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Quantifying axon diameter and intra-cellular volume fraction in excised mouse spinal cord with *q*-space imaging

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Abstract

Q-space magnetic resonance imaging (QSI) can quantify white matter (WM) axonal architecture at the cellular level non-destructively, unlike histology, but currently has several limitations. First, current methodology does not differentiate between diffusing molecules occupying extra- or intra-cellular spaces (ECS and ICS, respectively). Second, accurate assessment of axonal architecture requires high-gradient amplitudes not clinically available. Third, the only direct QSI marker of axonal architecture has been mean axon diameter (MAD), even though other direct markers would be valuable as well. The objective was to investigate three QSI-based methods that address the above limitations. Method 1 employs a two-compartment model to account for signal from ECS and ICS. Method 2 uses data only from low *q*-values thereby obviating the need for high-gradient amplitudes. Method 3 empirically estimates ICS volume fraction and provides an additional metric of axonal architecture. We implemented each method on data from excised healthy adult mouse spinal cords collected previously using a home-built 50T/m *z*-gradient yielding sub-micron displacement resolution. Through comparison with histology, each method was evaluated for accuracy in assessing axonal architecture. MAD measured with Methods 1 and 2 showed good correlation with histology ($R^2=0.99$ ($p<0.0001$), and 0.77 ($p<0.01$), respectively) and Bland-Altman analysis indicates that measurements from the two methods are not significantly different from histology. The third method measured ICS volume fractions (0.64 ± 0.07) that were highly correlated ($R^2=0.92$, $p<0.05$) with measurements from histology (0.68 ± 0.07). These methods may provide insight into axonal architecture in normal and abnormal WM tissue but additional validation with more samples will be needed.

Keywords

q-space imaging; white matter; C57BL/6 mice; axon diameter; intra-cellular volume fraction; axonal architecture

Introduction

Diffusion MRI techniques have proven to be an invaluable tool for non-invasive assessment of neural tissue micro-architecture. They have provided insight into numerous areas of study ranging from brain connectivity to white matter (WM) diseases such as multiple sclerosis

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(Basser and Jones, 2002; Horsfield and Jones, 2002). Diffusion weighted imaging (DWI) and diffusion tensor imaging (DTI) are the most widely used diffusion MRI techniques. However, both inherently assume diffusion to be Gaussian (i.e. free), which means that there are no barriers to molecular displacement, which is clearly erroneous in biological tissues. Q-space imaging (QSI), developed independently by Cory and Garroway (Cory and Garroway, 1990) and Callaghan et al (Callaghan et al., 1990), does not require any knowledge of properties of molecular diffusion. QSI, therefore, more accurately measures water diffusion in tissues and may potentially provide information on axonal architecture not amenable by DWI or DTI. For a review of QSI technique in the neurologic system, the reader is referred to a review by Cohen and Assaf (Cohen and Assaf, 2002) and a brief summary is given below.

Similar to conventional diffusion MRI techniques, QSI experiments utilize two magnetic field gradient pulses of amplitude G , and duration δ , separated by a diffusion time Δ , to sensitize the magnetic resonance signal to displacements due to molecular diffusion (Stejskal and Tanner, 1965). Due to diffusion, there is a loss of signal, which can be expressed as the echo attenuation, $E(q, \Delta)$, where $q = (2\pi)^{-1} \gamma G \delta$ and γ is the gyromagnetic ratio. The Fourier transform of $E(q, \Delta)$ results in a molecular displacement probability density function (PDF), which defines the conditional probability that a molecule is displaced a specific length along the gradient direction during the time, Δ .

The displacement PDF measured with QSI will be influenced by axonal architecture such as axon membranes and myelin sheaths acting as barriers to the diffusing molecules (e.g. water) and hence affect its displacement probabilities, e.g. higher probability of displacement parallel as opposed to displacement perpendicular to WM fiber tracts. The 1D QSI displacement profile, where the diffusion-sensitizing gradients are applied along a single direction, has a particularly simple interpretation as long as the gradients are applied perpendicular to the axon fibers, which can be thought of as having a tubular geometry (Avram et al., 2004). The width of the PDF, typically characterized by its full-width at half-maximum (FWHM), should correlate with the spacing between diffusion barriers. In WM, the dominant diffusion barrier is the axonal membrane (Beaulieu, 2002), and the spacing between barriers is simply MAD averaged over the imaging volume. Therefore, MAD can be estimated from the FWHM of the displacement PDF (Assaf et al., 2000; Chin et al., 2004).

