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White matter microstructure from nonparametric axon diameter distribution mapping

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Abstract

We report the development of a double diffusion encoding (DDE) MRI method to estimate and map the axon diameter distribution (ADD) within an imaging volume. A variety of biological processes, ranging from development to disease and trauma, may lead to changes in the ADD in the central and peripheral nervous systems. Unlike previously proposed methods, this ADD experimental design and estimation framework employs a more general, nonparametric approach, without a priori assumptions about the underlying form of the ADD, making it suitable to analyze abnormal tissue. In the current study, this framework was used on an ex vivo ferret spinal cord, while emphasizing the way in which the ADD can be weighted by either the number or the volume of the axons. The different weightings, which result in different spatial contrasts, were considered throughout this work. DDE data were analyzed to derive spatially resolved maps of average axon diameter, ADD variance, and extra-axonal volume fraction, along with a novel sub-micron restricted structures map. The morphological information contained in these maps was then used to segment white matter into distinct domains by using a proposed *k*-means clustering algorithm with spatial contiguity and left–right symmetry constraints, resulting in identifiable white matter tracks. The method was validated by comparing histological measures to the estimated ADDs using a quantitative similarity metric, resulting in good agreement. With further acquisition acceleration and experimental parameters adjustments, this ADD estimation framework could be first used preclinically, and eventually clinically, enabling a wide range of neuroimaging applications for improved understanding of neurodegenerative pathologies and assessing microstructural changes resulting from trauma.

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Keywords

MRI; Double diffusion encoding; Double pulsed field gradient; Axon diameter distribution; Pore size distribution; Nonparametric; Empirical; Average axon diameter

1. Introduction

The axon diameter distribution (ADD) is a key microstructural feature in the peripheral and central nervous systems. Conduction velocity scales with axon diameter (Tasaki et al., 1943; Waxman et al., 1995) and therefore provides an important functional marker that reflects information transmission in the nervous system. Conventional neuroanatomical methods applied to postmortem human or animal tissue have provided evidence that the ADD changes in neurological conditions such as amyotrophic lateral sclerosis (ALS) (Cluskey and Ramsden, 2001) and multiple sclerosis (MS) (Trapp et al., 1998; Evangelou et al., 2001; Lovas et al., 2000). In addition, several pathologies, including autism (Hughes, 2007), dyslexia (Njiokiktjien et al., 1994), schizophrenia (Randall, 1983), and even alcoholism (Livy and Elberger, 2008), have been associated with changes in the distribution of axon size. Changes in the number of axons and their diameters also take place throughout normal development accompanying the period of dynamic behavioral, cognitive, and emotional changes in childhood and adolescence (Yakovlev, 1967; Pfefferbaum et al., 1994; Gregg et al., 2007; Barnea-Goraly et al., 2005; Schlaug et al., 2009).

The biological significance of axon caliber is not limited to development or well-defined pathologies. Central nervous system neurites exhibit a beaded axonal morphology following mechanical, chemical, or metabolic insults (Ochs et al., 1997; Roediger and Armati, 2003). Beading is also seen in the peripheral nervous system when nerves are incrementally stretched and rapidly fixed by freeze-substitution or cold fixation (Ochs and Jersild, 1987). Recently, this mechanism of axonal beading was proposed to explain both the reduction in the mean apparent diffusion coefficient (ADC) during traumatic brain injury (TBI) and cerebral ischemia, and its re-elevation observed during recovery (Budde and Frank, 2010). Diffusion tensor imaging (DTI)-derived parameters, including fractional anisotropy (FA), while sensitive to these changes in axonal dimensions, do not provide information about specific microstructural or morphological changes or about their biophysical basis. Assuming that it is well-defined in the presence of beading, the ADD would directly reveal these underlying microstructural changes resulting from TBI or ischemia.

A common way to measure the ADD is by using optical or electron microscopy on fixed specimens. Measurement of postmortem histology-based ADD can be slow and tedious and is highly invasive. In addition, the examined tissue has to undergo a set of complex procedures that typically include extraction, freezing, cutting, dehydration, fixation, potting and mounting—processes known to change the cross-section area of fibers (Ohnishi et al., 1976). Moreover, cross-sectional slices from microscopy-based methods suffer from sampling only a small field of view (FOV). Measuring and mapping the ADD noninvasively using MRI presents several obvious advantages over traditional neuroanatomical methods: the procedure can potentially be performed on a live animal or human and over a large

volume of interest. Although in most cases the MRI voxel resolution is on the order of millimeters, the MR signal can be sensitized to the microscopic motion of diffusing water molecules and, indirectly, to the morphology of the restricting compartments that confine them, which are on the order of a few micrometers.

To achieve this degree of sensitivity, diffusion-weighted MRI can be used. A single pair of Stejskal Tanner (Stejskal and Tanner, 1965) pulsed-field gradients (PFG) is traditionally used to estimate certain microstructural features of porous materials (Callaghan et al., 1991) or macroscopic anisotropy (Basser, 1995). Single-PFG (recently termed single diffusion encoding, SDE, (Shemesh et al., 2015)) has also been used to estimate the ADD of white matter tissue (Assaf et al., 2008), by employing a parametric method that assumed the ADD is given by a gamma distribution. While such a parametric ADD may well describe healthy, normal neural tissue, a *nonparametric* ADD estimation would provide a more general and objective description of any axonal tissue, whether healthy or injured.

Although not developed for biological applications, an estimation scheme for nonparametric pore size distribution was originally suggested by Hollingsworth and Johns (Hollingsworth and Johns, 2003) for emulsions. Using an s-PFG NMR experiment and subsequently solving a linear regression problem, they estimated an empirical droplet size distribution. When this linear regression problem is solved, the design matrix (i.e., a matrix of values of explanatory variables, which in this case is the response to different droplet sizes) is highly ill-conditioned as a result of its intrinsically high multicollinearity (degree of correlation between the different independent variables). A similar problem arose when Assaf and Basser attempted to estimate the nonparametric ADD using a discrete histogram to represent this distribution (Assaf and Basser, 2007). In some cases, a solution is obtained by invoking certain assumptions about the character of the distribution. For instance, smoothness of the distribution is a common and usually valid assumption in natural porous materials (Song et al., 2002; Hollingsworth and Johns, 2003).

An extension of the s-PFG MR experiment, the double-PFG (d-PFG) (Cory et al., 1990; Mitra, 1995) experiment, employs two successively applied PFG pairs with amplitudes G_1 and G_2 , separated by a mixing time, τ_m , and a prescribed relative angle between them, ϕ (Fig. 1A). The d-PFG experiment, recently termed double diffusion encoding (DDE) (Shemesh et al., 2015), may be sensitized to the temporal correlations of molecular motions within the two diffusion periods and to the direction of the applied gradients (Feinberg and Jakab, 1990; Stapf et al., 1999). Using this experiment one can measure the average pore size (Özarslan and Basser, 2007; Koch and Finsterbusch, 2008; Komlosh et al., 2011) and the estimates of features of microscopic anisotropy (Lawrenz and Finsterbusch, 2011; Jespersen et al., 2013; Szczepankiewicz et al., 2015).

