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Rapid Assessment of Internodal Myelin Integrity in CNS Tissue

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Abstract

Monitoring pathology/regeneration in experimental models of de-/remyelination requires an accurate measure not only of functional changes but also of the amount of myelin. We tested whether x-ray diffraction (XRD), which measures periodicity in unfixed myelin, can assess the structural integrity of myelin in fixed tissue. From laboratories involved in spinal cord injury research and in studying the aging primate brain, we solicited “blind” samples and used an electronic detector to rapidly record diffraction patterns (30 minutes each pattern) from them. We assessed myelin integrity by measuring its periodicity and relative amount. Fixation of tissue itself introduced $\pm 10\%$ variation in periodicity and $\pm 40\%$ variation in relative amount of myelin. For samples having the most native-like periods the relative amounts of myelin detected allowed distinctions to be made between normal vs. demyelinating segments and motor vs. sensory tracts within the spinal cord, and aged vs. young primate CNS. Different periodicities also allowed distinctions to be made between samples from spinal cord and nerve roots, and between well-fixed vs. poorly-fixed samples. Our findings suggest that in addition to evaluating the effectiveness of different fixatives, XRD could also be used as a robust and rapid technique for quantitating the relative amount of myelin among spinal cords and other CNS tissue samples from experimental models of de- and remyelination.

Keywords

spinal cord injury; myelin injury; myelin repair; optic nerve; nerve roots; CNS; electron microscopy; x-ray diffraction; glutaraldehyde; paraformaldehyde; membrane structure; mouse; rat; primate

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INTRODUCTION

Classically used for periodicity measurements of internodal myelin in both PNS and CNS (Schmitt et al. 1935), we recently used X-ray diffraction (XRD) to determine the relative amounts of myelin (e.g., hypomyelination) and myelin membrane packing defects in freshly-dissected, unfixed peripheral nerves from transgenic mice engineered to model human peripheral demyelinating disorders (Avila et al. 2005; Wrabetz et al. 2006). In subsequent experiments, we also showed that XRD could rapidly and quantitatively distinguish PNS-like myelin in optic nerves from transgenic mice expressing the peripheral myelin protein P0 instead of the central myelin proteolipid protein (Yin et al. 2006), and hypermyelination in optic nerves from tg mice that over-expressed constitutively-active Akt in oligodendrocytes (Flores et al. 2008). XRD provided a quantitative validation of what was visually observed about internodal myelin from microscopy, and additionally revealed subtle differences not detected by electron microscopy. The XRD studies cited above were greatly facilitated by the ease with which fresh sciatic and optic nerves can be dissected from laboratory animals. Though more rarely analyzed by XRD, freshly-dissected spinal cord from mice or rats have also been examined—e.g., to compare the myelin structures between CNS and PNS (Schmitt et al. 1935), to explore how myelin structure responds to IDPN-induced axonal swelling induced by β,β' -iminodipropionitrile (IDPN) (Blaurock et al. 1991), and to characterize the differential effects of decompression on CNS vs. PNS myelinated tissue (Bond and Kirschner 1997). The use of spinal cord is challenging, owing to the difficulty of the dissection and the intrinsic fragility of the tissue, which lacks appreciable supporting connective tissue. As one of our long-term objectives is to assess myelin structural integrity in animal models of CNS injury and repair, we sought here to concentrate on samples prepared by laboratories that are already undertaking such research, and that are using microscopy to evaluate tissue ultrastructure. To avoid the problem of tissue deterioration during transit of material from to our laboratory, we analyzed CNS tissue that had been fixed in aldehyde according to the collaborating laboratories' protocols.

The samples examined showed a variety of fixation-dependent effects, as evidenced by the wide range in myelin periodicity (155–190 Å) and the broadened x-ray reflections. The different myelin periods and the dispersion in their measured values allowed us to distinguish spinal cord from spinal roots, and good fixation from poor fixation. For well-fixed spinal cords, the different relative amounts of myelin detected by diffraction allowed us to distinguish motor *versus* sensory tracts, and normal *versus* less myelinated segments. XRD was also able to differentiate between myelinated tissues from aged *versus* young primate brain. Our findings demonstrate that XRD can rapidly assess and quantitate the relative amount of myelin in whole segments of spinal cord after aldehyde-fixation. Application of this approach to evaluate different experimental animal paradigms of de- and remyelination (e.g., in spinal cord injury/repair, leukodystrophies, EAE, and EAN) should facilitate more rapid development of effective treatment strategies for humans.

