Probing axonal swelling with time dependent diffusion MRI

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Development of therapies for neurological disorders depends on our ability to non-invasively diagnose and monitor the progression of underlying pathologies at the cellular level. Physics and physiology limit the resolution of human MRI to millimeters, orders of magnitude coarser than cell dimensions. Bridging this resolution gap requires identifying specific cellular-level features that are reflected in the macroscopic MRI measurement. Here we uncover the sensitivity of diffusion MRI to the micrometer-scale axonal caliber variations or swelling, by identifying a specific power-law time-dependence of water diffusion coefficient along white matter axons. The power-law exponent 1/2 is found both numerically, using Monte Carlo simulations in realistic substrates created from 3-dimensional electron microscopy images of mouse brain corpus callosum, and experimentally, in 15 healthy subjects on a clinical scanner. Our statistical analysis confirms the short-range disorder in the swelling placement, which theoretically yields the observed value of the power-law exponent. By modifying our numerical substrates, we also exclude the competing effects of axonal undulations and of the mitochondria on the observed time dependence. Our results offer the exciting ability to probe micrometer-level axonal swelling and beading, a hallmark of pathological changes in diseases such as Alzheimer’s or traumatic brain injury, with a clinically feasible MRI technique with a millimeter-level resolution.

INTRODUCTION

Diffusion MRI (dMRI) is potentially sensitive to the micrometer length scale, and as such, is a promising in vivo technique for evaluating micrometer-scale structural features (the so-called tissue microstructure) of biological tissues. The sensitivity to tissue microstructure, however, is indirect, due to averaging of the local diffusion propagator over the millimeter sized MRI imaging voxel. Biophysical modeling of the diffusion signal in biological tissue is therefore essential for quantification of cellular parameters in order to gain specificity to cellular changes in development, aging and pathology. To robustly retrieve the salient features of cells or tissues across the gap of three orders of magnitude in spatial scales, it is critical to determine the essential assumptions to construct the most parsimonious biophysical models, thereby attaining highest precision without losing accuracy.

Here we show that the dMRI signal’s diffusion time-dependence in the direction along major fiber tracts is sensitive to the axonal swellings, a vital signature of normal axonal microgeometry. The sensitivity to this particular microarchitectural feature is proven by observing a specific power-law time-dependence of the axial diffusivity

\[ D(t) \approx D_\infty + c \cdot t^{-\theta}, \quad \theta = \frac{1}{2} \tag{1} \]

approaching its long-time limit \( D_\infty \). The overall strength \( c \) of the restrictions to axial diffusion, emerging from the randomly-placed axonal swellings, is the volume-weighted sum of individual axon’s corresponding quantities (Materials and Methods, Theory). To verify the power-law (Eq. (1)), we performed for the first time the 3-dimensional (3d) Monte Carlo (MC) simulations in a realistic microgeometry of the mouse brain WM (Figure 1a-b) for clinically relevant diffusion times. To explore the origin of the diffusion time-dependence along axons, we performed simulations in the realistic intra-axonal space (IAS) (1) with or (2) without considering short \( T_2 \) values in mitochondria (Figure 2a), and synthetic fibers with only (3) caliber variation or (4) undulation (Figure 3a). Our universal power-law (Eq. (1)) is robust to axonal orientation dispersion, Fig. 4. The specific numerical and analytical finding (Eq. (1)) is further confirmed by the standard pulse-field-gradient dMRI measurements in 15 normal human subjects on a clinical MRI scanner, Figure 5 and Supplementary Figure S2.

To put our results in context, axons in brain white matter (WM) are conventionally modeled as infinitely narrow featureless impermeable tubes — the so-called “sticks” — inside which diffusion is locally effectively one-dimensional and Gaussian along the axons, completely determined by a constant diffusivity that does not depend on the diffusion time. This simplified viewpoint — a cornerstone ingredient of the so-called WM Standard Model — approximates the net intra-axonal space within an MRI voxel as consisting of a collection of these sticks. In this picture, sticks are deemed non-exchanging with extra-axonal water, and their overall orientation is modeled either by a specific distribution function, such as the Watson distribution, or by using spherical harmonics up to a sufficient order, typically \( l_{\text{max}} = 4 \). Validation of assumptions made in WM diffusion modeling is crucial, yet challenging as the origins of the in vivo dMRI signal cannot be directly accessed non-invasively. While fit quality alone is insufficient to validate a given biophysical model, model selection becomes feasible based on testing their unique functional forms in the domain where the dependence on experimental parameters clearly reveals their assumptions. Borrowing this methodology from the physical sciences, the assumption of negligible exchange between sticks and extra-axonal water has recently been validated in vivo in human brain WM by observing the \( 1/\sqrt{b} \) scaling (ideal...
stick response) at very strong diffusion weighting \( t^{15,17} \). With more targeted measurements, the water exchange between intra- and extra-cellular spaces was suggested to be negligible for clinical TE in the human brain WM, where the exchange time between intra- and extra-cellular spaces was estimated to be 0.6-1.3 sec\(^{15,18}\), much longer than the TE in clinical (50-100 ms) and preclinical (< 150 ms) use.

Another key assumption underlying the WM stick model is that the diffusion along individual WM axons could be approximated as Gaussian, implying no noticeable time-dependence in the diffusion coefficient \( D(t) \) over the diffusion time range used in clinical or preclinical dMRI, and vanishing higher-order diffusion cumulants, such as kurtosis\(^{19}\), for each individual axon. In particular, dMRI measurements with varying diffusion times \( t \) can evaluate this assumption: the absence of time-dependence in \( D \) would signify Gaussian diffusion in every compartment, while the presence of time-dependence would reveal mesoscopic heterogeneity coarse-grained by diffusion in at least one of the compartments\(^{2,4,5,26}\).

To date, most of the WM studies focus only on the diffusion time-dependence perpendicular to axons to probe several microstructural length scales\(^{6,21} \), such as the inner axonal diameter\(^{22,23} \) and the packing correlation length of the extra-axonal space\(^{6,24,25} \). However, the time-dependence of the diffusion tensor eigenvalue parallel to major white matter tracts, recently observed in vivo in human brain WM using stimulated-echo dMRI, is found to be non-negligible, with about 10-15% change over the time range of 50-600 ms, and to follow a characteristic power-law (Eq. (1)).\(^{6} \) This non-trivial time-dependence along axons cannot be explained solely by overall fiber dispersion and/or the resulting contribution projected from the time-dependence perpendicular to axons. Rather, the observed non-Gaussian diffusion along axons suggests that the WM stick model should be augmented to incorporate micrometer scale restrictions \emph{along} the axonal bundle direction\(^{6} \).

To evaluate the effect of realistic axons on diffusion as compared to the employed sticks, ab initio Monte Carlo (MC) simulations of a random walk in substrates mimicking biological tissues are proposed here, that allow to calculate dMRI-related metrics, such as the cumulants of diffusion displacements, e.g., the diffusion coefficient and kurtosis, and subsequently focus on their unique functional forms. So far, numerical simulations for validating dMRI in brain microstructure (for an overview see ref\(^{26} \)) have been performed either in 2d or 3d simple geometries, or in combinations of those. In particular, the axonal shape is thereby typically modeled and simulated by artificial geometries\(^{27} \). Recently, benefiting from the advance in microscopy techniques, diffusion simulations have been performed in 2d realistic microgeometry of neural tissues reconstructed from light microscopy\(^{28} \) or electron microscopy (EM)\(^{29} \) and, furthermore, in 3d realistic microstructure of astrocytes reconstructed from confocal microscopy\(^{30} \). However, the crucial piece of the validation puzzle — simulations in 3d realistic EM-based microstructure of neuronal tissue (e.g., Figure 1a-b) — have been so far missing\(^{26} \). Having full control of the tissue microstructure also allows us to study the diffusion time-dependence along axons with or without axonal orientation dispersion. Our results for the first time elucidate the microstructural origins of non-Gaussian time-dependent diffusion along axons, and agree with human brain data acquired by using spin-echo diffusion pulsed-gradient sequences where the diffusion time is varied in the same range. The specific power law exponent \( \nu = 1/2 \) observed both in simulations and in human dMRI points at a remarkable sensitivity of a macroscopic dMRI measurement to the micrometer-scale axonal microstructure, offering a non-invasive imaging marker of axonal pathology three orders of magnitude below the nominal MRI resolution.