To address the problem of multicollinearity in ADD estimation with SDE MR we extended the method by using a DDE experiment (Benjamini et al., 2012). This adds an independent second dimension to the parameter space, namely the angle between the PFG pairs, which was shown to reduce the multicollinearity. The theoretical and experimental benefits of the DDE in the context of size distribution estimation were discussed and demonstrated in previous studies (Benjamini et al., 2012; Benjamini et al., 2014a) as were strategies for

optimal experimental design (Katz and Nevo, 2014). As a precursor to analyzing white matter microstructure, the DDE-based method was first validated on an ADD phantom using NMR (Benjamini and Nevo, 2013; Benjamini et al., 2014b) and later MRI (along with an experimental comparison between single- and double-diffusion encoding based methods) (Benjamini et al., 2014a).

In this study, a nonparametric DDE ADD estimation framework was developed, implemented, and tested on an ex vivo ferret spinal cord model. A voxelwise nonparametric ADD estimation provided maps such as average axon diameter and extra-axonal water fraction, along with unique ADD variance and sub-micron restricted structures map. The spatially resolved ADD map was then used to segment distinct domains in white matter on the basis of their statistical similarity. This method was further validated by a comparison of the ADD estimates with ADDs obtained from optical microscopy of the same specimen from different white matter tracts.

2. Theory

2.1. White matter model

In models of the microstructure and morphology of white matter, axons are usually idealized as infinite cylinders. White matter is commonly considered to comprised two compartments, each with distinct water diffusion properties. In one water is restricted, in the other diffusion is Gaussian (or hindered) (Assaf et al., 2004). The intra-axonal space is modeled as a finite and discrete distribution of impermeable parallel cylinders, in which water diffusion is restricted; the extra-axonal space is modeled as an “open space” in which water molecules experience hindered Gaussian diffusion. In this work we extend this model and include a third compartment, the sub-micron compartment, in which water molecules’ net displacement during the diffusion encoding period is smaller than the experimental sensitivity (i.e., unattenuated signal from highly restricted molecules). The acquired MR signal is assumed to be the superposition of the calculated signals originating from these non-exchanging compartments.

The signal attenuation from the extra-axonal compartment, E_{ex} , can be derived from Stejskal and Tanner (Stejskal and Tanner, 1965), and for the DDE pulse sequence (Fig. 1, note that we have chosen to use $G = |G_1| = |G_2|$, and assume that the diffusion gradients are perpendicular to the axons throughout this work) it is expressed as.

$$E_{ex}(G, \varphi, \Delta, \delta, D_{ex}) = e^{-\gamma^2 D_{ex} \delta^2 G^2 [2(\Delta - \frac{\delta}{3}) - (\frac{\delta}{3}) \cos(\varphi)]}. \quad (1)$$

where D_{ex} is the apparent diffusivity in the extra-axonal compartment, γ is the gyromagnetic ratio, G is the gradient amplitude, and φ is the relative angle between the two PFG pairs.

The intra-axonal signal attenuation, E_{in} , was calculated for every parameter set Ω (defined here as the combination of gradient amplitude, G ; its duration, δ ; and the relative angle between the two PFG pairs, φ , along with the diffusion period, Δ), diameter a , and intra-

axonal diffusion coefficient D_{in} , by using in-house software that implements the multiple correlation function (MCF) method (Grebekov, 2008; Özarslan et al., 2009). A benefit of using the MCF method in calculating the restricted diffusion signal is that it provides an exact solution with arbitrary timing parameters, e.g., finite δ and Δ .

Water diffusing in the sub-micron compartment was assumed to have a negligible attenuation under the current experimental conditions, and therefore the signal attenuation, E_{sm} , was set to be constant and equal to 1 (Alexander et al., 2010; Kroenke et al., 2005).

According to this model, the acquired signal attenuation for the j th experimental parameter set, Ω_j , can be written as

$$E^{data}(\Omega_j) = \sum_i \Psi(a_i) E_{in}(\Omega_j, a_i, D_{in}) + f_{ex} E_{ex}(\Omega_j, D_{ex}) + f_{sm} E_{sm}(\Omega_j), \quad (2)$$

where E^{data} is the measured signal attenuation; $\Psi(a_i)$ is the volume fraction contribution from the i th axon diameter, and f_{ex} and f_{sm} are the volume fractions of the extra-axonal and sub-micron compartments, respectively.

Different experiments, corresponding to different Ω s, are used to find these volume fractions, resulting in a system of linear equations, which can be written as the matrix equation

$$E^{data} = \mathbf{E}x, \quad (3)$$

where E^{data} is the experimental data vector, $\mathbf{E}$ is the design matrix, and x is the vector of volume fractions, i.e., Ψ , f_{ex} , and f_{sm} that are concatenated.

2.2. Data analysis

The coefficients vector, x , can be estimated by solving the following optimization problem:

$$x = \arg \min \| \mathbf{E}x - E^{data} \|, \\ x \geq 0, \sum x = 1 \quad (4)$$

where $\|\cdot\|$ is the 2-norm. As discussed earlier, Eq. (4) represents an ill-conditioned problem, i.e., a small change in E^{data} may result in large variations in x . The standard approach to ill-conditioned problems is to regularize them, especially when the solution is expected to be smooth (Lawson and Hanson, 1974; Fordham et al., 1995; Kroeker and Mark Henkelman, 1969). By slightly distorting the problem, a regularized problem has (by definition) a unique solution that depends smoothly on the data (Fordham et al., 1995). Furthermore, the solution to the regularized problem should be close to one of the infinity of feasible solutions of the original ill-conditioned problem. The regularized problem considered in this study is

$$x^{(\alpha)} = \arg \min_{x \geq 0, \sum x=1} \left(\| \mathbf{E}x - E^{data} \|^2 + \alpha \| \Psi \|^2 \right), \quad (5)$$

where the second term performs a Tikhonov regularization and its amplitude is controlled by the regularization parameter, α . Note that the regularization is performed on the ADD, Ψ , and not on the entire volume fractions vector, x , since the latter cannot be assumed to be smooth. The regularization term is a measure of the desired smoothness in Ψ , and although it makes the inversion less ill-conditioned, it may cause a bias.

Choosing the suitable value of α is still an open research question, with several approaches having been proposed (for a comprehensive review, please refer to (Mitchell et al., 2012)). The one used in this study, the S-curve method (Fordham et al., 1995), is based on the fit error, $\chi(\alpha)$,

$$\chi(\alpha) = \| \mathbf{E}x^\alpha - E^{data} \|, \quad (6)$$

as a function of α . The best α is chosen within a user-defined tolerance, TOL (Fordham et al., 1995; Song et al., 2002; Mitchell et al., 2012):

$$\frac{d(\log \chi)}{d(\log \alpha)} = \text{TOL}, \quad (7)$$

where a TOL=0.1 is a common choice for NMR applications (Fordham et al., 1995; Song et al., 2002), but to avoid over-regularization of the data, TOL=0.08 was used in this work.