Materials and Methods

Sample preparation for XRD

At the collaborators' labs, tissues were dissected from experimental animals (rat, Laboratories A and B; mouse, Laboratory C; rhesus monkey, *Macaca mulatta*, Laboratory D) fixed in aldehydes, and sent (coded) in fixative to the XRD laboratory in the Biology Department at Boston College. Depending on the protocol used by the collaborating laboratory, the cords were segmented, halved or quartered, or embedded in agar and cut into 500- μ m thick slabs using a vibratome (Laboratory B). Laboratory C had injected ethidium bromide (EB) to induce demyelination in spinal cord (Blakemore and Crang 1989; Woodruff and Franklin 1999). Samples from Laboratory D consisted of optic nerves and

corpus callosum dissected from fixed brains from both young and old monkeys. As fixed myelin is still osmotically active (Kirschner and Hollingshead 1980), the samples were always stored in the primary fixative. Upon receipt of tissues, the samples were loaded, in contact with the same fixative solution, into thin-walled capillaries (0.5–2.0 mm diameter, depending on sample width; Charles Supper Co., South Natick, MA), and stored at room temperature until x-ray data collection. Samples were mounted as straight as possible to ensure that the myelinated fibers would be parallel to the long dimension of the beam, and thus minimize artifactual broadening of the x-ray reflections. Samples much thicker than 1.5 mm absorb the scattered x-rays, so we trimmed over-sized samples sagittally and/or coronally. Table 1 summarizes the samples received from each laboratory and the chemical fixatives used, which included 2% glutaraldehyde (GA), 4% or 8% paraformaldehyde (PF), and 2% PF + 2.5% GA (PF+GA). Fig. 1 shows examples of samples as received and after insertion into x-ray capillaries.

X-ray diffraction analysis

Diffraction experiments were carried out using nickel-filtered, single-mirror focused CuK α radiation from a fine-line source on a 3.0 kW Rigaku x-ray generator (Rigaku/MSI Inc., The Woodlands, TX 77381-5209) operated at 40 kV by 14–22 mA. To record the x-ray patterns we used a linear, position-sensitive detector (Molecular Metrology, Inc., Northampton, MA 01060). Experimental details are elaborated elsewhere (Avila et al. 2005). To optimize the signal-to-background and obtain significant counting statistics using this detector, we recorded each XRD pattern for 30 min. As the beam is 3 mm high at the sample position, samples much longer than this along their neuraxis were shifted by ~3 mm to allow recording from a different region of the segment. Some segments were rotated 90° about their long axis in order to sample other volumes within the same segment.

Measurement of the positions of the reflections in the diffraction patterns give, from Bragg's law, immediate and direct information about the *periodicity* of myelin. To estimate the relative amount of multilamellar myelin in the volume of the sample that was subtended by the x-ray beam, we calculated the total integrated intensity (M) above “background” (B) using PeakFit (SysStat Software, Inc.) or in-house software (Inouye et al. 1989). The stability of the x-ray generator and its x-ray output, which declines very slowly over a period of years, obviated our need to continually monitor the intensity of the direct beam. The normalization, expressed by the quotient $M/(M+B)$, allowed us to compare the diffraction from samples having different x-ray absorptions owing to different thicknesses (e.g., whole cords, halves or quarter cords, vibratome slabs, and roots). As reported (Avila et al. 2005) and shown here, a scatter plot of the fraction of total scattered integrated intensity that is due to myelin ($M/(M+B)$) vs. myelin period (d) facilitated distinguishing these characteristics of internodal myelin among different samples. Statistical analysis of the data was carried out using the Student's t -test, and values of $p < 0.05$ were considered significant.

Results and Analysis

Extent of myelin preservation varied among samples

XRD is used typically to characterize the structural status of myelin in native, unfixed tissue; however, unlike optic and sciatic nerves which remain undamaged and coherent wholes when dissected, spinal cord is fragile and extremely difficult to manipulate after dissection. Hence, in the current study we used chemically-fixed cords, prepared according to protocols in the collaborating labs (Table 1). The most effective fixative would be expected to closely preserve (*i*) the native packing (period) of myelin membranes as measured by XRD from freshly-dissected, unfixed tissues of mice and rat—for CNS, this would be in the range of 154–160 Å, and for PNS, 173–178 Å (Avila et al. 2005; Kirschner and Blaurock 1992) (A.

Gilardini, unpublished observations), and (ii) the regularity of the membrane packing, as evidenced by retention of sharp reflections. Conversely, poor fixation would be indicated by major changes in myelin period and considerable broadening of the reflections, which would overlap if there were myelin arrays having different periods (Kirschner and Hollingshead 1980). As evident from the patterns recorded here from whole tissue segments (Fig. 2), and from quantitation of the patterns (Fig. 3), the extent of myelin preservation (i.e., period and relative amount of ordered arrays) varied from fixative to fixative, from laboratory to lab, and even within a lab (Table 1, Laboratories *A* and *B*). Nonetheless, within each batch of samples as described below, there was consistency among these metrics.