In recent work (Ong et al., 2008), using a custom built z-gradient coil and RF coil set (Wright et al., 2007) to optimize experimental conditions, the authors were able to differentiate WM tracts solely based on axon diameters derived from the FWHM of the displacement PDF and show significant correlations with histology. However, there are several limitations with this simple approach. First, QSI does not differentiate between diffusing molecules occupying either extra- or intra-cellular spaces (ECS and ICS, respectively) and the measured displacement PDF reflects displacements due to molecular diffusion in both compartments. Diffusion in the ECS and ICS is expected to be hindered and restricted (Assaf et al., 2004), respectively. As a result, the displacement PDF as measured in Ong et al. may be broader than the actual MAD due to the addition of displacements resulting from hindered diffusion in the ECS. This may explain our prior observations that the FWHM overestimated MAD calculated from histology by approximately 20% (Ong et al., 2008). Second, accurate assessment of axonal architecture with QSI requires sufficient displacement resolution, which, from its Fourier transform relationship, requires a high q -value. From the definition of q and constraints due to signal intensity and relaxation, high gradient amplitudes are needed to achieve sufficient displacement resolution. These gradient amplitudes are not clinically available, and necessitate custom hardware, thereby limiting applicability of the QSI methodology, particularly for *in vivo* studies. Third, an estimate of MAD by itself offers limited insight into axonal architecture. If it were coupled with information on ECS and ICS volume fractions, new information could be inferred such as an estimate of axon loss, which would affect the ECS and ICS volume

fractions, and demyelination, which would increase the FWHM of the displacement PDF. Both of these changes in axonal architecture are hallmarks of spinal cord injury and a variety of WM diseases from multiple sclerosis to Alzheimer's disease (Budde et al., 2007; Horsfield and Jones, 2002; Schwartz et al., 2005).

Despite the afore-mentioned limitations, QSI has been applied to various animal and human studies both *ex vivo* and *in vivo* (Cohen and Assaf, 2002; Farrell et al., 2008; Nilsson et al., 2007) (see Ong et al., 2008 for further references). While QSI has demonstrated improved sensitivity to changes in axonal architecture, information on specific changes such as axon loss is currently lacking.

The objective of the present work was to investigate three different QSI-based methods that address the current limitations of QSI due to (1) complications caused by signal arising from both ECS and ICS, (2) the need for high displacement resolution and high gradient amplitudes, and (3) the lack of axonal architecture metrics derived from QSI other than MAD. The first method, referred to as the 'displacement PDF method', employs a two-compartment model of the displacement PDF to account for signal from ECS and ICS. The second method, referred to as the 'low q -value method', extracts MAD information by fitting the echo attenuation at low q -values, which obviates the need for high-gradient amplitudes. The third method, referred to as the 'volume fraction method', empirically estimates ECS and ICS volume fractions, as opposed to current relaxometry (Whittall and MacKay, 1989) or modeling methods (Assaf et al., 2004) that require an ill-posed Laplace inversion and *a priori* assumptions, by analyzing echo attenuations as a function of diffusion gradient duration (Malmberg et al., 2006). We implemented each method on QSI data from healthy adult mouse spinal cords, and compared the results with histology. This data was collected previously and employed a custom-built 50 T/m z-gradient that allowed for sub-micron displacement resolution. Through comparison between experiments and histology, each QSI-based method was evaluated for accuracy in assessing axonal architecture.

Materials and Methods

As the work presented here reanalyzes previously published data, full details on animal preparation, QSI experiment protocol, and histology may be found in (Ong et al., 2008) and only a brief summary is given below, after which, a description of each QSI-based method will be given.

Animal preparation

Five C57 BL6 mice (8-9 months, 25-30 mg, Charles River, Wilmington, MA) were anesthetized and perfusion fixated following which the entire spinal cord was dissected out and postfixed for at least two weeks in a different fixing solution. Cervical C6/C7 sections (3-4 mm in length) were then cut from each spinal cord. After performing QSI experiments, cervical spinal sections that corresponded to the QSI slice were processed for optical histologic imaging as described below.