2.3. Volume and number weightings

Because the diffusion-weighted MR signal amplitude from an axon of diameter a is proportional to a^2 , the resulting diameter distribution is weighted by the volume fraction of each axon population. If axons are modeled as infinite cylinders, the vector $\Psi(a)$ describes the relative signal fractions from axons with different diameters, which are modulated by the cross-sectional area of the axons, as was previously suggested and employed (Packer and Rees, 1972). Transforming the volume fractions, $\Psi(a)$, into the number fraction (i.e., the relative number fraction of axons in each size group), $\Psi_{\sim}(a)$, involves dividing each of the volume fractions by its corresponding diameter squared, a^2 , and then normalizing them such that $\sum_i \Psi_{\sim}(a_i) = 1$.

Previous MRI studies that reported average axon diameter (Komlosch et al., 2013; Xu et al., 2014) were all based on the axon volume weighting, since there is no way to obtain a number weighting without knowing the ADD. The nonlinear transformation between $\Psi(a)$ and $\Psi_{\sim}(a)$, which is illustrated in Fig. 2, where an axon population comprised of 15 1 μm axons, 4 4 μm axons, and 1 7 μm axon, is shown. The resulting number-weighted (NW)

ADD, $\Psi(a)$, and the corresponding volume-weighted (VW) ADD, $\Psi(a)$, are then computed accordingly. Note that the nonlinear transformation between number and volume acts as a low-pass filter on the diameters. The NW ADD therefore emphasizes the smaller axons, while the VW ADD emphasizes the medium to large axons. These different ADDs result in two contrasts, which can be used to compute, for example, mean axon diameter maps.

3. Materials and methods

3.1. Specimen preparation

The animal used in this study was housed and treated at the Uniformed Services University of the Health Sciences (USUHS) according to national guidelines and institutional oversight. As part of standard necropsy for an unrelated study, a healthy adult male ferret was euthanized and underwent transcardial perfusion with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4, Quality Biological) followed by 4% paraformaldehyde (PFA, Santa Cruz Biotechnology, in PBS 0.1 M pH 7.4) at USUHS, according to standard methods. For the present study, the spinal cord tissue was extracted by careful surgical dissection. A portion of the spinal cord was then transferred to a PBS-filled container for an additional 7 days for washing, to ensure the removal of any residual PFA from the tissue. Before MRI data were acquired a portion of the spine was saved for subsequent histological analysis, while the rest was immersed in perfluoropolyether (Fomblin LC/8, Solvay Solexis, Italy) and inserted into a 5 mm Shigemi tube (Shigemi Inc., Japan) with a plunger matched to the susceptibility of water. The tube was placed and oriented along the direction of the main magnetic field in a 10 mm birdcage RF coil (M2M Imaging, Cleveland, OH) 7T Bruker wide-bore vertical magnet with an AVANCE III MRI spectrometer equipped with a Micro 2.5 microimaging probe and three GREAT 60 gradient amplifiers having a nominal peak current of 60 A per channel. This configuration can produce a maximum nominal gradient strength of $24.65 \text{ mTm}^{-1}\text{A}^{-1}$ along each of the three orthogonal directions. Sample temperature was set at 16.8°C .

3.2. Acquisition scheme and MRI protocol

All of the MR images were acquired on a 7 T Bruker wide-bore vertical scanner. DTI (Basser et al., 1994) was performed on the ferret spinal cord before the DDE protocol and was used to verify axonal fiber orientation and its coherence. DTI parameters were: $TR/TE=3000/58$ ms, matrix size of 128×128 , with a slice thickness of 3.5 mm, and $FOV=5$ mm, which led to a $0.039 \times 0.039 \times 3.5 \text{ mm}^3$ nominal resolution. DWI timing parameters were $\delta=3$ ms and $\Delta=50$ ms with 21 diffusion orientations and 2 b -values of 400 and 2000 s/mm^2 .

According to the current model, the spinal cord white matter is assumed to be comprised of elongated axons with a coherent orientation. It is therefore not necessary to account for orientation distribution in our theoretical model and possible to use a circular DDE gradient sampling scheme, as the specimen can be placed in the magnet so that its long axis is aligned with the main magnetic field (i.e., application of diffusion gradients solely in directions that vary perpendicular to the long axis).

In the DDE experiment (Fig. 1A), the gradients of the first PFG NMR block are fixed along the x -axis, perpendicular to the spinal cord's symmetry axis (z), while the orientation of the gradients in the second block is varied in the x - y plane by an angle ϕ with respect to the x -axis. The experimental parameter set, Ω , is defined here as a specific combination of gradient amplitude, G ; its duration, δ ; the relative angle between the two PFG pairs, ϕ ; and the diffusion period, Δ .

A thoughtful choice of experimental parameters improves the size distribution reconstruction (Benjamini et al., 2012; Katz and Nevo, 2014). Prior to the acquisition, we considered a 4-D parametric space comprised of $\phi=0-\pi$, $G=147-664$ mT/m, $\Delta=30-60$ ms, and $\delta=1.5-3.5$ ms (which led to a b -value range of 677-45,565 s/mm²). We then employed an iterative method to reduce the large number of possible experimental sets spanned by the 4-D space, which resulted in an optimal experimental design, by applying a previously suggested optimization framework (Katz and Nevo, 2014). At the end of the optimization process an experimental design composed of N experimental parameter sets, Ω , was determined.

We used $N=160$ such that the number of observations (i.e., Ω s) would be roughly 10 times the number of dependent parameters (i.e., diameters) in accordance with previous publications (Katz and Nevo, 2014; Benjamini and Bassar, 2014). The mixing time, τ_m , was kept at zero. To avoid a complicated 4-D display of the acquisition scheme, two of the parameters, G and δ , were expressed as the q -value (Callaghan et al., 1991), which is defined as $q \equiv (2\pi)^{-1}\gamma\delta G$, where γ is the gyromagnetic ratio. The optimized experimental design is shown in Fig. 1B. It is worth noting that since $\tau_m=0$, the second gradient of the first pair and the first gradient of the second pair are superposed, and their magnitude is equivalent to $2G$.

The DDE MRI sequence used in this study on the ferret spinal cord was constructed from a DDE NMR block followed by a spin echo MRI block. For these acquisitions, for which single lines were acquired separately in the k -space, the MRI parameters were: $TR/TE=3500/6.3$ ms, matrix size of 64×64 , acquiring separately single lines in the k -space. The imaging slice was selected to minimize nerve root entries, thus reducing the number of fibers perpendicular to the spinal cord main axis, with a thickness of 3.5 mm, and $FOV=5$ mm, which led to a $0.078 \times 0.078 \times 3.5$ mm³ nominal resolution. The number of averages of each image was 4, resulting in a total acquisition time of 40 h.

