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The role of myelination in measures of white matter integrity: Combination of diffusion tensor imaging and two-photon microscopy of CLARITY intact brains

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ABSTRACT

Diffusion tensor imaging (DTI) is used extensively in neuroscience to noninvasively estimate white matter (WM) microarchitecture. However, the diffusion signal is inherently ambiguous because it infers WM structure from the orientation of water diffusion and cannot identify the biological sources of diffusion changes. To compare inferred WM estimates to directly labeled axonal elements, we performed a novel within-subjects combination of high-resolution *ex vivo* DTI with two-photon laser microscopy of intact mouse brains rendered optically transparent by Clear Lipid-exchanged, Anatomically Rigid, Imaging/immunostaining compatible, Tissue hYdrogel (CLARITY). We found that myelin basic protein (MBP) immunofluorescence significantly correlated with fractional anisotropy (FA), especially in WM regions with coherent fiber orientations and low fiber dispersion. Our results provide evidence that FA is particularly sensitive to myelination in WM regions with these characteristics. Furthermore, we found that radial diffusivity (RD) was only sensitive to myelination in a subset of WM tracts, suggesting that the association of RD with myelin should be used cautiously. This combined DTI-CLARITY approach illustrates, for the first time, a framework for using brain-wide immunolabeling of WM targets to elucidate the relationship between the diffusion signal and its biological underpinnings. This study also demonstrates the feasibility of a within-subject combination of noninvasive neuroimaging and tissue clearing techniques that has broader implications for neuroscience research.

1. Introduction

Diffusion-weighted MRI is the only noninvasive method available for mapping fiber architecture of tissue *in vivo* (Le Bihan et al., 1986; Basser et al., 1994) and it is used frequently in both research and clinical settings to assess brain white matter (WM). Diffusion tensor imaging (DTI) is the most commonly employed diffusion model and utilizes the magnitude and orientation of anisotropic water diffusion to estimate the WM structure of brain tissue, thereby producing maps of macroscopic axonal organization in the brain (Basser and Pierpaoli, 1996; Le Bihan et al., 2001; Mori and Zhang, 2006). While DTI has provided extraordinary insights into the WM structure of the brain in both health and disease, the interpretation of diffusion data is often hampered by several important limitations and challenges (Beaulieu, 2002; Jones and Cercignani, 2010). Primary among these limitations is a lack of understanding of the actual biological sources underlying widely used DTI-derived measures.

DTI produces three scalar indices, or diffusivities (λ1, λ2, λ3) that describe the orientation-dependence of water diffusion within individual imaging voxels. These are used to calculate diffusion along the long axis of fibers, termed axial diffusivity (AD or λ||), diffusion perpendicular to the long axis termed radial diffusivity (RD or λ⊥), and mean diffusivity (MD) or the apparent diffusion coefficient (ADC), which is an average of the three diffusivities. A summary measure that reflects the orientation-dependence of diffusion is called fractional anisotropy (FA), which is significantly higher in brain WM due to the

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highly restrictive nature of coherent axonal bundles for water diffusion. FA is the most widely used invariant measure of anisotropy and is often used to assess so-called WM "integrity" (Jones et al., 2013). While FA is highly sensitive to microstructural changes, it is not specific to the types of change (e.g. AD or RD changes) and neither FA nor the diffusion tensor can unambiguously identify the underlying cause of anisotropy differences. This has led to considerable debate, since the inception of DTI, over the biological significance of these DTI-derived measures since WM integrity can be affected by many different parameters including axon packing density, axon caliber, myelination, microglia, inflammation, and tissue architecture (Mori and Zhang, 2006; Le Bihan and Johansen-Berg, 2012; Jones et al., 2013). The contribution of each of these parameters cannot be resolved using DTI alone, thus further investigation using other complementary multi-modal imaging techniques is imperative.

In the field of light microscopy, there have been several recent technical advances in tissue clearing that enable the molecular examination of entirely intact mouse brains without the need for physical tissue sectioning (Kim et al., 2013; Richardson and Lichtman, 2015). These intact-brain analyses are a vast improvement over traditional slide-based immunohistochemistry that involves sectioning and reconstructing tissue, which is time consuming, error-prone, and does not maintain the structural integrity of neural circuits (Jennings and Stuber, 2014). Clear Lipid-exchanged, Anatomically Rigid, Imaging/immunostaining compatible, Tissue hYdrogel (CLARITY; Chung et al., 2013) is a clearing technique that transforms intact brain tissue into nanoporous, lipid-free, hydrogel-tissue hydrids that are optically transparent but macromolecule permeable. CLARITY achieves tissue transparency to enable deep laser penetration in normally highly light-scattering mouse brains, while preserving proteins *in situ* and the native 3D structure of brain tissue. When coupled with two-photon or light-sheet laser microscopy, whole mouse brains can be imaged entirely intact with sub-cellular resolution (Tomer et al., 2014). This intact analysis approach can be especially beneficial for studying WM tracts within the brain, which have complex 3D architectures spanning multiple biological scales.

Therefore, to directly examine the contribution of myelination in DTI-derived metrics, we used a within-sample design combining *ex vivo* DTI at ultra high field (11.7 T) with CLARITY whole-brain immunolabeling of myelin basic protein (MBP; Baumann and Pham-Dinh, 2001; Boggs, 2006). We found that CLARITY MBP immunolabeling produced 3D whole-brain maps of myelinated WM structures and that MBP expression within these structures significantly correlated with DTI-derived FA measures. Moreover, when examining a subset of WM tracts, we found that the FA-MBP correlation was most robust specifically in WM regions known to have higher myelination and high fiber coherence, as measured by geometric diffusion tensor indices. Therefore, a brain-wide analysis of MBP indicates that FA is sensitive to axon myelination and that this multimodal approach incorporating 3D CLARITY analysis can be potentially used to resolve other molecular factors that contribute to the diffusion signal.