QSI Experiments

All QSI experiments on excised mouse cervical spinal cords were performed with a custom 50 T/m z-gradient coil/solenoidal RF coil (3 mm i.d. sample bore) set (Wright et al., 2007) designed for high-resolution QSI interfaced to a 9.4T spectrometer/microimaging system (Bruker DMX 400 with Micro2.5 gradients and BAFPA40 amplifiers, Karlsruhe, Germany).

A 2D diffusion-weighted stimulated-echo imaging sequence was used (matrix size = 64×64 , TE = 17.4 ms, Δ = 10 ms, δ = 0.4 ms, FOV = 4 mm, slice thickness = 1.0 mm). The diffusion

gradients were applied along the z-axis, which is perpendicular to the spinal cord longitudinal axis, in 63 increments in steps of $0.013 \mu\text{m}^{-1}$ yielding $q_{\text{max}} = 0.82 \mu\text{m}^{-1}$ (48 T/m maximum gradient strength). A second set of experiments needed for the method proposed by Malborg et al (Malmborg et al., 2006) was run with the same imaging parameters except: $\delta = 5 \text{ ms}$ (Δ remained 10 ms), and the diffusion gradients were again applied along the z-axis in 63 increments in steps of $0.013 \mu\text{m}^{-1}$ yielding $q_{\text{max}} = 0.82 \mu\text{m}^{-1}$ (3.84 T/m maximum gradient strength).

QSI Data Processing

Unless otherwise noted, all data analysis was performed in IDL (Interactive Data Language, Research Systems, Boulder, CO). Spatial reconstruction of the data yielded images in which each pixel contains the echo attenuation $E(q)$ for that location. For each pixel the resulting echo attenuation was normalized to the maximum value at the zero q -value. In order to compute a real Fourier transform, the echo attenuation plot was reflected about its origin ($q = 0$) to fill in the *negative* q -values, resulting in 127 total q -values (1 zero q -value, 63 positive, and 63 negative q -values). A displacement PDF was then computed by applying a 1D Fourier transform to the modified echo attenuation $E(q)$. Depending on the method used, either the echo attenuation, or the displacement PDFs were used for further processing as described below.

In order to compare differences between WM tracts, regions-of-interest (ROIs) of 20 pixels after zero-filling to 256×256 were manually selected in each of the seven WM tracts. ROI locations were chosen to match the histologic ROI location (as discussed below). For each ROI within a specimen, average q -space attenuation plots were recorded. For each specimen, q -space attenuation plots were obtained for each pixel, as described above and averaged within every ROI.

Optical Images of Mouse Spinal Cord

For comparison with the QSI data, light microscopic images of fixed mouse spinal cord sections embedded in epoxy (C6-C7) at seven WM tract locations reflecting a range of characteristic axonal architecture were obtained after staining for myelin with toluidine blue (Figure 1). Each image was digitized with a CUE-2 image analyzer (Olympus American, Melville, NY), in which an ROI was selected for detailed analysis. This ROI size approximated the pixel size of the experimental data before zero-filling.

These ROI images were segmented into ECS, ICS, and myelin compartments using a program written in Matlab (The Mathworks, Natick, MA). The segmented images were then used to calculate MAD (equating each ICS axon area to a circle, excluding the myelin), ICS volume fraction, and axon density (Table 1). ICS volume fraction was taken to be the area of both the ICS and myelin regions (see Discussion).

Displacement PDF Method: Estimating MAD and ICS Volume Fraction with a two-compartment PDF model

Since the displacement PDF contains signal from both the ECS and ICS, separation of these signals may lead to a more accurate MAD estimate as well as provide a means to estimate the ECS and ICS volume fractions. In this method, as developed independently by Nossin-Manor et al (Nossin-Manor et al., 2005), the displacement PDFs were fit to a two-compartment model. As ECS and ICS diffusion is expected to be Gaussian and restricted, respectively (Assaf et al., 2004), their displacement PDFs would be a Gaussian and an autocorrelation of the axon geometry, respectively. MAD was then estimated from the ICS displacement PDF FWHM. ECS and ICS volume fractions were estimated by normalizing each displacement PDF area by the overall fit area (ECS plus ICS).