\section*{RESULTS}

\subsection*{Structure analysis of axon caliber variation reveals short-range disorder}

To predict the power-law tail of diffusivity time-dependence based on the axonal micro-geometry, we estimated the auto-correlation function, i.e. power spectrum, of the actual axonal shape, using the radius variation along 227 segmented axons aligned with the \( z \)-axis (original radii in Figure 1c and normalized radii in Figure 1d, in seven selected axons). The power spectrum \( \Gamma_{1d}(k_z) \) along the concatenated axon with normalized radii (cf. Materials and Methods, Power spectrum) approaches a plateau at low \( k_z \), cf. Eq. (3), and indicates a structural exponent \( p = 0 \) (short-range disorder), predicting the dynamical exponent \( \nu = 1/2 \) in dimension \( d = 1 \) (Eq. (4)), and the time-dependence in Eq. (1). The low- \( k_z \) plateau demonstrates that restrictions along axons are randomly distributed with a finite correlation length (short-range disorder). The level of the plateau is determined by the mean \( \bar{a} \) and the variance \( \sigma_a^2 \) of the distance between restrictions (cf. Eq. [S13] and following derivations in the Supplementary Information of\(^{2} \)), as well as by the average restriction width \( \bar{\ell} \) (cf., Eq. (47) in\(^{24} \) with restriction “shape” \( v(k) \mid k \rightarrow 0 \rightarrow \bar{\ell} \):\(^{2} \)

\begin{equation}
\Gamma_{1d}|_{k_z \rightarrow 0, p=0} \approx \frac{\sigma_a^2}{\bar{a}^2} \cdot \frac{\bar{\ell}}{\bar{a}}.
\end{equation}

The normalized power spectrum in Figure 1f has a low- \( k_z \) plateau at \( \Gamma_{1d}(k_z) / \bar{a} \approx 0.36 \), corresponding to an average restriction width \( \bar{\ell} \approx 6.8 \) \( \mu m \), for which \( \bar{a} \approx 5.70 \) \( \mu m \) and \( \sigma_a \approx 2.88 \) \( \mu m \) (Figure 1e) are estimated by locating axon’s local swelling regions.

\subsection*{Monte Carlo simulations validate time-dependent diffusion due to axon caliber variation}

\emph{Mitochondria segmentation}

To evaluate the influence of mitochondria on the diffusion time-dependence, we manually segmented \( \sim 1300 \) mitochondria in 227 axons (Figure 2a). For individual axon, the inner axonal diameter approximately correlates with the mitochondrial volume per unit length via a quadratic function...
FIG. 1: Structural analysis of axons segmented from female mouse brain corpus callosum EM predicts short-range disorder along axons. (a) 3d EM image with segmented IAS of axons passing through the central slice. (b) 3d representation of the IAS segmentation yielding 227 axons that are long enough to pass through all slices (> 20 µm). (c) Radius variation r and (d) normalized radius variation ˜r along 7 selected axons. (e) Histogram of distances 2a between local swellings along all 227 segmented axons. (f) Power spectrum Φ1d(kz) along all axons shows a plateau at low kz, i.e. kzL → 1 (red dashed line), indicating a structural exponent p = 0 and a dynamical exponent θ = 1/2, which corresponds to short-range disorder in 1d (randomly positioned restrictions along axons) and leads to the diffusion time-dependence given by Eq. (1). Figure (a) and (b) are adapted from31 with permission from Springer.

Time-dependent diffusion coefficient

In the microstructure based on realistic IAS in Figure 3a, I-IV, the simulated overall D(t) (from all axons) exhibits a notable time-dependence, which scales as 1/√t (Figure 3b). This is consistent with the prediction of Eq. (1), corresponding to the dynamical exponent θ = 1/2 and the structural exponent p = 0, confirming our expectations that the restrictions to diffusion along axons are due to short-range disorder. The bulk diffusivity D∞ and strength of restrictions c of all axons in Eq. (1) and Eq. (8) (Table Ia) were estimated by using individual axon’s volume fraction f_i and parameters (D_i,∞, c_i) obtained by fitting individual axon’s D(t) to Eq. (7).

The simulated D(t) with or without considering the low T2 value in mitochondria (I and II) shows very small differences in diffusivity values and time-dependence (Figure 3b, Table Ia). Furthermore, compared with structure I, D(t) of axially symmetric cylinders with only caliber variation (III) has slightly larger diffusivity values and very similar time-dependence, and yet D(t) of undulating fibers with no caliber variation (IV) has much larger diffusivity values and smaller time-dependence.

For the micro-geometry I in Figure 3a, the radius variation along individual axon, i.e. coefficient of variation of radii CV(r), highly correlates with the relative diffusivity variation, i.e. ζ ≡ (D_0 - D_∞)/D_∞ with the intrinsic diffusivity D_0 = 2 µm^2/ms, via a quadratic function (R = 0.9147 in Figure 3d), a simple relation given by Eq. (14) in Supplementary Material.

In addition to the axonal shape, D(t) also depends on the fiber dispersion, modeled as an axially symmetric Watson distribution in simulations. For dispersion angles θ = 0°-45°, the simulated D(t) scales as 1/√t and decreases with the dispersion angle (Figure 4a), as manifested by the corresponding fit parameters D_∞ and c ∝ 〈cos^2 θ〉 (Eq. (9b) and Figure 4c-d). In particular, the estimate of c slightly deviates from this relation (Figure 4d), especially for high dispersion, due to an extra 1/t term contributed by the diffusion transverse to individual axons. Accounting for this small effect by using Eq. (10), the corrected value of c restores the relation.
than the axonal undulation, and low realistic axons largely depends on the caliber variation, rather than the axially symmetric cylinder with the same caliber variation. Furthermore, compared with micro-geometry I, the undulating fiber with no caliber variation (IV) shows much smaller kurtosis values and a totally different kurtosis time-dependence. These results indicate that the kurtosis time-dependence along the IAS (III) shows slightly smaller kurtosis values and similar kurtosis time-dependence. However, the undulating fiber with no caliber variation (IV) shows much smaller kurtosis values and a totally different kurtosis time-dependence. These results indicate that the kurtosis time-dependence along realistic axons largely depends on the caliber variation, rather than the axonal undulation, and low $T_2$ values in mitochondria generally has a small effect.

For a fiber bundle with the orientation dispersion, the simulated overall $K(t)$ increases with the dispersion angle (Figure 4b), especially for $\theta \gtrsim 30^\circ$.

In micro-geometry I without considering dispersion (blue data points in Figure 3c and 4b), the simulated overall $K(t)$ ($\sim 0.4$ at $t = 20-100$ ms) consists of two parts: (1) Inter-compartmental contribution originates from the diffusivity differences between multiple axons (first RHS term in Eq. (12b)), accounting for 23% to 36% of $K(t)$ at $t = 20-100$ ms; and (2) intra-compartmental contribution originates from individual axon’s axial kurtosis (second RHS term in Eq. (12b)), accounting for 77% to 64% of $K(t)$ at $t = 20-100$ ms.