Motor and sensory tracts can be distinguished by XRD

The four batches of spinal cord samples from Laboratory *A* yielded x-ray patterns that differed substantially among the batches: only one set gave sharp reflections (Fig. 2A, batch 2), while the others showed very broad reflections indicative of heterogeneity in period and/or disordered membrane packing. Two of the batches included samples that had been dissected to separate motor and sensory tracts from the spinal cord. The 8% PF-fixed cords were usually better preserved than 4% PF-fixed ones; however, other 8% PF cords more closely resembled the samples fixed at 4% PF or also had extremely broad reflections. The underlying reason for this batch-to-batch discrepancy among apparently identically fixed samples from this laboratory is not clear. The scatterplots, which correlate the relative amount of myelin $M/(M+B)$ vs. myelin period d (Fig. 3A), showed that there was, in fact, internal consistency of fixation within a particular set of samples. When the fixation was satisfactory, as evident from either the native-like period or sharp x-ray reflections, the fine-dissected myelinated tracts that were chiefly motor had stronger x-ray patterns than those that were chiefly sensory (Fig. 2A, batch 2). This can be accounted for by thicker sheaths in the motor fibers and/or a greater number of myelinated fibers within the tissue volume. Based on values for $M/(M+B)$ the relative amount of myelin in the sensory tracts was about one-half as much as in the motor tracts (Fig. 3A; sensory tract 0.10 ± 0.03 , motor tract 0.19 ± 0.02 , $N=6$ each, $p < 0.0004$).

Evaluation of vibratome slabs

The vibratome slabs prepared by Laboratory *B* from excised spinal cord showed very broad x-ray reflections (Fig. 2B) and substantial variations in period and relative amount of myelin (186.0 ± 5.0 Å; 0.14 ± 0.10 [$N=12$]) (Fig. 3B), even though all of these had been fixed identically (with 4% PF). Some of these samples were so swollen that there was overlap in both myelin period and relative amount of myelin between them and the spinal roots (see below). Measurements from different regions of the same slab showed large variations in both period and amount of myelin, which could be explained if the orientation of the myelinated fibers within the slab was not strictly parallel to the cut surfaces. Mis-orientation would result in geometric broadening of the reflections, indicating apparent membrane disordering and different amounts of myelin. (The agar by itself gave only low background scatter, and so did not interfere with the myelin scatter.) Once fixation is improved and the orientation problem solved, then standardization of vibratome-cut samples could become useful in analyzing more finely-defined columns or tracts within cords.

XRD distinguished hypomyelination in lesioned spinal cord

Samples from laboratory *C*, which were fixed with 2% GA, gave XRD patterns that were relatively weak ($M/(M+B)=0.07 \pm 0.02$, $N=44$) and had broadened reflections (indicative of disordering) (Fig. 2C); however, the myelin periods (162.5 ± 1.4 Å) were closer to native values and resembled those for some of the Laboratory *A* samples that had been fixed in 8% PF. The cords had been lesioned by injecting ethidium bromide (EB) into the right side of the cord (Blakemore and Crang 1989; Woodruff and Franklin 1999). Assessment of the right

and left halves of the rostral, central, and caudal segments revealed that the patterns from segments that were on the right (lesioned) side and at about the same level or caudal to the injection site were weaker and had a lower relative amount of myelin ($M/M+B=0.062\pm0.016$, $N=12$) than corresponding segments on the left (non-lesioned) side (0.086 ± 0.018 , $N=12$; $p<0.002$,) (Fig. 3C). Based on the $M/(M+B)$ values this corresponded to ~30% less myelin in the lesioned versus non-lesioned side. This indicated that XRD was able to distinguish the less myelinated fiber tracts from the normal ones.

XRD quantitated differences in periodicity and amount of CNS myelin in aging primates and aging samples

The samples from Laboratory *D* enabled us to test further our approach by using samples that had been previously analyzed by EM in a study on the effects of age on nerve fibers in a non-human primate (rhesus monkey) (Sandell and Peters 2001). Moreover, the samples provided allowed us to evaluate two very different fixatives on myelin preservation: 2% PF + 2.5% GA and 4% PF. The diffraction patterns differed substantially among the samples (Fig. 2D), which included both optic nerves and corpus callosum. A scatterplot of the relative amount of myelin *vs.* myelin period (Fig. 3D) showed quantitative fixative-related and age-related differences. Tissue fixed with PF+GA (irrespective of age of animal) gave diffraction patterns that had more native-like spacings ($d=158.3\pm2.7$ Å, $N=16$; optic nerve only) than those fixed with 4% PF ($d=181.4\pm5.0$ Å, $N=6$), indicating better preservation of the native myelin membrane arrays by PF+GA.

For the (PF+GA)-fixed optic nerves examined here, myelin period increased with animal age by ~1% (or <2 Å) per 10 years ($d = 0.1737t + 156.35$; $p<0.005$; Fig. 3E). The small increase detected by XRD here reflects changes in multilamellar, periodic myelin, and was much smaller in magnitude than the aperiodic splitting of the major dense and intraperiod lines detected by EM and reported as age-related alterations in internodal myelin of this tissue (Sandell and Peters 2001).