Fitting was done with a non-negative nonlinear minimization algorithm in Matlab. To find the exact ICS displacement PDF shape, an image-based finite difference diffusion simulation program (Hwang et al., 2003) was used to simulate the QSI echo attenuation based on WM tract histologic images as done previously (Ong et al., 2008). PDFs were simulated with signal only from the ICS (excluding myelin). The simulated PDFs were then fit to various peak shapes such as Gaussian, Lorentzian and others, and the goodness of fit was determined by the R^2 value.

Low q -Value Method: Estimation of MAD from low q -values

Axonal architecture information can also be obtained from the echo attenuation. Although WM is too heterogeneous to observe diffraction patterns in q -space (Chin et al., 2004), from a series expansion of the Fourier transform relationship, the initial echo attenuation at low q -values at a given diffusion time, Δ , where $q^{-1} \ll \text{MAD}$, can be expressed as

$$E(q_i, \Delta) = \exp(-2\pi^2 q_i^2 Z_{RMS}^2) \quad (1)$$

where Z_{RMS} is the root-mean-squared displacement during the diffusion time, i.e. the width of the displacement PDF (Callaghan, 1993). As long as the SGP approximation ($\delta \ll \Delta$) is fulfilled, Z_{RMS} may be used as an estimate of MAD by fitting the initial echo attenuation decay to Eq. 1. However, the q -space echo attenuation is comprised of both ECS and ICS signals. Similar to the displacement PDF method, a two-compartment version of Eq. 1 could be used to account for the ECS and ICS signals:

$$E(q_i, \Delta) = f_{ECS} \exp(-2\pi^2 q_i^2 Z_{ECS}^2) + f_{ICS} \exp(-2\pi^2 q_i^2 Z_{ICS}^2) \quad (2)$$

where f_{ECS} and f_{ICS} are the relaxation-weighted ECS and ICS volume fractions and Z_{ECS} and Z_{ICS} are the RMS displacements of diffusing molecules in the ECS and ICS.

Echo attenuations were fit to both Eqs 1 and 2 with a nonlinear minimization algorithm in Matlab. When fitting to Eq. 2, the following parameter constraints were applied: $f_{ECS} + f_{ICS} = 1$ and $Z_{ECS} < 8 \mu\text{m}$ (as RMS displacement in ECS cannot be larger than that of free water). WM tract MAD previously estimated from histology were used to identify the low q regime by only fitting $E(q)$ at $q < (\text{MAD}^{-1})/10$ (the first 11 to 5 q -values for the smallest to largest WM tract MAD, respectively). MAD, and ECS and ICS volume fractions were then estimated from Z_{ICS} , f_{ECS} and f_{ICS} , respectively.

Volume Fraction Method: Empirical estimation of ECS and ICS volume fractions

In addition to MAD estimates, ICS volume fractions were estimated using this third method, which is based on work by Malmberg et al (Malmberg et al., 2006) who proposed to empirically measure ICS volume fraction exploiting the differential behavior of the echo attenuation for restricted and free diffusion with respect to the diffusion gradient duration, δ . Under conditions of restricted diffusion, increasing δ for a given q -value and at constant diffusion time Δ , causes the echo to attenuate less (Mitra and Halperin, 1995), whereas for free diffusion the amplitude is not dependent on δ . Since diffusion in the ICS is restricted whereas in the ECS it is essentially free (Assaf et al., 2004), the echo amplitude from spins in each compartment should exhibit a different dependence on δ . From a comparison of echo attenuations obtained at the same q -values but with varying δ , the initial decay represents signal from ECS (Assaf et al., 2004) and should not vary with δ . However, at increasing q -value, the signal is primarily from the ICS

and will exhibit less attenuation with increasing δ . ICS volume fraction can then be inferred from the point at which the echo attenuations obtained at varying δ begin to diverge.