3.3. Voxelwise analysis

Voxelwise analysis was performed on the white matter of the spinal cord. Prior to analysis, the design matrix was calculated using in-house software that implements the multiple correlation function (MCF) method (Grebekov, 2008; Özarlan et al., 2009). The design matrix, $\mathbf{E}(\Omega, a, D)$, was generated with a cylinder diameter range of $1.3 \mu\text{m} \leq a \leq 12.5 \mu\text{m}$ with $0.70 \mu\text{m}$ steps, producing 17 different diameters. The theoretical signal attenuations were then calculated according to the previously selected 160 experimental sets. In other words, the rows of the matrix corresponded to signal attenuations arising from the optimal design of experimental parameters, while the columns corresponded to the chosen range of axonal diameters, resulting in a 160×17 design matrix.

To provide estimates of the intra- and extra-axonal diffusivities, along with the ADD, the extra-axonal and sub-micron water fractions, we use a heuristic approach, where 70 values of D_{in} in the range of $0.07\text{--}1.7\text{ }\mu\text{m}^2/\text{ms}$ are assumed, and the following process is performed for each of the white matter voxels:

For each iteration of $D_{in}^{(i)}$, the design matrix $\mathbf{E}(\Omega, a, D_{in}^{(i)}, D_{ex}^{(i)})$ is passed through a nonlinear optimizer (*Matlab* library function *lsqcurvefit*) to find the optimal $D_{ex}^{(i)}$ by solving Eq. (4) with $\mathbf{E}(\Omega, a, D_{in}^{(i)}, D_{ex}^{(i)})$.

At the end of the process, 70 pairs of $(D_{in}^{(i)}, D_{ex}^{(i)})$ are found, along with their residual error from E^{data} . The optimal $(D_{in}^{(opt)}, D_{ex}^{(opt)})$ pair that minimizes the residual error is then chosen.

The design matrix $\mathbf{E}(\Omega, a, D_{in}^{(opt)}, D_{ex}^{(opt)})$ is then used to solve Eq. (5), as described in the Theory section.

The total number of free parameters for the linear and nonlinear optimization problems was 19 and 1, respectively.

3.4. Clustering of distinct domains

The voxelwise analysis yielded a vector of specific physical attributes for each voxel, i.e., the ADD and the extra-axonal and sub-micron fractions. These attributes can be referred to as features for further cluster analysis with, for example, *k*-means (Lloyd, 1982), that will determine distinct domains in white matter.

A standard *k*-means clustering algorithm classifies the voxels independently, ignoring spatial considerations, such as contiguity and other known spatial correlations. Because of the somatotopic arrangement of axon pathways within the spinal cord (Dyce et al., 2009), neuronal structures with similar features are expected to have a spatial relationship and certain spatial correlations. Apart from the anatomical contiguity, the prior knowledge of left–right anatomical symmetry in the spinal cord of some pathways can be considered in the clustering algorithm.

Before segmenting the entire cross-section, we first examined the anatomical left–right symmetry by separately clustering the two halves for two reasons: (1) a similar clustering pattern from independently clustering both halves would point to the robustness of the analysis (i.e., a pseudo cross-validation), and (2) it would also support using the left–right symmetry as prior information. In addition, histological comparison of left and right lateral spinothalamic tracts (LSTT) (refer to Fig. 3 for location of the tracts) was performed (see Supplementary Material), revealing very similar ADDs.

Taking advantage of the symmetry, we designed and implemented a modified version of the *k*-means clustering algorithm, based on a previously proposed contiguity-enhanced *k*-means algorithm (Theiler and Gisler, 1997) with additional spatial symmetry constraints. The *k*-means algorithm was originally designed to minimize the average within-cluster variance, or

the compactness, of a clustering segmentation. In our modified version, voxels that are spatially contiguous or with a left–right symmetry are more likely to be in the same class than are random pairs of voxels. For a detailed description, refer to Appendix A. Unlike in the voxelwise analysis, in the clustering step we are not particularly concerned with voxel-by-voxel resolution or with finding small clusters. We were therefore able to use a 3×3 Gaussian smoothing kernel on the feature space prior to clustering (Hopfinger et al., 2000).

Lastly, to determine the number of clusters, we iterated the modified k -means algorithm with different k s and calculated the silhouette value (Rousseeuw, 1987), the Davies–Bouldin index (Davies and Bouldin, 1979), and the Dunn index (Dunn, 1973) for each number of clusters used. These three indices are data driven and considered to be internal evaluation. They all assign the highest score to the algorithm that produces clusters with the greatest similarity within a cluster and the least similarity between clusters. The resulting scores for each value of k were then standardized, averaged, and used to assess the optimal number of clusters, which yielded $k=6$ for both the NW and VW based feature spaces.

3.5. Histology

The fixed spinal cord was sliced into three 500 μm cross sections on a vibratome (Ted Pella, Inc., Redding, CA). Slices were equilibrated to 0.1 M sodium cacodylate buffer, pH 7.4, then post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 4 h at room temperature to create contrast in the sample. Following additional buffering and water rinses (30 min each), samples were dehydrated through a graded ethanol series to remove all traces of water, equilibrated to 100% propylene oxide, and infiltrated with increasing concentrations of Embed 812 resin. The cross sections were polymerized in 100% Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA) for 18 hours at 60°C. Thin sections (1 μm) from each of the 3 slices were cut on a Reichert Jung Ultramicrotome, (Ultracut-E), dried onto glass slides, then counterstained with toluidine blue. A cover glass was attached with Cytosol 60 permanent mountant (Electron Microscopy Sciences, Hatfield, PA) prior to imaging.

Transmitted light images were acquired using an Olympus IX81 wide-field microscope equipped with a 40X oil UPlanFLN, 1.30NA objective (Olympus) and an Orca Flash 4.0 camera (Hamamatsu) at $261 \times 261 \text{ nm}^2$ resolution. Multiple fields were acquired from five ROIs (Fig. 3), left anterior corticospinal tract (ACST), right lateral spinothalamic tract (LSTT), left anterior spinocerebellar tract (ASCT), right dorsal spinocerebellar tract (DSCT), and left gracile fasciculus (FG). All ROIs contained fields from three different cross-section slices.

An ROI-based analysis was performed to compare the ADDs measured by light microscopy- and MRI-based methods. ROIs that corresponded to the processed sections of the spinal cord were identified on a proton density image. Subsequently, a voxelwise analysis (as opposed to analyzing the average signal from the ROI) was performed on the voxels within each ROI, followed by the averaging of the resulting ADDs to an ROI ADD. This step ensured similar signal-to-noise-ratio levels in the voxelwise- and ROI-based analyses.