Samples from the younger animals (irrespective of fixative) gave stronger XRD patterns than those from the older ones (Fig. 2D). Quantitation of the relative amount of ordered myelin *versus* age of the animal showed that there was ~10% decrease per 10 years ($[M/M+B] = -0.002t + 0.20$; $p<0.005$; Fig. 2G). That this is due, at least in part, to a reduced packing density of fibers is suggested by morphometric analysis of rhesus optic nerve (Sandell and Peters 2001) which shows a ~16% decrease in packing density per 10 years.

Because some of the tissue had been stored in fixative for nearly 20 years, we determined whether storage time in the fixative affected either or both myelin period and relative amount of ordered membranes. Myelin period increased with storage time by ~2% (or ~3 Å) per 10 years ($d = 0.343t + 155.1$; $p<0.005$; Fig. 3F), and the relative amount of myelin diminished with storage by ~20% per 10 years ($[M/M+B] = -0.004t + 0.22$; $p<0.005$; Fig. 3H). That fixed, internodal CNS myelin retains a certain degree of structural plasticity in its membrane packing is consistent with previous results from PNS myelin, where fixation in either glutaraldehyde or osmium tetroxide does not prevent subsequent swelling of myelin when nerves are exposed to lowered ionic strength (Kirschner and Hollingshead 1980).

Spinal cord (CNS) was discriminated from nerve roots (PNS)

Dissected spinal cords may harbor adhering spinal roots, which are considerably thinner than cord. Although these can mostly be dissected away, there could still be some roots in the path of the x-ray beam through the sample. Thus, the question arose whether the presence of roots would interfere with the x-ray scatter from the cord, and thereby complicate interpretation of the data. In unfixed myelinated tissues, the ~20 Å-difference in

period between CNS and PNS myelins is readily detected by XRD (Kirschner and Hollingshead 1980); however, chemical fixation introduces modifications that might confound differentiating x-ray scatter between cords (CNS) and roots (PNS). The inclusion of nerve roots with the spinal cords from Laboratories *A* and *B* enabled us to address this question. (The roots were not distinguished as to ventral or dorsal.)

The x-ray patterns from the rat spinal roots of Laboratory *A* (Fig. 2A, upper spectrum) were clearly distinguished from those of cords (Fig. 2A, lower spectra): the peaks were centered at different channel positions compared to those from the CNS, indicating a different period ($d=195.4\pm2.5$, $N=4$ in two batches) than cord myelin ($d=160\pm6$, $N=12$ in two batches, different fixatives). The diffraction peaks from the roots tended to be stronger and sharper than those from the cord, indicating about twice as much myelin ($M/M+B=0.31\pm0.05$ vs. 0.14 ± 0.05) and myelin that was more ordered. As pointed out above, the rat spinal root samples from Laboratory *B* gave myelin diffraction patterns with periods ($d=190.9\pm3.8$, $N=4$) and amounts of myelin ($M/M+B=0.32\pm0.03$) that overlapped with some of those from the spinal cords ($d=184.7\pm4.8$; $M/M+B=0.15\pm0.09$, $N=17$), owing to the large amount of swelling in the latter (Fig. 3B); however, the distribution of scattering intensity among the reflections was always clearly differentiated between spinal roots and spinal cord (Figs. 3A,B), with about twice as much myelin in the former, based on $M/(M+B)$ values.

Discussion

In myelinated nerves conduction velocity depends on the structural integrity of the myelin—including internodal length, sheath thickness, and membrane packing (Waxman and Bangalore 2004). Myelin damage caused by disease or accident will result in conduction abnormalities and severe debilitation (Waxman et al. 1995). Reducing the time that is required by an investigator to analyze myelin structural integrity in a particular injury/repair model can help accelerate the pace of research and discovery, eventually impacting therapeutic advances and patient care. The objective of the current study was to test the use of XRD for assessing the preservation and amount of myelin in fixed CNS tissues under normal and pathological (e.g., demyelinating or remyelinating) conditions. In addition to evaluating the effectiveness of different chemical fixatives that are being used by different laboratories to preserve myelin structure, we demonstrated that XRD is able to quantitate rapidly the relative amount of myelin in spinal cord, optic nerve, and corpus callosum. Assessing myelin structural integrity in animal models of CNS injury and repair is one of our long-term objectives; hence, for this study we concentrated on samples received from three laboratories undertaking SCI research and one laboratory studying the aging primate brain. These samples were comparable to tissue samples routinely analyzed using histology and EM by the collaborating laboratories (Akiyama et al. 2002; Cao et al. 2005; Takami et al. 2002).