2. Materials and methods

2.1. Subjects

For initial pilot experiments to test the compatibility of performing the CLARITY protocol following *ex vivo* DTI in the same brains, we used three male *thy1*-eYFP-H mice (Jackson Laboratories, Bar Harbor ME), 3–6 months old (Chang et al., in press). For the main experiments, we used four male C57BL/6 J mice (Jackson Laboratories), 3–6 months old. Mice were housed under 12 h light/dark cycle with *ad libitum* access to food and water. All animal procedures were approved by the Feinstein Institute Medical Research Institutional Animal Care and Use Committee and maintained according to National Institutes of Health guidelines.

2.2. ex vivo *DTI*

Mice were transcardially perfused with a customized CLARITY liquid hydrogel (see Sec 2.3 for details) that also included 0.1 mM gadopentetate dimeglumine (Gd-DTPA; Magnevist). Brains were then removed and incubated in 4% PFA-liquid hydrogel for 3 days at 4 °C on a laboratory rocker. Prior to DTI scanning, the brains were immersed in a PBS-Magnevist solution for 48 h. Magnevist was used as a T1-shortening contrast agent for MRI to achieve shorter repetition times (TR), thereby allowing faster DTI acquisitions while maintaining a good signal-to-noise ratio (Aggarwal et al., 2010; Jiang and Johnson, 2011). For MRI, the brains were placed in 15 mm NMR glass tubes, which were filled with Fomblin® oil (Solvay Solexis, Thorofare, NJ), an MR-invisible liquid for susceptibility matching and limiting tissue dehydration. Diffusion-weighted images were acquired on an 11.7 T NMR spectrometer (Bruker BioSpin, Billerica, MA) using a three-dimensional gradient-and-spin-echo (3D DW-GRASE) sequence with twin navigator-echo phase correction (Aggarwal et al., 2010), along 15 independent directions and a *b*-value of 1500 s/mm^2. Total scanning time for each specimen was approximately 16.5 h. The temperature of the specimens was maintained at 28 °C during imaging in order to avoid polymerization of the CLARITY hydrogel.

DTI parameters were as follows: effective echo time (TE) of 31 ms, pulse repetition time (TR) of 800 ms, 4 signal averages with phase cycling, diffusion gradient duration/separation of 3/12 ms, and receiver bandwidth of 100 kHz. Typical imaging field of view and matrix size were 12.8×8.0×16.3 mm and 128×80×162, respectively. The native resolution was 100 μm isotropic. Images were reconstructed using IDL6.4 (ITT Visual Information Solutions, Boulder, CO) with zero-padding of the k-space data by a factor of 2. From the diffusion-weighted images, maps of FA, AD, RD, MD, C_l, and C_p were calculated using FSL (http://www.fmrib.ox.ac.uk/fsl) software as follows: AD=λ1, RD=(λ2+λ3)/2, MD=(λ1+λ2+λ3)/3, C_l=(λ1- λ2)/ λ1, C_p = (λ2- λ3)/ λ1, and FA = $\sqrt{\frac{3}{2}}\frac{\sqrt{(\lambda 1-\mathrm{MD})^2+(\lambda 2-\mathrm{MD})^2+(\lambda 3-\mathrm{MD})^2}}{\sqrt{\lambda 1^2+\lambda 2^2+\lambda 3^2}}$. TrackVis (http://www.trackvis.org) was used for post hoc calculations and visualization. DTI regions of interest (ROIs) were created in TrackVis with manually drawn ROIs using the Free Hand tool by E.H.C.

2.3. CLARITY whole-brain clearing and imaging

We performed the CLARITY procedure as previously described (Chung et al., 2013; Tomer et al., 2014; Yang et al., 2014) with custom modifications in order to maximize antibody penetration and minimize tissue expansion . We used a hydrogel containing 4% paraformaldehyde (PFA), 1.75% acrylamide, 0.01875% bis-acrylamide, and 0.25% VA-044 initiator. Prior to DTI, we perfused brains with this custom hydrogel solution, then performed the *ex vivo* DTI scanning. Following DTI, brains were then polymerized at 37 °C for 3 h to form the brain-hydrogel hybrid. There was only one perfusion and one CLARITY hydrogel used in this study (Fig. 1**A**). Tissue clearing was achieved with passive CLARITY (PACT; Tomer et al., 2014; Yang et al., 2014) until brains were optically transparent (30–40 days). While this passive technique is markedly slower, we found that it achieved excellent tissue transparency and structural preservation without tissue inflation. Transmittance through cleared whole brains was measured at three laser wavelengths (Chang et al., in press) using a fluorescence spectrometer (Public Lab, Cambridge, MA). Once transparent, the brains were washed in 1X PBS+0.1% Triton X for 2 days, changing the solution every 24 h. Brains were then incubated in primary antibody solution, anti-myelin basic protein (1:50, EMD Millipore), 0.5 M sodium borate solution, (pH 8.5) and 0.1% Triton-X (wt/vol) for 14 days at 37 °C on an orbital shaker. Brains were then washed for 7 days in 0.5 M sodium borate with 0.1% Triton-X on a shaker at 37 °C, followed by secondary antibody labeling with Alexa Fluor 633 (1:50, Life technologies) in 0.5 M sodium borate + 0.1% Triton-X for another 14 days at 37 °C on an orbital shaker. The brains were placed in a final wash for 7 days