The histological images were enhanced with adaptive histogram equalization. A total of 1000 axons or more were counted in each of the ROIs, by using a region growing algorithm for segmentation (*Matlab*). In the current nonparametric estimation framework the estimated ADD is discrete and assumed to be comprised of 17 diameter bins. Conversely, while being measured, the ADD from the microscopy images is not limited to any finite number of bins, resulting in a continuous distribution. For comparison and display purposes, the continuous values were discretized into the same 17 bins. Very few counted axons had a bigger diameter than the largest diameter considered in the MR ADD model range (12.5 μm , see Materials and Methods). These measurements were discarded, since it is assumed that these very large axons are captured by the extra-axonal volume fraction of the model.

The Jensen difference metric was chosen to provide a quantitative comparison between ADDs since it is a well-established method of measuring the similarity between two probability distributions (Endres and Schindelin, 2003). The Jensen difference metric is a symmetric version of the Kullback–Leibler divergence (Kullback and Leibler, 1951), which is bounded by 0 and 1, in which a lower distance value reflects higher similarity, and thus, higher quality of estimation. The difference between two distributions, Q and P , is defined as (Sibson, 1969).

$$d_{JD} = \sum_i \left[\frac{P_i \ln(P_i) + Q_i \ln(Q_i)}{2} - \left(\frac{P_i + Q_i}{2} \right) \ln \left(\frac{P_i + Q_i}{2} \right) \right]. \quad (8)$$

4. Results and discussion

4.1. Local microstructure

The VW ADD within each white matter voxel was estimated by solving Eq. (5), along with the extra-axonal and sub-micron fractions, and the intra- and extra-axonal diffusivities. The VW ADD can be subsequently transformed into NW ADD (see Theory). These values can be used to create maps in which D_{in} , D_{ex} , f_{ex} , and f_{sm} are assigned to each of the white matter voxels. Even more descriptive, moments of both VW and NW ADDs, e.g., mean, variance, etc., may be calculated and mapped with potentially revealing contrasts between regions.

Average axon diameter maps can therefore be calculated from the ADDs and are expected to yield different contrasts. The VW and NW average axon diameter maps are shown in Fig. 4A and B, respectively. As expected, the number weighting results in overall smaller average diameters (as illustrated in Fig. 2). In addition, the maps reveal two different contrasts, where, for example, looking at both sides of the LSTT, Fig. 4A is quite symmetric while 4B shows higher values on the right side.

The variance of the VW and NW ADDs was also used to create maps, shown in Fig. 4D and E, respectively. Here too, the difference between the maps is pronounced, in particular, the dynamic range of each of the maps: the VW variance is distributed between 2 and 18 μm^2 , while the range of the NW counterpart is only 2–8 μm^2 . Examination of Fig. 4 reveals that

smaller NW average axon diameters generally correspond to higher VW variances (Fig. 4B and D), especially evident in the left DSCT. Conversely, the contrast of the VW average axon diameter map and the NW variance map are similar (Fig. 4A and E). These correlations of first–second moments over the different weighting contrasts might provide important new insights and may change in degenerated or injured tissue. Conversely, traditional DTI-derived direction-encoded color and fractional anisotropy maps show no contrast within the spinal cord white matter (not shown here, but see (Komlosh et al., 2013)).

The extra-axonal water volume fraction map in Fig. 4C reveals fairly uniformly distributed values across the sample. The mean extra-axonal water fraction over the entire spinal cord white matter was 0.11 with a relatively small standard deviation of 0.06. Although in common use (Assaf et al., 2004; Komlosh et al., 2013; Avram et al., 2013), the diffusion model in the extra-axonal space (i.e., Gaussian diffusion) is the simplest one can apply. When employing multiple diffusion-times, as was done in the current study, a diffusion time-dependent model for the extra-axonal space might be more accurate, since the diffusion MR signal depends on the diffusion time. This time dependency was previously exploited and described by diffusion in disordered media and fractal spaces (Özarslan et al., 2006; Özarslan et al., 2012). In addition, it has been recently shown that the diffusion coefficient in extracellular space is time-dependent (Burcaw et al., 2015). Although the range of applied diffusion periods in this study is relatively small (30–60 ms), extending the current model to include such time dependency should be considered in future studies.

The sub-micron compartment was introduced into the white matter model to account for the population of highly restricted water molecules. Even when the maximal q -value is considered ($2q_{\max} = 1975 \text{ cm}^{-1}$), axons smaller than $1 \mu\text{m}$ (Hildebrand and Hahn, 1978) and local restrictions, e.g., water trapped within glial cells, at an even smaller scale are known to exist in white matter tissue (Stanisz et al., 1997; Clark et al., 2002). Under the current experimental conditions, water molecules trapped in such compartments would result in no significant signal attenuation (at the order, or less, of the noise level), even though they might comprise a significant fraction of the total water volume. Including their contribution in the model would lead to a more accurate description of the tissue, correct the bias toward the small axon diameters, and result in a sub-micron volume fraction map, which is presented in Fig. 4F. The contrast in this parametric map is unique, compared with Fig. 4A–E, and the mean sub-micron volume fraction over the entire spinal cord white matter was 0.14 with a relatively small standard deviation of 0.07, with a different model, a similar value was found in ex vivo baboon brain white matter (Kroenke et al., 2005). The presence of a relatively large sub-micron component within white matter might suggest a relationship to water molecules trapped within the lipid layers of myelin (Kroenke et al., 2005).

Considering the extra-axonal and sub-micron fractions, the average intra-axonal fraction (i.e., $1 - f_{\text{ex}} - f_{\text{sm}}$), is 0.75, which is in very good agreement with values obtained in previous brain extracellular space studies (Nicholson, 1965; Syková and Nicholson, 2008), and also MR studies (Burcaw et al., 2015).

To compare the current average axon diameter map with previous diffusion MR-based studies, one should use the VW map (Fig. 4A). Matched with previous studies on rodent (Xu

et al., 2014) and porcine (Komlosh et al., 2013) spinal cords, the estimated average axonal diameter maps have very similar contrasts, while the current analysis resulted in consistently larger diameters. This size discrepancy should be expected because of three major differences between the previous and current studies. (1) The sources of the specimen (rat, pig, and ferret) differ. (2) In the previous studies a single axon diameter in each voxel, not a distribution, was assumed. This assumption made the model simpler but also introduced an inevitable error because of the deviation from biological reality. (3) The purpose of the inclusion of a sub-micron compartment is to avoid the bias toward the small axon diameters that are below the experimental sensitivity. This model is therefore expected to result in larger average axon diameters. Because of these differences one should not expect reproducibility between the current and previous studies. Therefore, any comparison of the average diameter maps should be qualitative.

A theoretical framework for obtaining the moments of a general distribution of isolated pores with characteristic shapes (planar, cylindrical, or spherical) was suggested by Özarslan et al. (Özarslan et al., 2011). While in that work the estimation of the first moment and the standard deviation proved to be fairly accurate, measures that depend on the higher-order differences between these moments are not, and are not expected to be, reliably estimated from real MR data (Özarslan et al., 2011). Conversely, higher moments (e.g., third moment, skewness) can be directly derived from the ADDs and be used as additional different contrasts. Since this study serves as proof of concept and does not include abnormal tissue in comparison with control, such contrasts were not necessary.