Monitoring the structural damage to myelin following spinal cord injury (Totoiu and Keirstead 2005) and repair (McDonald and Belegu 2006) requires quantitative, analytical tools for assessing the *extent of remyelination*. Conventionally, such tools include light microscopy of sections stained with myelin-specific reagents (e.g., lipophilic compounds, or antibodies to myelin proteins), and electron microscopy. Light microscopy gives a gross measure of myelin amount and its distribution among different fiber types, but no information about either packing defects or stability. Electron microscopy provides ultrastructural details about myelination: e.g., how extensive it is, whether the period looks normal, whether there is any swelling (dyscompaction), what is the distribution of fiber types in the nerve, and whether the morphology of differentiated regions (such as nodes and paranodes) appear normal. Chemical processing for microscopy, however, introduces considerable structural artifacts (Kirschner and Hollingshead 1980). Further, the

ultrastructural analysis involves selective sampling because the microscopist tends to examine only those regions of thin-sections where multilamellar myelin is most regular. These regular membrane arrays are regarded as indicative of the *in vivo* structure and are typically used to guide further experiments; however, the disordered or dyscompacted arrays that might be present could signal pathology. Thus, focal abnormalities in tissue architecture detected by EM may relate to myelin pathology, including edema, inflammation, and degeneration. Here we explored an alternative method, complementary to electron microscopy, that can quantitatively and more rapidly (within 30 min) provide much needed information about the extent of myelination—*X-ray diffraction* (XRD), which has classically been used for over 70 years for measuring myelin periodicity (Schmitt et al. 1935). That XRD can also be used to measure the relative *amount* of internodal myelin in whole tissue was only recently demonstrated (Avila et al. 2005; Wrabetz et al. 2006). Validation of this approach is provided by parallel morphological (g-ratio) and XRD measurements on hypermyelinated optic nerves from 2 month-old tg mice that over-express constitutively-active Akt in oligodendrocytes (Flores et al. 2008). The increase in g-ratio for the tg mice compared to wildtype mice corresponded to a 30% increase in myelin thickness, which resembled the 33% increase in the relative amount of myelin measured by XRD. Further validation is shown in a separate study where the changes in g-ratios, myelin basic protein levels, myelin yield, and myelin lipid levels all closely parallel the change in the relative amount of myelin as quantitated by XRD (Agrawal et al. 2009). In the current study, the greater relative amount of myelin in motor *versus* sensory tracts provided the basis for distinguishing these tracts from one another. Whether this difference arose from thicker sheaths or a greater number density of fibers in the motor tract cannot be discriminated by XRD; however, microscopy readily shows that motor axons are larger and have thicker myelin sheaths.

One great advantage of XRD is that once the fixed tissue is available, the data about myelin structural integrity in whole tissue can be collected within minutes using an electronic detector and conventional x-ray generator (Padron et al. 1979) or within seconds using the detector with a synchrotron x-ray source (De Felici et al. 2008), and subsequently quantitated. By comparison, undertaking electron microscopy requires several days to a week or more to complete tissue processing, sectioning, examining ultrastructure in tissue sections, and morphometry. Moreover, depending on beam size, XRD can probe in a single exposure a tissue volume that is 2–30 million times greater than that of EM and containing hundreds or thousands of nerve fibers (Avila et al. 2005; Mateu et al. 1996).

XRD can detect ~ 1 Å-changes in period, which is a tenfold finer resolution than detected by EM—e.g., the ~ 15 – 20 Å-difference in period between CNS and PNS myelins. Periodicity measurements for nerves where the myelin diffraction patterns have sharp reflections are accurate to ± 0.5 Å (Blaurock 1967; Blaurock and Worthington 1969; Finean 1960; Finean 1961; Kirschner and Sidman 1976; Schmitt et al. 1935; Schmitt et al. 1941). Under apparently identical conditions—e.g., animal species and strain, pH, ionic strength, temperature, medium composition—sample-to-sample differences in period are about ± 1 – 2 Å for myelins having the greatest packing regularity (Caspar and Kirschner 1971; Hedley-Whyte and Kirschner 1976; Kirschner and Sidman 1976; Mateu et al. 1996). Different native periodicities among wildtype animals of the same species can be accounted for by different genetic backgrounds (Avila et al. 2005; Mateu et al. 1996). For less well-ordered myelin which yields broadened reflections—such as found in dysmyelination or after fixation—the periodicity is within ± 3 of the mean value (see, for example, (Kirschner and Hollingshead 1980)). Sample mis-orientation can also result in broadened reflections and apparent heterogeneity in periodicity and lower amounts of myelin, as shown in the current study when vibratome slabs were used. Overcoming this problem could be achieved by either more careful orientation of the agar-embedded tissue in the vibratome, or by trial-and-

error repositioning of the cut slab in the x-ray beam. That XRD can rapidly evaluate myelin structure in such a large sample of fibers and with such high precision and reproducibility provided the rationale for exploring its potential usefulness in research involving myelin damage and repair, where being able to quantitate the amount of myelin is important.