**In vivo MRI observation of power law tail in the diffusion time-dependence due to axon caliber variation**

The time-dependent axial diffusivity $D(t)$, measured by mono-polar PGSE in the human brain WM (Figure 5a-b), were averaged over 5 healthy subjects and plotted with respect to $1/\sqrt{t}$. In all WM ROIs, the axial diffusivity time-dependence demonstrates a $1/\sqrt{t}$ power-law relation in Eq. (1) (P-value $< 0.05$, Table II), indicating that the univer-

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**TABLE I: (a) Fit parameters of the time-dependent axial diffusivity $D(t)$ in our simulations in four micro-geometries (Figure 3a-b), including 3d realistic IAS reconstructed from EM of mouse brain genu of CC with (I) $T_2a$ relaxation time $T_{2a} = 80$ ms in cytoplasm and $T_{2m} = 20$ ms in mitochondria, and (II) $T_{2a} = T_{2m} = 80$ ms in IAS, and two kinds of synthetic fibers with different salient features, such as (III) the axially symmetric cylinder with the caliber variation, and (IV) the undulating fiber without caliber variation. All fibers are aligned to the z-axis, and no dispersion is considered. (b) Fit parameters of the time-dependent axial diffusivity $D(t)$ in our simulations in 3d realistic IAS reconstructed from EM of mouse brain genu of CC for different dispersion angles (Figure 4a). Simulation results for dispersion angles $\theta = 15^\circ$.30$^\circ$ are consistent with the human brain PGSE data in genu, cf. Table II.**

| Dispersion angle $\theta$ ($^\circ$) | $D_{\infty}$ ($\mu$m$^2$/ms) | $c$ ($\mu$m$^2$-ms$^{-1/2}$) |
|-----------------------------------|-----------------|-----------------|
| 0                                 | 1.35            | 0.503           |
| 15                                | 1.26            | 0.473           |
| 30                                | 1.00            | 0.392           |
| 45                                | 0.67            | 0.287           |

(a) Fit parameters in Figure 3b

(b) Fit parameters in Figure 4a

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**FIG. 2: Mitochondria segmentation and characterization of the relation between mitochondria and axonal diameter: (a) ~1300 mitochondria (red) in the 227 axons (gray) of Figure 1a-b were manually segmented. (b) The inner axonal diameter $r$ approximately correlates with each axon’s mitochondrial volume per unit length ($V_{mito}/L$) via a quadratic function. (c) Axonal diameters in cross-sections where mitochondria are present (red) are significantly larger, compared to cross-sections where mitochondria are absent (blue) (P-value < 0.001, one-sided Wilcoxon sum-rank test).**

The finite value of time-dependence amplitude $c$ corresponds to about 4.4% diffusivity change over $t = 20-100$ ms time range. In particular, the diffusivity change $\Delta(1/\sqrt{t}) \sim \Delta t \cdot t^{-3/2}$ is even larger at short diffusion times. Including time-dependence for the intra-axonal compartment is therefore especially important for animal imaging and for human dMRI at relatively short diffusion times, achievable on high-gradient systems.

**Time-dependent Kurtosis**

In realistic IAS (Figure 3a, I and II), the simulated overall $K(t)$ slightly decreases due to the short $T_2$ values in mitochondria (Figure 3c), with almost the same kurtosis time-dependence. Furthermore, compared with micro-geometry I, the axially symmetric cylinder with the same caliber variation as the IAS (III) shows slightly smaller kurtosis values and similar kurtosis time-dependence. However, the undulating fiber with no caliber variation (IV) shows much smaller kurtosis values and a totally different kurtosis time-dependence. These results indicate that the kurtosis time-dependence along realistic axons largely depends on the caliber variation, rather than the axonal undulation, and low $T_2$ values in mitochondria...
FIG. 3: Exploring the origin of the diffusion time-dependence along axons using Monte Carlo simulations in the IAS of 227 axons segmented from EM (Figure 3a, I): (a) Four kinds of micro-geometries are created for each axon as follows: I. Original IAS from EM with $T_2$ relaxation time $T_{2a} = 80$ ms in cytoplasm (gray) and $T_{2m} = 20$ ms in mitochondria (red). II. Simulated IAS with mitochondria removed ($T_{2a} = T_{2m} = 80$ ms). III. Simulated axially symmetric IAS with the same caliber variation as the original IAS, but no undulation. IV. Simulated IAS that includes the undulation and volume from the original IAS, but no caliber variation. (b) (c) For all 227 axons, the simulated overall $D(t)$ for scenarios (I-IV) is plotted as $1/\sqrt{t}$, showing a linear scaling, which points to short-range disorder in $1d$ along axons. Dashed lines are predictions of $D(t)$ time-dependence based on Eq. (1) and Eq. (8), with fit parameters $D_\infty$ and $c$ shown in Table 1a. Interestingly, the time-dependence of $D(t)$ is most strongly influenced by caliber variation, as it becomes much weaker in scenario IV with only undulation, and no caliber variation. (c) The simulated overall $K(t)$ for scenarios (I-IV) is plotted as $1/\sqrt{t}$, showing a non-monotonic change with $t$. While removing undulation (scenario III) slightly lowers the kurtosis, it is mainly when axon caliber variation is removed (scenario IV), that the kurtosis drops to close to 0 and time-dependence alters. Both (b) and (c) indicate that axon caliber variation is causing the along axon time-dependence as observed in vivo in brain white matter (Figure 5). (d) In the original IAS, scenario I, the radius variation along each individual axon, $CV^2(r)$, and corresponding relative diffusivity variation, $\zeta \equiv (D_0 - D_{i,\infty})/D_{i,\infty}$, highly correlate with each other, and obey a simple quadratic relation in Eq. (14), demonstrated by plotting $\zeta$ versus $CV^2(r)$, consistent with the theory in Supplementary Material.

Furthermore, in WM ROIs, the axial kurtosis from the same in vivo measurements (Figure 5c-d) is $\sim 0.8$ and varies over diffusion time in some ROIs, demonstrating non-Gaussian diffusion along axons.

Time-dependent parameters in CC of each subject

Across the 9 sub-regions of CC (G1/G2/G3 for genu, B1/B2/B3 for midbody, and S1/S2/S3 for splenium in Figure 6a), time-dependent parameters estimated based on in vivo brain data measured by PGSE show similar patterns between each subject. The bulk diffusivity $D_\infty$ in $t \to \infty$ limit has a high-low-high pattern in genu-midbody-splenium in all subjects (Figure 6b), whereas the strength $c$ of restrictions along axons has a low-high-low pattern in most of the subjects (Figure 6c).
FIG. 4: Exploring the effect of dispersion on the diffusion time-dependence along axons using Monte Carlo simulations in the IAS of 227 axons segmented from EM (Figure 3a-I), whereby each axon’s main direction is chosen such that the overall orientation distribution is characterized by a Watson distribution with concentration parameters \( \kappa = [\text{Inf}, 15.4, 4.7, 1.65] \), corresponding to dispersion polar angles \( \theta = [0^\circ, 15^\circ, 30^\circ, 45^\circ] \). (a) The simulated \( D(t) \) scales as \( 1/\sqrt{t} \) and decreases with the dispersion angle. The dashed lines are predictions based on Eq. (1) and Eq. (8), with parameters \( D_\infty \) and \( c \) shown in Table I b. (b) The simulated \( K(t) \) increases with the dispersion angle. (c) The bulk diffusivity at \( t \to \infty \) limit, \( D_\infty \), is \( \propto \langle \cos^2 \theta \rangle \), Eq. (9b). (d) The strength of restrictions, \( c \), slightly deviates from this proportionality relation in Eq. (9b) (blue) due to an extra \( 1/t \) term contributed by the diffusion transverse to individual axon, especially for the high dispersion case (large \( \theta \), small \( \langle \cos^2 \theta \rangle \)). Accounting for the \( 1/t \) term in Eq. (10), the corrected value of \( c \) restores the simple relation (red).