The voxelwise ADD maps create a three-dimensional space in which each voxel has its own microstructural signature, consisting of the VW and NW ADDs, f_{ex} , and f_{sm} . Because these maps contain abundant information it is prudent to refine and compress them to a more compact representation, which would allow visualization and spatial contrast within the white matter.

4.2. Segmentation according to microstructural similarity

The position of white matter tracts in spinal cord atlas reference data (Standring, 2008) can be used to segment the spinal cord into regions, according to distinct white matter pathways (Benhamou et al., 2014). Other methods take a more data-driven approach to segmenting the spinal cord by using image intensities obtained from different staining agents (e.g., H&E and oligodendrocytes markers (Assaf et al., 2008)) or different MRI contrasts (e.g., T_1 and T_2 (Yovel and Assaf, 2007)) as features in a clustering algorithm. A third approach uses the microstructural information to segment the spinal cord, as was done in the AxCaliber study (Assaf et al., 2008). We adopted the latter concept; however, instead of using the parameters from the a priori assumed shape of the ADD as features in a k -means algorithm, we used the nonparametric ADD, which makes no a priori assumptions regarding its shape. The ADD is a morphological feature of the tissue. Different white matter fascicles often differ in their diameter distribution as well as in other microstructural features, such as myelin content or cell density (Barnea-Goraly et al., 2005; Gregg et al., 2007; Imfeld et al., 2009; Schlaug et al., 2009). To derive the dissimilarities of the different voxels, we considered two sets of

feature spaces, VW and NW, by using the VW and NW ADDs, $\Psi(a)$ and $\tilde{\Psi}(a)$, along with the extra-axonal and sub-micron volume fractions.

Subsequently, six clusters were identified ($k=6$) according to the internal clustering evaluation score described earlier. This time, the entire white matter of the sample was clustered by using the proposed modified k -means algorithm on each of the VW and NW based feature spaces, resulting in the clustering pattern shown in Fig. 5A and C, respectively. Shown in Fig. 5B and D are the mean ADDs from each of the VW and NW clusters, respectively, with error bars that reflect a 95% confidence interval across voxels, along with the extra-axonal and sub-micron volume fractions.

In both feature spaces, analogous colors were assigned to clusters with similar ADDs (e.g., light vs. dark blue), while high contrast colors indicated different ADDs (e.g., red vs. blue). The separate clustering of both types of weightings emphasizes the distinct information representation each of them contains, which leads to a different contrast. As mentioned earlier, the effect of transforming the VW ADD to a NW ADD can be thought of as a low-pass filter on the diameters, which preserves and therefore emphasizes how the lower axon diameters are distributed. Conversely, the VW ADD contains more detail regarding the larger axon diameters. The clusters from the NW feature space (Fig. 5C) are generally bigger and more contiguous than the clusters obtained from the VW feature space (although equal importance for contiguity was imposed, see Appendix A).

Upon inspection of the spinal cord segmentation (Fig. 5A and B) the ASCT and DCST could be generally identified (dark and light blue, respectively) in both VW and NW clustered maps, indicating that the segmentation is indeed meaningful. Other tracts, such as the FG and ACST, can also be identified, although with less specificity. While not indicative of a specific tract, the green cluster in the NW feature space seems to be the general area of the motor (efferent) pathways (Sengul et al., 2013). And indeed, when looking at this cluster's corresponding NW ADD (Fig. 5D), cluster number 6 has the largest average axon diameter. Since conduction velocity scales with axon diameter (Tasaki et al., 1943; Waxman et al., 1995) axons of motor pathways are expected to be large.

The main advantage of this proposed ADD estimation framework over previous ones is that it uses a nonparametric approach, therefore making it suitable to analyze abnormal tissue, a case for which there is no basis for using a parametric modeling of the ADD. In the present study, a healthy normal tissue was used to illustrate the potential of the method, and therefore left-right anatomical symmetry and contiguity could have been assumed and incorporated in the clustering process. We expect that for abnormal tissue (e.g., developing or degenerated), these assumptions should be relaxed.

4.3. Comparison with microscopy

Multiple microscopy images of five toluidine blue-stained ROIs from three axial slices were acquired. A high-resolution proton density MR image is shown in Fig. 3, along with the locations of the five ROIs: left-ACST, left-ASCT, right-LSTT, right-DSCT, and left-FG. Representative microscopy images and the corresponding segmented masks used for axon counting of the four white matter tracts, fused to a mask-based image overlay, are shown in

the top row of Fig. 6. The scale (orange) bars represent 10 μm . The two bottom rows of Fig. 6 show the corresponding VW and NW ADDs from each of the ROIs. In each distribution, solid red lines and blue bins are the microscopy- and MRI-based ADDs, respectively.

An inspection of the microscopy- and MRI-based ADDs indicates good agreement between the two methods. By comparing the VW and NW distributions the importance of each of the weightings becomes clear. In the FG, a minor error in the smallest diameter in the VW ADD led to a more significant error in the NW ADD. More notable is how discrepancies in the large axon diameter range in all of the VW ADDs disappeared after being transformed into NW.

The values of the average and standard deviations obtained from the microscopy- and MRI-based ADDs were calculated for each of the ROIs and are presented in Table 1. The agreement between the averages and standard deviations derived from both the VW and NW ADDs is excellent, although slightly better for the latter. These results are consistent with the low-pass filter notion, where after application of such “filtering” the NW ADDs contain less fine detail. The discrepancy between MR and microscopy results can be attributed to the effect of shrinkage during the preparation of tissue for histological analysis. The multiple steps in the staining protocols, especially tissue dehydration, are known to reduce the cross-section area of fibers and thus directly affect the validity of a comparison of microscopy- and MRI-based results (Gusnard and Kirschner, 1977; Strausfeld, 1983).

To quantify the accuracy of the method we calculated the Jensen differences between all pairs of microscopy and MR regions, both for the NW, VW, and the two combined. Tables 2 and 3 are distance tables of the VW and NW ADDs, respectively. As expected, the distances derived from the NW ADDs are smaller. The diagonal in each of the tables designates matching regions in MR and microscopy, therefore representing the correct anatomical designation for an MRI region. With the exception of ASCT-L and ACST-L, given the MR-based VW ADD, the correct actual matching region is found, as demonstrated by the minima (in each of the columns) of the Jensen distances in the diagonal cells of Table 2. Proving more robust (although less detailed), the Jensen distances of the NW ADDs were all predictive of the correct MR and microscopy pairs, as can be seen by the diagonal values in Table 3.

To help visualize the accuracy according to the Jensen distances, a joint VW–NW distance matrix was built by first normalizing and then averaging Tables 2 and 3. The resulting matrix is shown in Fig. 7, with relative distances as grayscale values (black—0, white—1). Compared with their row and column, the diagonal values were consistently lowest, pointing to the good agreement between MR- and microscopy-based ADDs.