Neither chemical fixation nor pre-treatment (such as by rapid-freezing) is required by XRD. Moreover, the chemical processing steps required for thin-sectioning introduces gradual changes in periodicity, membrane packing, and packing disorder (Kirschner and Hollingshead 1980). Rapid freezing of tissue is thought to minimize structural artifacts, but the use of frozen sections still requires an initial fixing of the tissue, followed by cryo-protection (for example, by infiltrating sucrose), and embedding in OCT. Whereas even minimal fixation with aldehydes changes myelin structure, such treatment nonetheless does provide tissue (such as spinal cord) that is less fragile, easier to manipulate, storable for considerable lengths of time with little or no degradation (Avila 2007), and more convenient to ship from the originating laboratory to the XRD lab. Whole tissue that has been examined by XRD can subsequently be further processed and examined by EM in thin-sections to visualize details about certain of the abnormalities detected by XRD. The current study demonstrated that not only in freshly-examined fixed tissue but also in fixed tissue that has been stored for an extended duration (~20 y), myelin period and amount could be quantitated by XRD. The latter finding suggests that myelin structural integrity in appropriately-fixed samples from tissue banks could also be evaluated.

We were surprised here to discover the large variation in myelin periodicity and relative amount among the samples provided by the collaborating laboratories. This was immediately evident from the variation in quality of the myelin diffraction. In the case of the PNS samples (spinal roots) received from two different laboratories using three different fixatives (Laboratories A and B), the relative amount of myelin was in the range previously observed for unfixed, wildtype mouse sciatic nerve myelin (Avila et al. 2005; Mateu et al. 1996). However, the ~185–196 Å-periods measured for these samples were ~10–20 Å larger than those measured from unfixed nerves. Similar effects were observed with the CNS tissues, in that the relative amounts of myelin were similar to those previously reported for unfixed, mouse optic nerve myelin (Avila et al. 2005), whereas the periods ranged from near-native values in PF+GA to periods swollen by variable amounts, up to ~20 Å in PF alone. These findings suggest that while the fixatives can induce substantial swelling of the myelin arrays, they do not tend to produce changes in the relative amounts of myelin—a finding that validates the use of XRD for detecting hypomyelination (owing to demyelination or remyelination) in fixed CNS myelin. The large variation in period emphasizes not only the importance of fixative choice, but also the need for non-pathological or normal samples, prepared in parallel, for each batch of tissue to be examined by XRD. While long-term storage of samples produced a gradual increase in period and decrease in relative amount of myelin—as observed for the non-human primate tissues—such changes were tenfold smaller than the fixative-dependent or batch-to-batch variation in period measured for tissues examined shortly after fixation.

We emphasize that XRD—by contrast with morphometric analysis—is unable to analyze the relative amount of internodal myelin in plastic-embedded tissue. The chemical processing required for thin-sectioning produces structural artifacts in internodal myelin that result in the diffraction peaks becoming very weak and significantly broadened while the background scatter increases (Avila et al. 2005; Kirschner and Hollingshead 1980). Nonetheless, myelin periodicity can be obtained by XRD using such preparations, as this measurement is based on the positions of the intensity maxima.

Recent synchrotron XRD studies report using myelinated tissues from human surgical biopsies (Falzon et al. 2007) or autopsies (De Felici et al. 2008); however, in the first study the tissue had been snap-frozen in liquid nitrogen and in the second it had been fixed in formaldehyde. As the periodicities were not reported in the first study, the extent of structural preservation upon freezing in the absence of cryoprotectants, which themselves can induce transient phase separations in myelin (Hollingshead et al. 1981; Kirschner et al. 1979), remains to be evaluated. The second study reports a detailed analysis of the myelin diffraction patterns, but did not recognize the extent to which the membrane packing had been modified by the formaldehyde treatment (Kirschner and Hollingshead 1980). In fact, the reported ratio of the 2nd to 4th order intensities differs by a factor of ten from previously published data from *unfixed, unfrozen* human autopsy material (Chandross et al. 1978), and the intensity of the 3rd order increased from undetected (in the unfixed, unfrozen) to being greater than that of the 4th order (after formaldehyde)—both changes indicative of major changes in the interactions between the pairs of membranes constituting the repeating unit of myelin. These two recent studies, which utilized state-of-the-art XRD sources and detectors, substantiate how sensitive and rapid the XRD approach can be; however, the studies also demonstrate how important is the sample preparation, as internodal myelin is acutely responsive to its environment.

Conclusions

Our findings demonstrate that the x-ray diffraction technique applied to tissues from the CNS (or segments thereof) can not only evaluate the effectiveness of different chemical fixatives on preserving internodal myelin structure, but also quantitate the relative amount of internodal myelin among the various samples. We suggest that this technique can be exploited with great advantage in spinal cord injury research as well as in other research areas of white matter disease/disorder where it is important to characterize demyelination and remyelination (Table 2). As shown here, analysis of aldehyde-fixed tissue can provide useful metrics about the preservation of myelin structural integrity. Because sufficient amounts of myelin having the correct membrane packing are essential for normal conduction and nerve function, then being able to quantitate accurately these characteristics of myelin will enlighten us about both demyelinating and remyelinating states in animal models of disease (such as EAE and leukodystrophies) or injury (such as SCI), and help guide translational research focused on evaluating and improving therapies for humans.

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Abbreviations

CNS	central nervous system
EM	electron microscopy
GA	glutaraldehyde
MBP	myelin basic protein

PF	paraformaldehyde
PLP	proteolipid protein of CNS myelin
PNS	peripheral nervous system
P0	protein zero glycoprotein of PNS myelin
SCI	spinal cord injury
tg	transgenic
WT	wildtype
XRD	x-ray diffraction

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Figure 1.