Two echo attenuation plots were recorded where δ was changed from 0.4 to 5 ms ($E(\delta=0.4\text{ms})$ and $E(\delta=5\text{ms})$, respectively). The signal attenuation value at which $E(\delta=0.4\text{ms})$ and $E(\delta=5\text{ms})$ begin to deviate from each other, P_d , (as observed by Malmberg et al when $E(\delta=5\text{ms})/E(\delta=0.4\text{ms}) > 1.2$) provides a relaxation-weighted estimate of ICS volume fraction. However, unlike in (Malmberg et al., 2006), P_d was taken as the value of $E(\delta=5\text{ms})$ immediately prior to where the ratio $E(\delta=5\text{ms})/E(\delta=0.4\text{ms}) > 1.2$. This was done because we observed that the echo attenuation curves had already deviated when $E(\delta=5\text{ms})/E(\delta=0.4\text{ms}) > 1.2$.

Results

Figure 2a shows a simulated PDF from only the histologic ICS region used for the displacement PDF method. The peak shape of the ICS displacement PDF was determined empirically by fitting the simulated PDFs from above to various peak shapes such as Gaussian, Lorentzian and others. As shown in Figure 2, the peak shape that gave the best fit as determined by the R^2 value was a decaying exponential reflected about the origin ($R^2 > 0.99$). Figure 2b shows sample experimental displacement PDFs fitted to a weighted sum of Gaussian and decaying exponential. Figure 2c shows a plot of average WM tract MAD calculated from histology versus average experimental ICS displacement PDF FWHMs. A Bland-Altman plot (not shown) yielded a 95% confidence interval from -0.07 to $0.03 \mu\text{m}$. Also shown in Figure 2c is the ICS volume fraction calculated from both histology and experiments averaged over all specimens. There was no difference in the mean ICS volume fraction between tracts determined histologically (see Table 1). Therefore, a single ICS volume fraction was calculated for each specimen by averaging over each WM tract. There was no correlation between ICS volume fractions measured from histology and the displacement PDF method. No significant correlation was found between the ECS displacement PDF FWHM and histologic MAD, and the average FWHM of the ECS displacement PDF was $4.5 \pm 2.1 \mu\text{m}$.

Figure 3a shows sample fits of experimental data with Eq. 1 and 2 using the low q -value method. Figure 3b shows a plot of experimental MAD estimated from one- and two-compartment fits (Z_{RMS} and Z_{ICS} , respectively) vs histologic MAD averaged for each WM tract. An outlier Z_{ICS} value for the FC WM tract was removed as its value was less than the smallest MAD observed histologically. A Bland-Altman plot between the Z_{ICS} and histology was generated (not shown) and the 95% confidence interval was from -0.11 to $0.24 \mu\text{m}$. As defined in Eq. 2, f_{ICS} is used to estimate the ICS volume fraction. Similar to the displacement PDF method, a single ICS volume fraction estimate was obtained for each specimen by averaging over the WM tracts, because the ICS volume fractions calculated from histology showed no significant differences among WM tracts. There was no correlation between f_{ICS} and histologic ICS volume fraction. The average f_{ICS} was 0.89 ± 0.01 , which is higher than the average histologic ICS volume fraction of 0.68 ± 0.07 . No significant correlation was found between Z_{ECS} , the RMS displacement of water molecules in the ECS, and histologic MAD, and average Z_{ECS} was $6.8 \pm 1.2 \mu\text{m}$.

Figure 4a shows sample q -space echo attenuations ($E(\delta=0.4\text{ms})$ and $E(\delta=5\text{ms})$) from the VST WM tract of a single specimen used for the volume fraction method and how the ratio $E(\delta=5\text{ms})/E(\delta=0.4\text{ms})$ is used to determine ICS volume fraction from P_d . Figure 4b shows a plot of histologic vs experimental ICS volume fractions. As described above, for each specimen, in both experiments and histology, a single ICS volume fraction was calculated by averaging all WM tract ICS volume fractions. A Bland-Altman plot between the experimental and

histologic ICS volume fractions (not shown) indicates the 95% confidence interval to fall in the range from -0.01 to -0.06 .

Discussion

Displacement PDF Method

The excellent correlation between histologic and experimental MAD (Figure 2b) and the Bland-Altman results suggest good agreement between MAD measured with histology and the displacement PDF method. Compared with the authors' previous work (Ong et al., 2008) where, as mentioned above, ECS and ICS signals were not separated, MAD estimates from the displacement PDF method better match those measured by histology. In addition, while there was no correlation between histologic and experimental ICS volume fractions, both values averaged across specimens fall within the range of 60-80% reported for the rat corpus callosum (Sykova and Nicholson, 2008). Finally, MAD estimates from the displacement PDF method were within 4% of histologic MAD. In addition, the average ECS displacement PDF FWHM of was $4.5 \pm 2.1 \mu\text{m}$, which is below the expected RMS displacement of free water with a diffusion time of 10 ms, and the lack of correlation with histologic MAD support our model of water molecules in the ECS experiencing hindered diffusion.