4.4. Limitations and translational potential

As mentioned earlier, the spinal cord axons are assumed to possess the same orientation and, therefore, an orientation distribution function was not incorporated into the model. Despite the highly organized structure within the spinal cord, potential misalignment between fibers and the z -axis (perpendicular to the diffusion gradients), both *ex* and *in vivo*, presents more challenges. In the likely event that spinal cord axons are parallel to each other but with a

varying orientation over different voxels, it is essential to adopt an alternative acquisition scheme. Generally, what is required is a 3-D gradient sampling scheme that involves the application of the current circular DDE acquisition in different orientations, similar to the “isotropic” design of DTI experiments (Jespersen et al., 2013). In a slightly different approach, the principal fiber orientation in each voxel is calculated from a subset of these measurements (or from a previous DTI scan), and the projection of the data onto the desired plane can be used and analyzed (Komlosh et al., 2013). This framework can also be used in specific regions of brain white matter, such as the corpus callosum, optic tracts, and other pathways, where axons generally exhibit a coherent orientation.

As opposed to the spinal cord, brain white matter tissue axons may take on all possible orientations within an imaging volume. Unless the orientation distribution of the axons (within a voxel) is assumed to be uniform (i.e., random orientation), this added complexity requires a modification of the theoretical model, in addition to the aforementioned alterations to the acquisition scheme. In this case, the theoretical model would include a fiber orientation distribution along with the diameter distribution. The orientation distribution may be based on a parametric model (e.g., spherical harmonics (Özarslan and Bassar, 2008)) or data driven (i.e., nonparametric, empirical). The latter approach would add the orientation distribution weights as free parameters to the model, which, given the same number of free parameters, would reduce the stability of the estimation. Practically, adding an orientation distribution in addition to a size distribution might prove challenging, since axon orientation and size introduce competing effects on the DW signal attenuation.

The strong assumption of restricted diffusion within cylinders should also be discussed. While considered to be an acceptable simplification of the underlying microstructure of white matter, axons are not perfect cylinders of infinite length. As their actual cross-section deviates from a circle, an error is introduced into the size estimations. In addition, the existence of non-axonal cells, which cannot be generally modeled by a simple geometry, should be taken into account. The latter caveat was partially alleviated by incorporating the sub-micron compartment in the model, which potentially accounted for highly restricted water in glial cells. Including this compartment in the model should prove even more important in *in vivo* studies. In clinical studies the maximal diffusion weighting is generally lower than the one used in the current study, thus leading to an even larger fraction of unattenuated DW signal from highly restricted molecules. Without a model for this effect, water diffusing experiencing sub-micrometer restriction would strongly bias the estimates.

The long scan time (40 h) in the current study is more than an order of magnitude longer than what is required for a preclinical or clinical scan. However, the use of fixed tissue in this study allowed signal averaging and the acquisition of individual *k*-space lines. For *in vivo* tissue, motion and flow prevent the use of such an acquisition protocol, and fast imaging readout blocks, such as echo planar imaging (EPI), are required. In our case, 64 phase encoding steps could be scaled down by using a single-shot echo planar sequence, significantly reducing the scan time.

The use of a DDE diffusion MRI sequence for clinical purposes was recently demonstrated (Koch and Finsterbusch, 2011) and was followed by an *in vivo* multiple-PFG diffusion (or

multiple DE) experiment that provided improved sensitivity to small axon diameters and reduced diffusional attenuation (Avram et al., 2013). Using clinically applicable diffusion gradients Avram et al. were able to reach q -values ($2q$ and $4q$ in the d- and q-PFG, respectively) of about 1000 cm^{-1} , which resulted in the detection of axons in the range of 1–10 μm . These clinical implementations of the multiple-PFG pulse sequence could be used with the presented framework. Specifically, because our implementation of the MCF method can account for arbitrary experimental parameters (e.g., long diffusion gradient durations) or for the effect of imaging gradients on the signal attenuation, nonparametric ADD estimation using DDE MRI has the potential for preclinical and clinical applications. Moreover, the microstructural information our method provides, even from ex vivo samples, is unique and cannot be obtained by histological methods on large-scale tissue sections (i.e., information from the entire volume of the tissue).

5. Conclusions

In this study we have used a nonparametric method to estimate on a voxel-by-voxel basis the volume-weighted ADD within a spinal cord specimen. We have shown how the VW ADD can be transformed to NW ADD, and how these two weightings produce different contrasts within the spinal cord white matter. A sub-micron compartment was incorporated in the model to account for highly restricted water molecules that result in an unattenuated DW signal above the noise floor. The new white matter model alleviates the under-estimation bias of axon diameter, while providing a unique parametric map of the sub-micron volume fraction in the tissue, which may be indicative of water molecules trapped within the lipid layers of myelin (Kroenke et al., 2005) or in glial cells (Stanisz et al., 1997).

The VW and NW ADDs, along with the extra-axonal and sub-micron volume fractions, were used to segment the spinal cord into domains with similar morphological features, resulting in different clusters for volume and number weightings. Histological measures of the ADD from five ROIs were compared with the estimations, exhibiting a very good agreement between the two. Both the clustering and comparison with microscopy revealed the underlying difference between volume and number weighting. While the VW ADD generally contains greater detail, especially in the medium to high diameter range, it is also more susceptible to errors in estimation. On the other hand, the NW ADD is more robust (because of the low-pass “filtering” effect of the VW to NW transformation) and accurate when compared with microscopy, although the arguably important information contained in the mid-high diameter range is lost. Since by obtaining the VW ADD one can very easily compute the NW ADD, we suggest that both these complementary distributions be used side by side.

Apart from transmitting sensory and motor information between the periphery and the cerebral cortex, the characterization of features of spinal cord microstructure has great clinical value; for instance, spinal cord lesions in MS patients appear to be symptomatic more often than brain lesions (Trop et al., 1998) and correlate better with the degree of physical disability (Nijeholt et al., 1997). Previous MRI-based methods used a gamma function to model the ADD (Assaf et al., 2008; Barazany et al., 2009). More recent attempts to derive the parametric form of the ADD used biomechanical (Gov, 2009) or

neurophysiological (Pajevic and Basser, 2013) principles, based on minimizing energy consumption or optimizing information transmission, respectively. These new distributions are based on principles of optimization that the nervous system is implicitly assumed to follow. Although these assumptions might be valid for healthy tissue, a more general approach, which would account for changes in development, degeneration, disease, and trauma, is to estimate the ADD directly without making any such a priori assumptions.

Unlike SDE techniques such as AxCaliber (Assaf et al., 2008), or ActiveAx (Alexander et al., 2010), DDE MRI is inherently more robust to ADDs (Özarslan and Basser, 2007; Benjamini et al., 2014a). With further acquisition acceleration and experimental parameter adjustments, this ADD estimation framework could be preclinically validated, enabling a wide range of neuroimaging applications for improved diagnosis of neurodegenerative pathologies and assessing microstructural changes resulting from trauma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Spatially constrained k-means algorithm

In this study we designed and implemented a modified version of the k -means clustering algorithm, based on a previously proposed contiguity-enhanced k -means algorithm (Theiler and Gisler, 1997), with additional spatial symmetry constraints.

