integrity even in combination with harsh immunohistochemical procedures. (A) Overnight incubation of immunostained vibratomed sections in cacodylate buffer followed by next day osmium immersion results in significant damage to myelin integrity. While the electron-dense DAB reaction product can be detected (arrowheads), myelin integrity is extremely poor. Further, parenchymal damage is significant and mitochondrial and ribosomal ultrastructures are severely compromised. (B) Rapid fixation of immunostained vibratomed sections with osmium immersion in the presence of potassium ferrocyanide results in significant preservation of myelin integrity. Furthermore, electron-dense DAB reaction product can be detected (arrowheads) within transplanted human cells.

#### 4. Discussion

Potassium ferrocyanide is used frequently as a histochemical tool to detect electron-dense reaction products [20]. In 1977, ferrocyanide was identified as a useful technique to stain nodes of Ranvier in mammalian peripheral nerves [21]. Since then, only a few groups have utilized this technique for myelin fixation within peripheral tissue [22] and spinal roots [17]. However, it is not clear whether similar technical procedures would result in efficient myelin fixation in the CNS. Interestingly, studies have shown distinct ultrastructural, as well as molecular, differences between peripheral and central myelin where smaller periodicity of myelin lamellae in central, as opposed to peripheral, myelin reflects greater abundance of short-chain fatty acids and thus, less fixation stability [23]. To our knowledge, use of potassium ferrocyanide for CNS myelin fixation has not been previously reported. Our data suggest that potassium ferrocyanide during lipid fixation is critical to preservation of myelin integrity.

Characterization of the stage in the preparation procedure during which damage to myelin is produced has been previously published [24]. Condie et al. indicated that damage to myelin integrity can be indirectly traced to the nature of polymerization of methacrylate used during the embedding procedures. Therefore, use of alternative embedding media that do not distort myelin ultrastructure such as Vestopal W was recommended [24]. Presently, use of methacrylate for fixation of biological specimens is rare and epoxy resins have become the most common media for electron microscopy procedures. All of our specimens in the present study were embedded using Epon, which provides uniform hardening with minimal tissue shrinkage. Thus, our embedding procedures are consistent with those previously recommended and are unlikely to account for the differences in myelin ultrastructure.

One of the key findings in the present study was the importance of rapid fixation procedures. In fact, in a number of other conditions not discussed here, we have found that increasing the delay in lipid fixation further exacerbates CNS myelin integrity. These data are consistent with the prediction that CNS myelin is less stable than PNS myelin, and suggests that acquisition of fine myelin ultrastructure is significantly dependent on rapid tissue fixation. Incubation of tissue in buffers or fixatives overnight is highly discouraged, as myelin is predicted to rapidly degenerate during these incubation periods. While Liu et al. have recently described an optimization EM protocol for embedded human brain specimen with long-term fixations and frozen sections, the efficacy of this technique in preservation of myelin integrity in immuno-EM remains unclear [25].

This systematic comparative study characterizes a highly modified immuno-EM procedure that can preserve CNS myelin integrity to a great extent while still allowing antigen detection at the immunohistochemical level. This methodology can be especially useful to investigators interested in the study of myelin ultrastructure, mechanisms of myelination/remyelination, and problems associated with demyelination, such as in injury or disease.

**Acknowledgements** We would like to thank Dr. Desiree Salazar for her contribution of NOD-*scid* transplanted animals. We would also like to thank our collaborators at StemCells, Inc., in particular, Dr. Nobuko Uchida, Dr. Stan Tamaki, and Dongping He for maintenance and culturing of human neural stem cells. We would like to acknowledge the technical advice of Dr. Charles Ribak and in particular, his EM technician, Zhiyin Shan.

**Funding** This study was funded by NIH grant (NS49885 to AJA).

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