It should be noted that there is an apparent inconsistency in how the ICS volume fraction and MAD were calculated from histology; the myelin area was included in the calculation of ICS volume fraction, but not for MAD. The decision to include or exclude myelin was motivated by differences in the distance between diffusion barriers in the ICS and myelin spaces. In ICS, the distance between diffusion barriers would be the diameter of the axon, excluding myelin. In myelin, the distance between diffusion barriers would be the spacing between the lipid bilayers ($<0.1 \mu\text{m}$). At sufficiently long diffusion times, both ICS and myelin are expected to exhibit restricted diffusion. Since the ICS displacement PDF results from molecules exhibiting restricted diffusion in the proposed model, it should reflect contributions from water diffusion in myelin. Therefore, for accurate comparison with the displacement PDF results, the ICS volume fraction measured from histology was defined as the sum of the ICS and myelin areas. However, as the spacing between the lipid bilayers in myelin is $<0.1 \mu\text{m}$, our displacement PDF resolution is not high enough to resolve the restricted diffusion in the myelin and the FWHM may primarily reflect ICS. Therefore, for proper comparison with the results from the displacement PDF method, histology-derived MAD was computed by excluding the myelin region. The inadequate displacement resolution may also explain the discrepancies between the ICS volume fraction measured with the displacement PDF method and histology (see volume fraction method).

As previously mentioned, Nossin-Manor et al (Nossin-Manor et al., 2005) also used a two-compartment model to analyze QSI data. Displacement PDFs measured from excised rat spinal cords were fitted to a bi-Gaussian model to characterize fast and slow diffusion components. When the diffusion gradients were applied perpendicular to the long axis of the spinal cord, the mean displacements measured from both the slow component of the bi-Gaussian model and the basic single-component QSI analysis exhibited restricted diffusion behavior and were in good agreement with each other. The authors concluded that the basic single-component QSI experiment described water diffusion perpendicular to the WM tract nearly as well as the two-compartment model, which conflicts with the results reported here. Several factors can help explain this discrepancy. First, the displacement resolution was only $3.9 \mu\text{m}$ in Nossin-Manor et al compared with $0.6 \mu\text{m}$ in the work reported here. Since axon diameters are on the order of $1-2 \mu\text{m}$, the low displacement resolution found in Nossin-Manor may have blurred the discrimination between diffusion in the ECS and ICS. Second, the diffusion time in Nossin-Manor et al is much longer than the one used here (50-250 versus 10 ms). Thus, the longer diffusion time may emphasize the ICS signal through the greater attenuation of the ECS signal

due to unrestricted diffusion. Third, Nossin-Manor et al assumed a bi-Gaussian model, whereas only the ECS displacement PDF was modeled as a Gaussian in the work here. The ICS displacement PDF is the auto-correlation function of the pore geometry that, as our simulations suggest, is not Gaussian. While Nossin-Manor et al reached different conclusions, differences in data acquisition and analysis do not make their results inconsistent with those reported here.

Low q -Value Method

Histology derived MAD correlated with corresponding values from both one- and two-compartment low q -value methods (Figure 3b). The two-compartment fit of the echo attenuation has a higher average R^2 (0.96 versus 0.88) suggesting that it may be a better model for the echo attenuation at low q -values (Figure 3a). The MAD estimate from the two-compartment fit, Z_{ICS} , does show closer correspondence with histology as evidenced by the linear regression slope and intercept of close to one and zero, respectively. The addition of the Bland-Altman results between Z_{ICS} and histology suggests good agreement between MAD measured with histology and the two-compartment low q -value method.

The lack of correlation between f_{ICS} and histologic ICS volume fraction coupled with an average f_{ICS} that is higher than the average histologic ICS volume fraction suggests that f_{ICS} does not accurately measure ICS volume fraction. Furthermore, according to our model that water molecules in the ECS experience hindered diffusion, Z_{ECS} should not be correlated with MAD and have a value less than the RMS displacement of freely diffusing water molecules. As expected, no significant correlation was found between Z_{ECS} and histologic MAD. However, the average Z_{ECS} was within expected range of the RMS displacement of free water at a diffusion time of 10 ms.