DISCUSSION

By performing MC simulations inside axons from 3D EM images of the brain, we demonstrated that the diffusion along axons is not Gaussian, with the time-dependent diffusivity obeying a specific power-law tail. Our simulation results are consistent with in vivo human brain data measured by using time-dependent dMRI. Further, we evaluated how theory matches simulations and experiment. Below we discuss the agreement between simulation, measurement and theory, and address limitations in our simulation pipeline.

MC simulations inside axons reveal a specific power-law tail as hallmark of short-range disorder

Our simulation results (Figures 3 and 4) are consistent with in vivo measurements and the corresponding theoretical predictions: diffusion along axons is characterized by short-range disorder in \( 1d \) and the dynamical exponent \( \theta = 1/2 \) for Eq. (1), which are also demonstrated by the power spectrum analysis (Figure 1f) based on the actual axonal shape. Since the time-dependence of diffusivity along axons is proportional to \( (1/\sqrt{t}) \sim \Delta t \cdot r^{-3/2} \), it is negligible only when the time range \( \Delta t \) is small (e.g., \( \Delta t < 5 \) ms), or the diffusion time is long (e.g., \( t > 200 \) ms).

Based on simulation results in four types of micro-geometries in Figure 3a-I-IV, the origin of diffusivity and kurtosis time-dependence along axons is due to the caliber variation, rather than the axonal undulation, as demonstrated in Figure 3b-c: The low \( T_2 \) values in mitochondria has a very small effect on the simulated diffusivity and kurtosis (cf. I and II). Further, the axially symmetric cylinder with caliber variation (III) has the diffusivity and kurtosis time-dependence similar to the realistic IAS (I and II). In contrast, the undulating fiber with no caliber variation (IV) shows not only the smallest diffusivity time-dependence (i.e. smallest \( c \) in Table 1a) but also a different kurtosis time-dependence from others cases (I-III).

For diffusion solely within IAS, the specific power-law relation of diffusivity time-dependence along axons is very robust even for highly dispersed fiber bundles (Figure 4a, \( \theta = 45^\circ \)) since, in IAS, the diffusion displacement perpendicular to axons is relatively small and negligible (cf. inner axonal diameter ~1 \( \mu m \), compared with diffusion along axons (cf. axon segment length > 18 \( \mu m \)). However, in realistic brain tissues, the diffusivity time-dependence perpendicular to axons is dominated by extra-axonal space, where the diffusion displacement perpendicular to axons could be comparable to the diffusion along fiber bundles. Therefore, this spe-
FIG. 5: (a-b) Time-dependent axial diffusivity $D(t)$ measured in vivo in brain WM of 5 control subjects using monopolar PGSE. In all WM ROIs, the experimental axial diffusivity scales as $1/\sqrt{t}$ (P-value < 0.05, Table II), manifesting that the universality class along WM axons is short-range disorder in 1d, corresponding to a power-law tail with $\vartheta = 1/2$\textsuperscript{5}. The fit parameters are summarized in Table II. (c-d) Time-dependent axial kurtosis $K(t)$ measured in vivo in brain WM of 5 control subjects using monopolar PGSE. The in vivo measured $K(t)$ is not zero, signifying the non-Gaussian diffusion along WM axons in the human brain. The error bar indicates the standard error of 5 subjects. (ACR/SCR/PCR = anterior/superior/posterior corona radiate, ALIC/PLIC = anterior/posterior limb of the internal capsule, genu/midbody/splenium of CC)

cific time-dependence along axons (short-range disorder in 1d) would be less robust for highly dispersed fibers if diffusion simulations were performed not just in IAS but also in extra-axonal space.

**In vivo human brain data agree with simulation results**

The diffusivity time-dependence of in vivo human brain data measured by PGSE in Figure 5a-b is consistent with the simple power-law in Eq. (1) in all ROIs. Particularly, in genu, the fit parameters of PGSE measurements (Table II) are in the same scale of those in MC simulations of dispersion angles $\theta = 15^\circ$-$30^\circ$ (Table Ib), consistent with the fiber dispersion $\approx 20^\circ$ observed in histology\textsuperscript{31,36}. Furthermore, the value of axial kurtosis in PGSE measurements in Figure 5c-d is non-negligible in WM ROIs.

Compared with our simulation results and human brain PGSE measurements, in vivo human brain data measured by STEAM shows a stronger time-dependence, manifested by the larger value of $c$ (strength of restrictions) in Table 2 of\textsuperscript{6} than that in Tables Ib and II of this study. Similarly, another ex vivo study of spinal cord WM measured by STEAM also shows a much stronger time-dependence along axons, i.e. larger $c$ in Table 1 of\textsuperscript{16}, than the one observed in this study. This overestimated diffusion time-dependence is potentially caused by the water exchange between intra-/extra-axonal water (fast diffusion, long $T_1$, $T_2$ values) and myelin water (slow diffusion, short $T_1$, $T_2$ values) during the mixing time of STEAM sequence\textsuperscript{37}.

In addition to the WM, the mean diffusivity time-dependence of $1/\sqrt{t}$ was also observed in the brain gray matter\textsuperscript{5,38,39}, suggesting that the characteristics of short-range disorder due to neurite swellings or beadings and dendritic spines are universal in neuronal tissues.
Impact on biophysical modeling of brain white matter

Our simulations indicate that the intra-axonal axial kurtosis is non-negligible at clinical diffusion times. In contrast, the standard WM model\textsuperscript{1} assumes Gaussian diffusion along the sticks, corresponding to a negligible $K$ along axons.

In the clinically relevant time range $t \approx 20-100$ ms, the intra-axonal kurtosis along axons is $\sim 0.7$ for $\theta = 30^\circ$ based on simulations (Figure 4b), and the axial kurtosis measured by monopolar PGSE is $\sim 0.8$ in the human brain WM (including intra- and extra-axonal spaces) (Figure 5c-d). The measured kurtosis in experiments is slightly larger than the intra-axonal kurtosis in simulations, probably due to the lack of simulations in extra-axonal space.

Besides the general value of axial kurtosis, the observed time-dependence is also non-trivial. Based on our simulations in IAS, axial kurtosis have 7% changes over the clinical time range $t = 20-100$ ms.

In simulations of varying the fiber dispersion, the intra-axonal $K(t)$ increases with the dispersion angle, especially for $\theta \gtrsim 30^\circ$ (Figure 4b). This is because that, for highly dispersed fibers, individual axon’s diffusivity projected to fiber bundle’s main direction varies in a wide range, leading to a large contribution to the overall $K(t)$, i.e. the first right-hand-side term in Eq. (12b). In other words, the higher order cumulant of intra-axonal signal is very sensitive to the fiber dispersion (e.g., type and degree of orientation distribution), which should always be considered in biophysical modeling of WM.

Neurite caliber variation: clinical significance

Based on numerical simulations in realistic axonal shape (Figure 3), the diffusivity time-dependence along axons is much more sensitive to the caliber variation than the axonal undulation and low $T_2$ values in mitochondria.