The k -means algorithm was originally designed to minimize the average within-cluster variance, or the compactness, of a clustering segmentation. In our case, clustering N voxels into K groups, $x_i = (x_{i1}, \dots, x_{ip})^T$ denotes p features measured in the i th voxel. We then minimized the within-cluster sum of squares, i.e., noncompactness, or minimizing.

$$V = \frac{1}{N} \sum_{k=1}^K \sum_{i \in \mathbf{I}_k} (x_i - c_k)^2, \quad (\text{A.1})$$

where c_k is the center of mass of the k th cluster, and $\mathbf{I}_k$ denotes the set of i s which belong to the k th cluster. V is then normalized by the overall variance of the data such that its value varies from zero to one, with smaller values indicating better clustering.

In our modified version, voxels which are spatially contiguous or with a left–right symmetry are more likely to be in the same class than are random pairs of voxels. We therefore define the discontinuity at a certain voxel as the fraction of its neighbors and their symmetric counterparts that are not in the same cluster as

$$\Gamma_i = \frac{\text{Number of adjacent voxels } j \text{ for which } k(i) \neq k(j)}{\text{Number of adjacent voxels}}. \quad (\text{A.2})$$

It is worth noting that the difference between the current algorithm and the one used by Theiler and Gisler (Theiler and Gisler, 1997) is in the definition of adjacent voxels. To account for left–right symmetry we not only considered the 8 neighboring voxels to be adjacent, but also their symmetric counterparts. The global discontinuity can also be defined as.

$$\Gamma = \frac{1}{N} \sum_{k=1}^K \Gamma_k. \quad (\text{A.3})$$

The clustering algorithm then minimizes a linear combination of noncompactness and discontinuity, where the user has to specify a parameter λ to define the relative importance of each property. In other words, its objective is to minimize

$$y = (1 - \lambda)V + \lambda\Gamma. \quad (\text{A.4})$$

To determine the λ parameter, the L-curve method (Miller, 1970; Mitchell et al., 2012), which is commonly used to establish the regularization parameter when solving the Fredholm equation, can be easily adopted and applied in our case. To create such an L-curve we solve Eq. A.4 iteratively with a range of λ parameters, and plot the noncompactness, V , versus the discontinuity, Γ . In this way, the L-curve clearly displays the compromise between minimization of these two quantities, which is the heart of the proposed clustering method. The schematic illustration in Fig. A.1 clearly displays the trade-off between minimizing the noncompactness and the discontinuity.

An important quality of the L-curve is that the optimal regularization parameter is not far from the regularization parameter that corresponds to the L-curve's corner (Hansen, 1992). We extended this method to our current problem, and by locating the corner of the L-curve we found an approximation of the optimal λ parameter, which in turn, solved Eq. A.4 with a good balance between the two error types. Using this method, with $k=6$ and for the VW feature space, resulted in $\lambda=0.23$. The same λ parameter was then used to cluster the NW feature space to ensure a fair comparison.

Fig. A.1.

A schematic illustration of an L-curve. Plot of noncompactness versus discontiguity, as a function of λ . As λ increases, the discontiguity decreases while the noncompactness increases.

Appendix B. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neuroimage.2016.04.052>.

Fig. 1. MRI protocol and acquisition scheme. (A) The DDE pulse sequences used in the present study. Note that $\tau_m=0$, $\delta=\delta_1=\delta_2$, and $\Delta=\Delta_1=\Delta_2$. For a given gradient amplitude, δ , and Δ , the relative angle between the gradients, ϕ , is varied. (B) Optimized acquisition scheme that includes 160 data sets from the 4-D parameter space spanned by $q=(2\pi)^{-1}\gamma\delta G$, ϕ , and Δ . Note that both G and δ are varied and provide two dimensions of the 4-D parameter space.

Fig. 2.
An illustration of the nonlinear transformation between the VW ADD, $\Psi(a)$, and the NW ADD $\tilde{\Psi}(a)$.

Fig. 3.
A high-resolution proton density MR image along with the locations of the five regions of interest, left ACST, right LSTT, left ASCT, right DSCT, and left FG.

Fig. 4.

Spinal cord maps computed according to the estimated structural information. Average axon diameter (first moment) derived from (A) the volume and from (B) the number-weighted ADDs. Axon diameter variance (second moment) derived from (D) the volume and from (E) the number-weighted ADDs. (C) Extra-axonal volume fraction (f_{ex}) map, and (F) sub-micron fraction (f_{sm}) map.

Fig. 5.

Microstructural similarity patterns after segmentation with $k=6$. The clustering pattern using the (A) VW and (C) NW feature spaces. (B) and (D) show the mean ADD and the extra-axonal and sub-micron volume fractions from each of the clusters in (A) and (C), respectively. The error bars reflect a 95% confidence interval across voxels.

Fig. 6.

Microscopy images and the corresponding segmented masks used for axon counting of the five white matter tracts, fused to a mask-based image overlay, are shown in the top row. The scale (orange) bars represent 10 μm . The two bottom rows shows the corresponding VW and NW ADDs from each of the ROIs; solid red lines are the microscopy-based ADDs, and the blue bins are the MRI-based ADDs.

Fig. 7.
The joint VW–NW distance matrix showing relative distances between all pairs of microscopy and MR regions.

Table 1

Average and standard deviations from histology- and MRI-based ADDs.

ROI	NW Hist.	NW ADD	VW Hist.	VW ADD
ACST	3.62±2.08	3.51±2.17	6.26±3.06	6.57±3.34
ASCT	3.72±1.92	3.72±1.76	5.72±2.27	5.46±2.30
DSCT	2.61±1.52	2.70±1.66	4.99±3.26	5.66±3.79
FG	3.56±1.90	3.61±1.85	5.75±2.65	5.61±2.56
LSTT	3.18±1.98	3.16±2.03	5.89±2.84	6.10±3.08

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Table 2

Jensen distance table for all pairs of microscopy and MR regions (VW).

Hist\MR	ACST-L	ASCT-L	DSCT-R	FG-L	LSTT-R
ACST-L	.017	.027	.021	.114	.013
ASCT-L	.071	.021	.040	.199	.044
DSCT-R	.027	.010	.011	.126	.014
FG-L	.085	.098	.075	.062	.082
LSTT-R	.025	.025	.029	.122	.007

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Table 3

Jensen distance table for all pairs of microscopy and MR regions (NW).

Hist\MR	ACST-L	ASCT-L	DSCT-R	FG-L	LSTT-R
ACST-L	.004	.009	.006	.056	.016
ASCT-L	.015	.006	.009	.079	.027
DSCT-R	.007	.006	.005	.057	.019
FG-L	.038	.079	.059	.010	.019
LSTT-R	.011	.035	.024	.031	.001