It should be emphasized that the experimental protocol was not optimized for fitting $E(q)$ at low q -values as the data was initially collected to maximize the displacement resolution. As a result, only the first 5-11 points of the q -space echo attenuation fulfilled the low q -value condition, $q^{-1} \ll \text{MAD}$, depending on the WM tract. Using a relatively small number of data points significantly limits the degrees of freedom for fitting, especially for the two-component fit. This may explain the higher estimate of ICS volume fraction, ECS RMS displacement and lower R^2 . In an experimental protocol optimized for the low q -value method, q -values greater than $\sim 0.1 \mu\text{m}^{-1}$ would not be necessary to fulfill the low q -value condition. The time saved could be used to improve the fitting by sampling more low q -value points or averaging the signal.

Volume Fraction Method

Unlike the displacement PDF method, histologic and experimental ICS volume fractions are well correlated (Figure 4b). Furthermore, all experimental ICS volume fractions fall within the expected range of 60-80% (Sykova and Nicholson, 2008). The Bland-Altman results suggest that the volume fraction method slightly underestimates the histologic ICS volume fraction. Nevertheless, our data indicate that this method may provide ICS volume fraction estimates in WM in fair agreement with those observed histologically thereby validating the prior results by Malmberg et al (Malmberg et al., 2006).

It should be noted that this method only provides a relaxation-weighted ICS volume fraction estimate from the point of deviation, P_d . The exact relaxation weighting effect on P_d is unclear. Nevertheless, as a result of the short TE used here, ECS and ICS signals did not decay significantly, errors from different T_2 relaxation times in the two compartments should be small (ECS and ICS T_2 s have been reported as 78 and 300 ms, respectively (Peled et al., 1999)). Further, as the technique only differentiates between restricted and free diffusion, histologic ICS volume fraction included both the ICS and myelin regions since the myelin water is

restricted by the myelin sheaths as discussed earlier. Unlike the displacement PDF method, there is no need for sufficient displacement resolution to resolve the restricted diffusion in myelin as the volume fraction method uses the echo attenuation in q -space. This may explain why the ICS volume fraction measured with this method, and not the displacement PDF method, showed correlation with histology. Finally, due to the cylindrical geometry of axons, the observed fraction of restricted diffusion, and hence ICS volume fraction, depends on the orientation of the applied diffusion gradients as discussed by Malmberg et al. For example, if the gradients were applied parallel to the axon direction, then the ICS signal would not be restricted. True ICS volume fraction requires the diffusion gradients to be applied orthogonal to the axon. While the choice of spinal cord tissue allowed the diffusion gradients to be oriented orthogonal to the WM tracts, the volume fraction method can be readily applied to tortuous WM tracts in the brain with a tensor analysis (Assaf et al., 2002) so that the q -space echo attenuation from diffusion orthogonal to the WM tract can always be computed.

Limitations and Potential Applications

The results presented here suggest that the displacement PDF and low q -value methods can provide estimates for MAD, while the volume fraction method can provide estimates of ICS volume fraction, in good agreement with histology. Due to the small sample size ($N = 5$), however, and the fact that the experimental protocol used here was not optimized for the low q -value and volume fraction methods, further validation of and protocol optimization for each method is needed and currently underway. The small sample size may also explain the lack of correlation between ICS volume fractions measured with the displacement PDF or low q -value methods and histology.

A major limitation of the methods reported here is that data acquisition required specialized hardware not commercially available in order to fulfill the SGP approximation. Hardware limitations in micro-imaging and clinical systems would demand either operation outside the SGP approximation or at lower displacement resolution, both affecting accuracy and complicating applications *in vivo*. The displacement PDF method, in particular, demands acquisition of signal at high q -values for sufficient displacement resolution. Given the current hardware setup, the displacement PDF method may find applications in magnetic resonance microscopy of neural tissues as a basic science tool to study axonal architecture non-destructively (Ong et al., 2008).