Axonal varicosities, or axonal beading along axons, can be a pathological change caused by accumulation of transported materials in axonal swellings after traumatic brain injury (TBI)\textsuperscript{41,42}; it has been observed that varicosities arise during dynamic stretch injury, caused by microtubule breakdown and partial transport interruption along axons. Furthermore, varicosities due to ischemic injury to WM axons can be caused by Na\textsuperscript{+} loading of the axoplasm, which leads to a lethal Ca\textsuperscript{2+} overload through reversed Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange\textsuperscript{43}. Hence, the average distance between varicosities is potentially a biomarker for axonal injury in TBI and ischemia, facilitating evaluation of the effectiveness of treatment and rehabilita-

Time-dependent parameters in CC have similar patterns between each subject

Time-dependent parameters estimated based on in vivo brain data measured by PGSE show similar patterns in CC between each subject. The bulk diffusivity $D_\infty$ in $t \to \infty$ limit shows a high-low-high pattern in all subjects (Figure 6b), consistent with the pattern of axonal density in CC observed in histology\textsuperscript{40}. This is because that the intra-axonal axial diffusivity is larger than the extra-axonal axial diffusivity\textsuperscript{13,14}. On the other hand, the strength $c$ of restrictions along axons shows a low-high-low pattern in most of the subjects (Figure 6c), indicating that the midbody has the largest caliber variation or the width of swelling, i.e. $c \propto \Gamma_{1a}(k_z)^5$ in Eq. (2).

The time-dependent tissue parameters have similar patterns between subjects, especially in CC composed of highly aligned axons, demonstrating the potential of clinical applications in the future, cf. Discussion, Neurite caliber variation: clinical significance.
tion services. Since the average distance between varicosities along axons is of a scale of ten micron\textsuperscript{41–43}, much smaller than the resolution of most of the clinical imaging techniques, dMRI is the method of choice to in vivo estimate the pathological change of TBI\textsuperscript{44,45} and of ischemic stroke\textsuperscript{46}. In particular, by measuring time-dependent DTI, it may become possible to evaluate the correlation length of varicosities along axons\textsuperscript{4}, related with the average distance between varicosities, a potential biomarker for monitoring TBI and ischemic stroke patients.

Besides the axonal beading in WM, the ubiquitous $1/\sqrt{t}$ time-dependence along neurites in gray matter suggests possible applications in other neurodegenerative diseases. For instance, reduced axonal varicosity was observed in the human superior frontal cortex of mild Alzheimer disease\textsuperscript{47}; decreased dendritic spine density was observed in the human prefrontal cortex of Schizophrenia\textsuperscript{48}; and the increase of axonal varicosity was observed in injured dopaminergic neurons in the rat substantia nigra, an animal model of Parkinson’s disease\textsuperscript{49}. The ability to evaluate restriction changes along neurites opens a door to monitoring the progression and therapy response of these diseases.

Outlook

In simulations of realistic IAS (Figures 3 and 4), the maximal diffusion time is limited by the length of segmented IAS along the $z$-axis. In this study, the axon length $L_{ax} \sim 18 \mu m$ corresponds to a length-related correlation time $\tau_{ax} = L_{ax}^2/(2D_0) = 81 ms$ for $D_0 = 2 \mu m^2/ms$. When applying a diffusion time $t$ longer than $\tau_{ax}$, the simulated diffusion metrics could be sensitive to the periodicity of axonal length, resulting in an unexpected time-dependence for $t > \tau_{ax}$. To solve this problem, segmenting a longer IAS in a larger tissue sample enables diffusion simulations at longer diffusion times.

In this study, we only focus on 3d reconstructions of the intra-axonal geometry of myelinated axons in WM. However, other structures, such as unmyelinated axon, extraxonal space, glia cell and blood vessel, also have non-trivial contributions to the diffusion time-dependence and need to be considered when building the numerical micro-geometry.

Future work will focus on diffusion simulations in the tissue microstructure of a large sample size\textsuperscript{50}, as well as including other cell types and the extra-axonal space. The simulation pipeline is applicable not only to the WM but also to the gray matter, facilitating the biophysical modeling of tissue pathological changes in neurodegenerative diseases.

CONCLUSIONS

By fully controlling the micro-geometry of numerical phantoms, MC simulations provide the flexibility to evaluate the influence of the axonal microstructure on diffusion time-dependence along axons. Out of many possible causes tested with numerical simulations, the diffusion time-dependence along axons generally originates from the caliber variation along axons, and yet the low $T_2$ values in mitochondria and axonal undulations have relatively small effects. Based on the power-spectrum analysis and simulation results of the time-dependent diffusion within realistic IAS segmented from the mouse brain WM, we demonstrated that the positions of restrictions (e.g., swelling) along WM axons are random, characterized by the short-range disorder in $1d$ and a finite correlation length. Our results are consistent with the in vivo human brain data measured by PGSE. And the estimated tissue parameters in CC have similar patterns between each subject.

This study provides biophysical metrics of restriction changes along neurites evaluated via dMRI, revealing the potential of non-invasive imaging markers for monitoring ischemia and neurodegenerative diseases.

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MATERIALS AND METHODS

All procedures performed in studies involving animals were in accordance with the ethical standards of New York University School of Medicine. All mice were treated in strict accordance with guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental procedures were performed in accordance with the Institutional Animal Care and Use Committee at the New York University School of Medicine. All procedures performed in studies involving human participants were in accordance with the ethical standards of New York University School of Medicine. All protocols were approved by the local institutional review board (New York University School of Medicine). Informed consent was obtained from all individual participants included in the study.

Electron microscopy and intra-axonal space (IAS) segmentation

The brain tissue from a female 8-week-old C57BL/6 mouse’s genu of corpus callosum (CC) was processed and analyzed with a scanning electron microscope (SEM) (Zeiss Gemini 300 SEM with 3View). Part of the data was discarded due to unstable quality, leading to a volume (Figure 1a) of $36 \times 48 \times 20 \ \mu m^3$. To segment long axons passing through all slices, we employed a simplified seeded region growing algorithm$^{31,51}$. The segmented axons (Figure 1b) shorter than $20 \ \mu m$ were discarded, leading to 227 long axons ($\geq 20 \ \mu m$ in length). More details were reported in our previous work$^{31}$.

The intra-axonal space (IAS) segmentation was down-sampled into a voxel size of $(0.1 \ \mu m)^3$. The effect of orientation dispersion was controlled by subsequently realigning axons along the $z$-axis (Figure 3a). The aligned axons were truncated at both ends by $1 \ \mu m$ to avoid oblique end faces, resulting in axons of about $18 \ \mu m$ in length.

To further evaluate the influence of mitochondria on the diffusion time-dependence, we manually segmented $\sim 1300$ mitochondria in the 227 axons (Figure 2a), and reported statistical results in mitochondrial morphometry (Supplementary Material).

Power spectrum

The power-law tail of the diffusion time-dependence is determined by the structural universality class of the tissue microstructure$^5$. To determine the structural universality class of the micro-geometry along axons, we calculated the $3d$ density-density correlation function

$$\Gamma_{3d}(r) = \frac{1}{V} \langle \rho(r_0 + r) \rho(r_0) \rangle,$$

where $\rho(r)$ is the $3d$ binary mask of an axially symmetric cylinder with normalized radius variations along axons, and $V$ is the total volume of $\rho(r)$; the average $\langle \ldots \rangle$ is performed over the point $r_0$.

First, each axon’s radius variation was normalized by the mean and the standard deviation of radii of all axon segments (standard score). Next, the normalized radius variations are randomly concatenated along the $z$-axis. Finally, the concatenated normalized radius variation was rotated around the $z$-axis to generate an axially symmetric $3d$ binary mask $\rho(r)$. The $1d$ power spectrum $\Gamma_{1d}(k_z)$ along the $z$-axis is further calculated by the $1d$ Fourier transformation of $\Gamma_{3d}(r)$ along the $z$-axis and summations over $x$- and $y$-axis:

$$\Gamma_{1d}(k_z) = \int \Gamma_{3d}(r) e^{-i k_z z} \, dz.$$

The $1d$ power spectrum is in the units of length, and is further normalized (divided) by the average distance $\bar{r}$ of restrictions along axons. The restrictions in general can be provided by any kind of microstructural inhomogeneity, and here are interpreted as coming from the local swellings or local narrowing regions along axons. The power spectrum can be always represented by a power-law at low $k_z$:

$$\Gamma_{1d}(k_z)_{k_z \to 0} \sim (k_z)^p,$$

with a structural exponent $p$ determining the universality class of the micro-geometry$^5$, which has been related to the dynamical exponent of diffusion in $d$ spatial dimensions:

$$\vartheta \equiv \frac{p + d}{2}.$$

For the short-range disorder introduced in Materials and Methods, Theory, the corresponding exponent $p = 0$ yielding $\vartheta = 1/2$ in 1-dimension ($d = 1$)$^5$, predicting the diffusion time-dependence in Eq. (1) and Eq. (7), that we are going to test with simulations.