Examples of samples examined in this study. (**Top**) Samples (provided by *Laboratory A*) after inserting into x-ray capillaries, and maintained in contact with fixative. The piece of cord (2 mm capillary) is much thicker than the spinal nerve root (1 mm capillary). To appreciate how much of the specimen is “sampled” by the x-rays, consider that the beam is ~3 mm long (parallel to the long axis) $\times$ 200 μ m wide. The right hand end of the root’s capillary is sealed with wax and nail polish. (**Middle**) Intact spinal cords (as received from *Laboratory C*) with their dorsal aspects facing the viewer (* dorsal horns). The heavy arrows show where we trimmed to avoid distortion damage from the pins holding the cords to the wax. The heavy brackets (and red marking) indicate the lesioned regions. The boxes indicate where we divided the cord into rostral, caudal, and lesioned segments before inserting left and right halves into x-ray capillaries. (**Bottom**) Cross-section of adult rat spinal cord (at T10) showing the motor pyramidal and extrapyramidal tracts, and sensory pathways of the dorsal columns.

Figure 2.

XRD data (intensity shown as counts *vs.* channel number) recorded from aldehyde-fixed samples from four different laboratories (indicated by A–D). The spectra shown are representative patterns, corresponding to the mean values measured with respect to myelin period and to the relative amount of myelin (see below, Fig. 3). To more clearly distinguish among the spectra, the x-ray patterns have been shifted vertically relative to the lowest pattern within each panel.

(A) Data from tissue *batches 2* and *3* are illustrated. The nerve roots (upper spectrum)) show a very different pattern than the cords, with the intensity maxima at different channel positions. For *batch 2*, the patterns from the motor tracts are significantly stronger than from the sensory tracts (*solid line vs. dashed line*), indicating a greater relative amount of myelin. Another set of samples (*batch 3*), also fixed in 8% PF, did not replicate these findings owing to poorly preserved myelin, as indicated by the larger period, broader reflections, and weaker intensity. (B) Data from tissue *batch 2* is shown. The rat tissue samples were fixed with 4% PF, and show similar periods for root and cord myelins: whereas the roots are similar in period to those from *Laboratory A* (~190 Å), the cords differ considerably in period between the two batches (see Fig. 3B). (C) Spinal cords from mice that had been lesioned on the right side with ethidium bromide (EB), and perfused with 2% GA after 10 d. The cords were subdivided as shown above (Fig. 1, bottom) and into left and right halves. We detected a greater relative amount of myelin in left (unlesioned) *vs.* right (lesioned) segments, as evident from the stronger patterns, particularly for the **caudal**, and somewhat for the lesioned (**center**) regions (Fig. 3C). The 2% GA fixative used here was not as effective in preserving myelin period as 8% PF; and the increased disordering of membranes obscured but did not prevent detecting a lower amount of myelin on the lesioned (right) side. (D) Of the four optic nerves shown here, two were from young animals, and two from old; and two had been fixed with PF + GA (2% PF + 2.5% GA) and two with 4% PF. Both “young” nerves had stronger myelin diffraction than the corresponding “old” ones that had been fixed similarly; and the myelin periods from the (PF+GA)-fixed nerves were more native-like than those in 4% PF.

Figure 3.

Scatter plots of the relative amount of myelin ($M/M+B$) vs. myelin period (d) for all of the samples received from the four different laboratories (A–D). Representative x-ray spectra are shown in Fig. 1.

(A) Data from the different batches of samples tend to cluster, indicating internal consistency within each batch, but variation batch-to-batch, even for samples stabilized with the same fixative (*batches 1–3*). Data from motor vs. sensory tracts (*batch 2*), dissected from cords as indicated in Fig. 1 (bottom), are distinguished from one another. The data in the upper right hand corner are from spinal roots, and are clearly separated from the cord data. (B) Whereas the cord (■) and root (◆) data for *batch 1* are differentiated from one another, the data from the vibratome slabs (■, ●, ▲) (*batch 2*) had so much scatter that some of the measurements overlapped with the root data (◆). The myelin periodicity is substantially larger than those measured for the samples from laboratory A. (C) Comparison of right vs. left segments reveals a noticeable reduction in the relative amount of myelin (see *Inset* on expanded scale) in regions caudal to (△ vs. ▲) and near to (□ vs. ■) the lesion on the right-hand side but not on the left hand, non-lesioned side. The period in these 2% GA-fixed nerves, and hence the myelin preservation, can likely be improved by using PF+GA. (D) Summary of data for both optic nerve and corpus callosum samples from non-human primate. The samples show substantially better myelin preservation with PF+GA fixative than with 4% PF (closed and open symbols vs. crosses) with respect to period but not necessarily with respect to the relative amount of myelin. The values in parentheses at each data point indicate the age (in years) of the animal when sacrificed and the time (in years) of storage of the tissue. Because samples had been stored in fixative for times ranging from 5–20 years, we examined correlations between myelin period and relative amount of myelin vs. age of animal and tissue storage time (E–H). Whereas period increased with age of animal and with tissue storage time, the relative amount of myelin decreased with age and with storage. For each of these linear correlations, $p < 0.005$ (see text for details).