However, the low q -value and volume fraction methods do not have the same strict restrictions on gradient strength as the displacement PDF method, or QSI data collection in general. The low q -value method, while requiring that the SGP approximation be fulfilled, only needs data to be acquired at low q -values. Given that MAD in WM is less than $10 \mu m$, only q -values less than $\sim 0.1 \mu m^{-1}$ would be needed, which is achievable with more conventional gradient technology. The volume fraction method does not even need operation within the SGP limits. As long as the diffusion gradient duration is varied, the point of deviation between echo attenuations should remain provided SNR is adequate. While there may be an optimal range, there is no inherent need for high gradient amplitudes with this method. Thus, the low q -value and volume fraction methods are not limited by the typical hardware constraints imposed by QSI, which would facilitate translation of both methods to the evaluation of the human spinal cord such as in trauma or demyelinating disease. Recently, Farrell et al (Farrell et al., 2008) and Assaf et al (Assaf et al., 2002) applied QSI to study multiple sclerosis in human brain and cervical spinal cord *in vivo*.

Conclusions

The present study evaluated the potential for three QSI-based methods, referred here as the displacement PDF, low q -value and volume fraction methods, as means to estimate MAD and

ICS volume fractions in mouse spinal cord WM tracts. The displacement PDF and two-compartment low q -value methods measured MAD in good agreement with histology. The volume fraction method measured ICS volume fractions in good agreement with histology. Collectively, all three methods may provide insight into axonal architecture in healthy and diseased or injured WM tissue but more work, on larger sample sizes, will be needed to evaluate the methods' full potential.

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Figure 1.

Top Image: Optical image of C6/C7 mouse cord section showing WM tract locations: A) dorsal corticospinal (dCST), B) gracilis (FG), C) cuneatus (FC), D) rubrospinal (RST), E) spinothalamic (STT), F) reticulospinal (ReST), G) vestibulospinal (VST). The spinal cord is approximately 3 mm wide. Middle Images: Optical images of WM tracts from mouse spinal cord C6/C7 section. Each image is 700×700 with a pixel resolution of $0.1 \times 0.1 \mu\text{m}$. Bottom Images: Sample q -space echo attenuation plots and displacement PDFs for seven WM tract ROIs.

Figure 2. Displacement PDF Method

a) Sample simulated PDF on histologic ICS region only (blue points) with exponential decay peak fit (red line) to determine ICS displacement PDF shape. All fits had $R^2 > 0.99$. b) Sample experimental PDF (blue points) with overall fit (red line) and ECS and ICS displacement PDF fits (green lines). All fits had $R^2 > 0.99$. c) Plot of WM tract histologic vs experimental MADs with equation of line of best fit and mean ICS volume fraction values.

Figure 3. Low q -Value Method

- a) Sample fits of a normalized signal decay curve from dCST WM tract (diamonds). Only the first 11 points were used for fitting (solid diamonds) in order to fulfill the low q -value condition.
- b) Plot of average WM tract histologic vs experimental MADs (upper curve (orange triangles): one-compartment model; lower curve (green squares): two-compartment model) with equation of line of best fit.

Figure 4. Volume Fraction Method

a) Sample VST $E(\delta=0.4ms)$ and $E(\delta=5ms)$ curves on logarithmic axes from a single specimen. $R = E(\delta=0.4ms)/E(\delta=5ms)$. Left arrow indicates the $E(\delta=5ms)$ value used to estimate ICS volume fraction. b) Plot of individual specimen histologic vs experimental ICFs with equation of line of best fit and mean values.

Table 1
Average Regional White Matter Tract Histologic Characteristic

	dCST	FG	RST	ReST	FC	STT	VST
Axon Diameter (µm)	0.81 ± 0.06	0.99 ± 0.06	1.16 ± 0.04	1.36 ± 0.09	1.40 ± 0.14	1.44 ± 0.11	1.82 ± 0.26
ICS Volume Fraction	0.65 ± 0.10	0.69 ± 0.07	0.69 ± 0.07	0.68 ± 0.06	0.73 ± 0.05	0.65 ± 0.11	0.69 ± 0.12
Axon Density (10 ⁵ /mm ²)	3.42 ± 0.55	2.25 ± 0.30	1.62 ± 0.25	1.18 ± 0.16	1.21 ± 0.20	0.89 ± 0.14	0.59 ± 0.16