Numerical simulation in realistic microstructure

Numerical simulations have been widely used to validate dMRI in brain microstructure, either in simple geometries$^{20,21,23,24,52–58}$ or in their combinations$^{59–61}$. In particular, the axonal shape is modeled by artificial geometries$^{27,62–66}$, or based on realistic shapes obtained from microscopy images$^{28–30}$. Here, we validate the diffusion time-dependence along axons via Monte-Carlo simulations in realistic axonal shape from a mouse brain SEM data.

To explore the origin of the diffusion time-dependence along axons, we designed four kinds of micro-geometries for each IAS: (1) The realistic IAS with $T_2$ relaxation time $T_{2a} = 80 \ \text{ms}$ in cytoplasm$^{14}$ and $T_{2m} = 20 \ \text{ms}$ in mitochondria$^7$ is closest to the reality and serves as the main result (Figure 3a, I). (2) The realistic IAS with $T_{2a} = T_{2m} = 80 \ \text{ms}$ has no $T_2$ value differences within axon (Figure 3a, II). For the first two cases, the mitochondrial membrane is assumed to be highly permeable based on the previous study$^7$, ideally with an infinitely large permeability in simulations, i.e. permeation probability = 1. To further decompose the complicated axonal shape, we designed two types of synthetic fibers with different salient features: (3) The axially symmetric cylinder with caliber variation has circular cross-sections and the same variation of cross-sectional area as the realistic IAS (Figure 3a,
III). (4) The undulating fiber with no caliber variation has the same volume and undulation (axonal skeleton) as the realistic IAS (Figure 3a, IV). The axonal skeleton was constructed by connecting the center of mass of each cross-section, and smoothed along the axon by a Gaussian filter of a standard deviation \( \sigma = 1 \, \mu m \). All fibers are aligned to the \( z \)-axis, and the axonal orientation dispersion is not considered when comparing the above four cases.

Further, to evaluate the effect of fiber orientation dispersion, segmented axons in Figure 3a, I \((T_{2a} = 80 \, ms, T_{2m} = 20 \, ms)\) were oriented based on a Watson distribution with concentration parameters \( \kappa = [\inf, 15.4, 4.7, 1.65] \) for the cases of no dispersion up to high dispersion, corresponding to the overall polar dispersion angles \( \theta = [0^\circ, 15^\circ, 30^\circ, 45^\circ] \), defined by \( \theta \equiv \cos^{-1} \left( \frac{\cos^2 \theta}{2} \right)^{13,31} \).

MC simulations of random walkers were implemented in CUDA C++ for diffusion in a continuous space. \( 2.27 \times 10^9 \) walkers in total were employed inside 3d segmentations of 227 IASs, with \( 1 \times 10^7 \) walkers per IAS. Each particle diffused over \( 5 \times 10^3 \) steps with a duration \( \delta t = 2 \times 10^{-4} \, ms \) and a length \( \sqrt{6D_0 \delta t} = 0.049 \, \mu m \) for each step, where the intrinsic diffusivity in IAS, \( D_0 = 2 \, \mu m^2/\,ms \), is taken to agree with recent experiments\(^{13,15}\). Maximal diffusion time in simulations is \( t = 100 \, ms \). Total calculation time was \( \sim 1.5 \) days on \( \sim 20 \) Nvidia Tesla V100 GPU on the NYU Langone Health, Bigpurple high-performance-computing cluster.

The \( i \)-th axon’s moment tensors \( \langle x_{ij} x_{ik} \rangle_i \) and \( \langle x_{ij} x_{jk} x_{ik} x_{ik} \rangle_i \) are calculated based on the simulated diffusion displacement \( x_{ij} \) (\( i, j = 1, 2, 3 \)), and their projections yield the axon’s apparent diffusivity \( D_i(t, \hat{n}) \) and apparent kurtosis \( K_i(t, \hat{n}) \) in the direction \( \hat{n} \)\(^{16,68}\):

\[
D_i(t, \hat{n}) = \frac{\langle s^2 \rangle_i}{2t}, \quad (5a)
\]

\[
K_i(t, \hat{n}) = \frac{\langle s^4 \rangle_i}{\langle s^2 \rangle_i^2} - 3, \quad (5b)
\]

where

\[
\langle s^2 \rangle_i = n_{ij} n_{ik} \langle x_{ij} x_{ik} \rangle_i,
\]

\[
\langle s^4 \rangle_i = n_{ij} n_{ik} n_{jk} n_{ik} \langle x_{ij} x_{jk} x_{ik} x_{ik} \rangle_i.
\]

For an axon oriented into the direction \( \hat{n}’ \), the axon’s axial diffusivity and axial kurtosis defined along the \( z \)-axis direction \( \hat{z} \) were calculated based on the estimated moment tensors, yielding \( D_i(t, \hat{n}) \) and \( K_i(t, \hat{n}) \), where \( \hat{n} = 2(\hat{n}’ - \hat{z}) \hat{z} - \hat{n}’ \).

In vivo MRI

dMRI measurement were performed on five healthy subjects (4 males/1 female, 21-32 years old) using a monopolar PGSE sequence provided by the vendor (Siemens WIP 919B) on a 3T Siemens Prisma scanner (Erlangen Germany) with a 64-channel head coil. For each subject, we varied the diffusion time \( t = [22, 28, 34, 40, 50, 60, 70, 80, 90, 100] \, ms \) and fixed the diffusion gradient pulse width \( \delta = 15 \, ms \). For each scan, we obtained three \( b = 0 \) non-diffusion weighted images and 64 diffusion weighted images (DWIs) of \( b \)-values \( b = [0, 1, 1.5] \, ms/\,\mu m^2 \) along \( [12, 20, 30] \) gradient directions for each \( b \)-shell, with an isotropic resolution of \( (3 \, mm)^3 \) and a field-of-view of \( 210 \times 204 \, mm^2 \). The whole brain volume was scanned within 30 slices, aligned parallel to the anterior commissure-posterior commissure line. GRAPPA with acceleration factor \( = 2 \) and multiband with acceleration factor \( = 2 \) were used. All scans were performed with the same TR/TE = 4000/139 ms. Total acquisition time is \( \sim 60 \, min \) for each subject. In the main text, we will focus on this dataset. The data of 10 additional subjects scanned with a smaller voxel size, exhibiting similar outcomes, are shown in \textit{Supplementary Material}.

Image processing

Our image processing DESIGNER pipeline is based on\(^{89}\) and includes five steps: denoising\(^{70,71}\), Gibbs ringing elimination\(^{72}\), eddy-current and motion correction\(^{73}\), Rician noise correction\(^{74}\), and diffusion tensor estimation\(^{75}\).

For each voxel, we fitted dMRI data to the diffusion and kurtosis tensor using weighted linear least square (WLLS)\(^{75}\), and calculated eigenvalues of the diffusion tensor (in the order of \( \lambda_1 \geq \lambda_2 \geq \lambda_3 \)) and the fractional anisotropy (FA) accordingly\(^{76}\). Experimental axial diffusivity is defined by \( D \equiv \lambda_1 \), and experimental axial kurtosis is defined by the apparent kurtosis along the principal axis of the diffusion tensor.