Summary of Results

Table 1

Laboratory	Tissue Batch	Samples ^{a,b,c,d}	Fixative	Myelin Metric ^f		
				Period (Å)	Relative amount	N
A (rat)	1	a cords;	4% PF	174.6±3.0	0.13±0.05	2
		roots		196.7	0.26	1
	2	a cords	8% PF	157.6±1.3	0.14±0.05	12
				(159.6±1.5) ^g	(0.12±0.06) ^g	
		roots		195.0±2.8	0.33±0.05	3
	3	a cords	"	170.5±2.5	0.10±0.04	30
	4	a cords	"	155.4±1.0	0.16±0.05	19
B (rat)	1	b cords;	4% PF	181.4±2.0	0.17±0.03	5
		roots		185.3	0.29	1
	2	b cord slabs;	"	186.0±5.0	0.14±0.10	12
		roots		192.7±0.8	0.33±0.02	3
C (mouse)	1	c cords	2% GA	162.5±1.4	0.07±0.02	44
D (monkey)	1	d optic nerve	2% PF+2.5% GA;	159.7±8.8 ^h	0.16±0.05	33
		corpus callosum	2% PF+2.5% GA			

^aFischer rats;

^bSprague-Dawley rats;

^cC57BL/6 mouse, some samples of which had been lesioned with ethidium bromide;

^drhesus monkey (*Macaca mulatta*). No attempt was made here to distinguish ventral from dorsal roots.

^eThe detailed fixation procedures for each laboratory are as follows:

Laboratory A: Adult female rats, under general anesthesia, were perfused transcardially with 100 ml of cold 0.1 M phosphate-buffered saline (PBS) at pH 7.4, followed by vascular perfusion with 300 ml of either 4% or 8% PF in a Sorensen phosphate solution at pH 7.2. Spinal cords were removed, and postfixed and stored at 4°C in the same fixative solution until used for XRD.

Laboratory B: The rats were anesthetized deeply with ketamine (75 mg/kg) and xylazine (10 mg/kg), and perfused through the heart with 4% PF in 0.14 M Sorensen's phosphate buffer at pH 7.4. The spinal cords were excised and stored in the same fixative. For one experiment, the cord was trimmed and embedded in agar, from which vibratome slabs were prepared.

Laboratory C: The mice were deeply anesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.1 M PBS at pH 7.4, followed by of 2% GA in 0.1 M PBS. Spinal cords were dissected, mounted on strips of dental wax, postfixed with the same solution as used for perfusion, and stored at 4°C until further use

Laboratory D: The monkeys were perfused intracardially with a warm solution containing 1% PF and 1.25% GA in a 0.1 M cacodylate or phosphate buffer at pH 7.4. The brains were then removed and one hemisphere from each brain was stored in a cold solution of 2% PF and 2.5% GA in 0.1 M buffer of the same type used for the perfusion. Some tissues were stored in 4% PF. Samples for XRD were dissected as needed from the stored material, and maintained in the same fixative.

f For each complete batch of samples, upper value is for CNS, and *italicized* value is for PNS; *N* is number of measurements. "Relative amount" is the relative amount of myelin determined by the quotient $M/(M+B)$. See Materials and Methods for details.

g CNS samples re-examined three months later.

h The large standard deviations were owing to the fact that some samples had been fixed in PF while the others had been fixed in PF+GA. See Fig. 3D-H to see how the samples actually grouped by fixative and by age of animal.

Table 2

How XRD will inform quantitatively about demyelination & remyelination

Variable	Relative Amount of Myelin Measured by $M/(M+B)^a$: Expected Changes in its Value (<i>with explanation</i>)
Age of animal before maturity	Increases with development (<i>Sheaths become thicker</i>)
Cord or root size	Increases (<i>If fraction of tissue volume that is myelin increases</i>)
Hypomyelination	Less than (age/weight-matched) control (<i>Less myelin per tissue volume</i>)
Demyelination	Decreases (<i>Delamination of sheaths; and accumulation of debris between fibers reduces number density of sheaths</i>)
Hypermyelination	Greater than (age/weight-matched) controls ^b (<i>More myelin per tissue volume</i>)
Remyelination	Increases (compared to demyelinated sample) (<i>As myelin sheaths thicken and number density of myelinated fibers increases</i>)

^aThis is determined by calculating the total intensity in the myelin diffraction peaks (M) above the background intensity (B), after excluding the small-angle region of the pattern around the beam stop.

^bThis is observed for PLP-Akt overexpressor mice, which show nearly twice the relative amount of myelin in tg mice at 6 months of age (Flores et al. 2008).