Regions of interest (ROI)

Each subject’s mean FA map, averaged over all diffusion time points, was registered to FSL’s standard FA map with FMRIB’s linear and non-linear registration tools (FLIRT, FNIRT)\(^{77,79}\). We retrieved the transformation matrix (FLIRT) and the warp (FNIRT) to inversely transform John’s Hopkins University (JHU) DTI-based WM atlas ROIs\(^{80}\) to the individual space. Cerebrospinal fluid (CSF) mask was segmented by FSL, FAST\(^{81}\) and expanded by 1 voxel to exclude WM voxels close to CSF. We focused on main WM tracts, such as anterior corona radiata (ACR), posterior corona radiata (PCR), superior corona radiata (SCR), anterior and posterior limb of the
internal capsule (ALIC, PLIC), genu, midbody, and splenium of the corpus callosum.

To further discuss the variation of tissue properties in CC, we divided CC ROIs defined in JHU DTI atlas into 9 sub-regions in total (Figure 6a), such as G1, G2, G3 for genu, B1, B2, B3 for midbody, and S1, S2, S3 for splenium. The 9 sub-regions are then co-registered and transformed to individual subject’s space by using FSL.

Theory

The time-dependent diffusion along WM tracts probes the geometry and the length scale of restrictions along axons. The assumption of randomly-positioned restrictions along an individual axon (short-range disorder in 1d) yields the following functional form for the axial diffusivity in a given axon

$$D_i(t) \simeq D_{i,\infty} + c_i \cdot \frac{1}{\sqrt{t}},$$

with dynamical exponent $\vartheta = 1/2$ signifying the diffusion time-dependence: the power-law tails $D_i(t) - D_{i,\infty} \sim t^{-\vartheta}$. Here, $D_{i,\infty}$ is the bulk diffusivity at $t \to \infty$, and $c_i$ is the strength of restrictions for a given axon.

Substituting Eq. (7) into Eq. (12a), we obtained the axial diffusivity of all axons in Eq. (1), where the overall bulk intra-axonal diffusivity $D_{\infty}$ (i.e., extrapolated to an infinitely long time) and overall strength of restriction $c$ are the volume-weighted sum of individual axon’s corresponding quantities, $D_{i,\infty}$ at $t \to \infty$ and $c_i$:

$$D_{\infty} \equiv \langle D_{i,\infty} \rangle, \quad c \equiv \langle c_i \rangle.$$  

In what follows, we use the time interval $t = 20$-80ms to fit $D_{i,\infty}$ and $c_i$ from MC simulations of individual axon, i.e. fitting $D_i(t)$ to Eq. (7), and employ these parameters to predict the axial diffusivity $D(t)$ of all axons in Eq. (1) and Eq. (8). The maximal diffusion time used for fitting is bounded by the axonal length of the EM substrate $L_{ax} \sim 18 \mu$m: $L_{ax}^2/(2D_0) \approx 80$ ms for $D_0 = 2 \mu$m$^2$/ms.

In particular, for the microstructure I in Figure 3a with no dispersion, we correlated the relative diffusivity variation $\zeta \equiv (D_0 - D_{i,\infty})/D_{i,\infty} \simeq \delta D_i/D_{i,\infty}$ with the axonal shape, which can be parameterized into the radius variation along each axon (coefficient of variation of axonal radius, $CV(r)$). In general, we empirically observed that the axons with a more inhomogeneous shape, e.g., with higher caliber variations, usually have larger values of $CV(r)$ and of the corresponding $\zeta$.

Considering a fiber bundle with the orientation dispersion, the diffusion displacement within an axon (dispersed into $\theta_i$) is generally along the axon due to the thin size. Its projection to the fiber bundle’s main direction leads to a contribution to the second order cumulant $\langle s^2 \rangle_{i} \propto \cos^2 \theta_i$ along the fiber bundle. As a result, the overall diffusivity and corresponding parameters are given by

$$\frac{D(t)}{D(\theta=0)} \simeq \langle \cos^2 \theta \rangle, \quad (9a)$$

$$\frac{D_{\infty}}{D_{\infty,\theta=0}} \simeq \langle \cos^2 \theta \rangle, \quad \frac{c}{c_{\theta=0}} \simeq \langle \cos^2 \theta \rangle. \quad (9b)$$

However, for a highly dispersed fiber bundle (e.g., $\theta \approx 45^\circ$), some axons are oriented roughly perpendicular to the fiber bundle’s main direction; these axons’ radial diffusivity can be projected to the main direction, resulting in a small contribution to the overall axial diffusivity $D(t)^8$, biasing the estimate of $c$. To account for this contribution, a correction term is added to the overall $D(t)$ in Eq. (1):

$$D(t) \simeq D_{\infty} + c \cdot \frac{1}{\sqrt{t}} + c' \cdot \frac{1}{t}, \quad (10)$$

where $c'$ is related with the caliber variation$^{24}$ and the undulation$^{83}$.

Code availability

The source codes of Monte Carlo simulations can be downloaded on our github page (https://github.com/NYU-DiffusionMRI).

Data availability

The SEM data and axon segmentation can be downloaded on our web page (http://cai2r.net/resources/software). All human brain MRI data for this study are available upon request.
SUPPLEMENTARY MATERIAL

How to combine individual axon’s diffusivity and kurtosis?

At diffusion time $t$, the total diffusion signal can be approximated by

$$S(b, t) \approx e^{-b D(t)+b^2 D^2(t) K(t)} + O(b^3) = \sum_i f_i \cdot e^{-b D_i(t)+b^2 D^2_i(t) K_i(t)} + O(b^3), \quad (11)$$

where $D(t)$ and $K(t)$ are overall diffusivity and kurtosis, and $D_i(t)$ and $K_i(t)$ are diffusivity and kurtosis of individual compartment. Performing the Taylor expansion of Eq. (11) up to $b^2$ terms, we obtained

$$D(t) = \sum_i f_i \cdot D_i(t), \quad (12a)$$

$$K(t) = \frac{1}{D^2(t)} \sum_i \left[ 3 f_i \cdot (D_i(t) - D(t))^2 + f_i \cdot D^2_i(t) K_i(t) \right]. \quad (12b)$$

The relation between metrics of axonal shape and the relative diffusivity variation in Figure 3d

In Figure 3d, the metrics specifying the axonal shape (CV($r$)) and the the relative diffusivity variation ($\zeta$) highly correlate with each other. Interestingly, the coefficient of variation of axonal radius variation, CV($r$), is calculated solely based on axon’s 3d micro-geometries; in contrast, the relative diffusivity variation $\zeta \equiv (D_0 - D_{i,\infty})/D_{i,\infty}$ is estimated based on simulation results. To explain this observation, we derived here a simple relation to link the two metrics.

Considering an axon composed of multiple axonal segments, for the $i$-th axon, the diffusivity of the $j$-th axonal segment is $D_{ij} = D + \delta D$, where $D$ is the coarse-grained average of diffusivity, and $\delta D$ is the local fluctuation, such that $\langle \delta D \rangle = 0$. The assumption of the local fluctuation $\delta D \ll D$ indicates that the theory only applies to the case of $\zeta \sim \delta D/D \ll 1$. In particular, the local fluctuation of diffusivity $\delta D \approx (\partial D/\partial n) \delta n$ is proportional to the local fluctuation of restriction density $\delta n$, with $n$ the mean density. It is then straightforward to calculate individual axon’s bulk diffusivity $D_{i,\infty}$, given by

$$\frac{1}{D_{i,\infty}} = \langle \frac{1}{D_{ij}} \rangle \approx \frac{1}{D} \left[ 1 + \langle \frac{\left( \delta D \right)^2}{D^2} \rangle \right]$$

$$\approx \frac{1}{D} \left[ 1 + \left( \frac{\partial \ln D}{\partial n} \right) \langle \left( \delta n \right)^2 \rangle \right],$$

simplified as

$$\frac{D - D_{i,\infty}}{D_{i,\infty}} \propto \langle \left( \delta n \right)^2 \rangle. \quad (13)$$

The cross-sectional area (CSA) variation $A(x)$ along an axon can be expressed as the convolution of restriction density $n(x)$ and shape function of a restriction $v(x)$, i.e. $A(x) = n(x) \ast v(x)$. In long time limit, the mesoscopic restrictions are coarse grained by diffusion, and the local CSA variation can be approximated by its Fourier quantity at low $k \cdot \bar{l} \ll 1$:

$$\delta A \sim \frac{1}{L} \cdot \delta \bar{A}(k) |_{k\rightarrow 0^+} = \frac{1}{L} \cdot [\delta \bar{n}(k) \cdot \bar{v}(k)] |_{k\rightarrow 0^+} \sim \delta \bar{n} \cdot v_0,$$

where $L$ is the axon length, $v_0 = \bar{v}_{k=0} \sim \bar{A} \cdot \bar{l}$ is the restriction power (e.g., single bead volume), $\bar{A}$ is the mean CSA, and $\bar{l}$ is the mean restriction width. If the value of $\bar{l}$ is similar between different axons, the local fluctuation of restriction density along an axon is proportional to the local CSA variation normalized by the mean CSA, i.e. $\delta \bar{n} \propto \delta \bar{A}/\bar{A}$. Substituting into Eq. (13), and approximating the local average diffusivity by $\bar{D} \sim D_0$, we obtained

$$\zeta \propto CV^2(r), \quad (14)$$

which is demonstrated by plotting $\zeta$ versus $CV^2(r)$ in Figure 3d, where the correlation coefficient $= 0.9147$ is high, and a small intercept $= 0.009$ verifies this simple relation.

Eq. (14) is applicable when only considering the diffusion along axons in intra-axonal space, and is difficult to be observed in real tissues, where other effects may pronounce (e.g., hindered diffusion in extra-axonal space, water exchange between compartments, CSF signal contamination), resulting in a different value of $\zeta$.

Statistics in mitochondrial morphology

For segmented mitochondria in Figure 2a, the mitochondrial surface area is $2.26 \pm 2.11 \text{ mm}^2$, and the mitochondrial volume is $0.21 \pm 0.25 \text{ mm}^3$; for individual axon, the number of mitochondria per unit IAS volume is $0.32 \pm 0.14 \text{ mm}^{-3}$, the ratio of mitochondrial surface area to IAS volume is 0.67 $\pm 0.32 \text{ mm}^{-1}$, and the volume fraction of mitochondria to IAS is $6.0 \pm 3.0\%$, with histograms shown in Figure S1. The above values are consistent with the previous histological study in mouse optic nerve.

Although the mitochondrial distribution along axons correlates with the axonal caliber variation and potentially has an effect on the diffusion time-dependence, the small mitochondrial volume ($~6\%$ of the IAS volume) suggest that the mitochondria have relatively small effect on the dMRI measurements, as shown in Figure 3b-c.
FIG. S1: Mitochondrial morphometry based on the segmentation in Figure 2a: (a) Histogram of the mitochondrial number per unit IAS volume for each axon. (b) Histogram of the ratio of mitochondrial surface area to IAS volume for each axon. (c) Histogram of the volume fraction of mitochondria to IAS for each axon. (d) Histogram of mitochondrial surface area of all segmented mitochondria. (e) Histogram of mitochondrial volume of all segmented mitochondria.

Human brain data of ten additional subjects

The dMRI measurement was performed on ten healthy subjects (7 males/3 females, 23-30 years old) by using a monopolar PGSE sequence provided by the vendor (Siemens WIP 511E) on a 3T Siemens Prisma scanner (Erlangen Germany) with a 64-channel head coil. For each subject, we varied diffusion time \( t = [21.2, 22, 24, 26, 28, 30, 40, 50, 75, 100] \) ms and fixed diffusion gradient pulse width \( \delta = 15 \) ms. For each scan, we obtained one \( b = 0 \) non-diffusion weighted image and 64 DWIs of \( b \)-values \( b = [0.1, 0.4, 1, 1.5] \) ms/\( \mu m^2 \) along [4, 10, 20, 30] gradient directions for each b-shell, with an isotropic resolution (2 mm)\(^3\) and a field-of-view (216 mm)\(^2\). The scanned brain volume was a slab of 15 slices, aligned parallel to the anterior commissure to posterior commissure line. The CC was in the middle of the slab for covering the entire CC. All scans were performed with the same TR/TE = 5000/150 ms. Total acquisition time is \( \sim 65 \) min for each subject.

Image processing pipeline and chosen ROIs are the same as the one in the main text.

The time-dependent axial diffusivity \( D(t) \), measured by monopolar PGSE in the human brain WM (Figure S2a-b), were averaged over 10 healthy subjects and plotted with respect to \( 1/\sqrt{t} \). In all WM ROIs except Midbody of CC, the axial diffusivity time-dependence demonstrates a \( 1/\sqrt{t} \) power-law relation in Eq. (1) (P-value < 0.05, Table S1), indicating that the universality class along WM axons is the short-range disorder (randomly distributed tissue inhomogeneity) in 1d, corresponding to a dynamical exponent \( \vartheta = 1/2 \). The fitted parameters \( (c, D_{\infty}) \) are shown in Table S1.

Furthermore, the axial kurtosis in WM is \( \sim 0.8 \), demonstrating the non-Gaussian diffusion along axons (Figure S2c-d).

| ROI         | P-value | \( D_{\infty} \) (\( \mu m^2/\text{ms} \)) | \( c \) (\( \mu m^2/\text{ms}^{-1/2} \)) |
|-------------|---------|------------------------------------------|------------------------------------------|
| ACR         | 1.3e-2  | 1.23                                     | 0.333                                    |
| SCR         | 5.6e-4  | 1.31                                     | 0.396                                    |
| PCR         | 2.6e-3  | 1.43                                     | 0.289                                    |
| PLIC        | 7.9e-7  | 1.51                                     | 0.436                                    |
| Genu        | 2.3e-2  | 1.43                                     | 0.383                                    |
| Midbody     | 0.43    | -                                        | -                                        |
| Splenium    | 1.8e-2  | 1.73                                     | 0.289                                    |
| ALIC        | 2.3e-2  | 1.36                                     | 0.269                                    |

TABLE S1: Fit parameters of the time-dependent axial diffusivity \( D(t) \) in human brain data measured using monopolar PGSE (Figure S2a-b) (ACR/SCR/PCR = anterior/superior/posterior corona radiate, ALIC/PLIC = anterior/posterior limb of the internal capsule, genu/midbody/splenium of CC).
FIG. S2: (a-b) Time-dependent axial diffusivity $D(t)$ measured in vivo in brain WM of 10 control subjects using monopolar PGSE. In all WM ROIs except Midbody of CC, the experimental axial diffusivity scales as $1/\sqrt{t}$ (P-value < 0.05, Table S1), manifesting that the universality class along WM axons is short-range disorder in $1d$, corresponding to a power-law tail with $\vartheta = 1/2^5$. The fit parameters are summarized in Table S1. (c-d) Time-dependent axial kurtosis $K(t)$ measured in vivo in brain WM of 10 control subjects using monopolar PGSE. The in vivo measured $K(t)$ is not zero, signifying the non-Gaussian diffusion along WM axons in the human brain. The error bar indicates the standard error of 10 subjects. (ACR/SCR/PCR = anterior/superior/posterior corona radiate, ALIC/PLIC = anterior/posterior limb of the internal capsule, genu/midbody/splenium of CC)