Development and Evaluation of Relaxation-Based Measures of Myelin Content and Microstructure in Rodent Brains

By

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CHAPTER 1

INTRODUCTION

Rodent models of white matter disease are of great interest in neurobiology and neurodevelopment to increase the understanding of pathology, disease progression, and treatment options. While histology is the current gold standard for determining the degree of myelination and microstructural characteristics in white matter, it is limited to ex vivo tissue with small coverage area, requires many tissue-preparation steps and is relatively time-consuming. In contrast, imaging, specifically magnetic resonance imaging (MRI), provides an opportunity to directly assess myelination, and potentially microstructure either non-invasively (in vivo) or non-destructively (ex vivo) across whole brain in a more time-efficient manner. In addition, imaging methods have the ability to link rodent studies to clinically relevant human diagnosis and monitoring.

While two quantitative relaxation-based MRI techniques display correlative measures to myelin content, neither can provide an absolute measure of the volume of myelin similar to histology. In addition to the myelin volume, histology provides further microscopic information such as axon size and myelin thickness. However, MRI is inherently macroscopic with resolution ~ 100X greater than the average axon size. Previous work has shown the potential for obtaining microscopic information using MRI, but there has been a lack of validation to histology. Therefore, the work in this dissertation aims to fill the gaps of both macroscopic and microscopic imaging of myelin with MRI by:

1. Developing MRI and histology methods for rodent brain
2. Validating myelin volume imaging in multiple animal models

3. Evaluating microstructural information from g-ratio imaging
CHAPTER 2

BACKGROUND

2.1 WHITE MATTER AND MYELIN

The mammalian nervous system is mainly comprised of two types of tissue, gray and white matter. Neurons are the major cellular component of the central and peripheral nervous systems (CNS and PNS, respectively) that transmit and receive signals to and from the rest of the body. Axons extend as long projections from the neuronal cell body and provide the pathway for signal transmission. In white matter, to increase conduction speed along the axon, myelin surrounds the axons in a sheath-like structure, seen in Fig 2.1 (1–3).

Myelin is composed of ~ 80% lipid and 20% protein and forms in a lamellar, membranous structure around the axon. The layers of myelin run in a nodal fashion along the axon, creating nodes of Ranvier, which are small breaks in the myelin. In order to increase the speed of action potential conduction down the axon, myelin electrically insulates the axons between nodes. This induces salutatory conduction from one node of Ranvier to the next instead of traversing the entire length of the axon. This is a vital aspect of complex neuronal sensory, motor, and behavioral functionality (4). The myelin bilayers wrap around the axons leaving space between layers, which is filled with water as seen in Fig 2.1. This water makes up ~ 40% of myelin weight and is called myelin water (1).
Since myelin can increase conduction speed 10 to 100X, demyelinated axons or damage to the myelin layers can drastically inhibit neuronal health. Decreased speed of signal transmission has the largest effect on complex motor and sensory signaling, which leads to neuronal diseases. This can be seen in many neurodegenerative disease, such as Multiple Sclerosis (MS) and developmental disorders such as Tuberous Sclerosis Complex (TSC). These diseases have been shown to be directly related to the health of the myelin content and structure (5–7). Consequently, the ability to quantitatively monitor myelin content and structure would be quite advantageous to assess disease progression as well as treatment efficacy (4).

2.1.1 Axon And Myelin Geometry

Myelin is produced by oligodendrocytes in the CNS and Schwann cells in the PNS. Oligodendrocytes are branched and myelinate multiple axons while Schwann cells myelinate only one axon. The thickness of myelin is largely believed to be dependent on the size of the axon (8). This is not surprising since axon size and myelin thickness are inversely proportionate
to conduction velocity along the axon. We can quantify this relationship by the g-ratio, the ratio of axon radius to myelinated fiber radius as shown in Fig 2.2.

![Diagram of g-ratio](image)

\[ \text{g-ratio} = \frac{r}{R} \]

**Figure 2.2.** Diagram of the g-ratio of myelinated axon.

The g-ratio is known to change as myelination occurs during development as well as demyelination disease processes. Therefore, this quantitative metric has been strongly associated as a more specific measure of white matter and myelin health (9).

### 2.2 MYELIN DEVELOPMENT AND TRANSGENIC MODELS

The PI3K/Akt/mTOR (mammalian target of rapamycin) pathway has proven to be a key pathway of myelination through displayed involvement in myelin development and differentiation of oligodendrocyte precursor cells (OPCs). In this work, several transgenic mouse models are used to interrogate the role of different players in the PI3K/Akt/mTOR pathway shown below in Fig 2.3 with a star signifying the genes targeted (10).
Figure 2.3. Diagram of the PI3K/Akt/mTOR pathway. Stars signify the three genes targeted in conditional knockout models in this work (10).

All transgenic models were conditional knockouts, mutating the gene of interest only in oligodendrocytes and oligodendrocyte precursor cells (OPCs). This was done using the Cre-recombinase technique and an Olig2 promoter.

The first model targets the \textit{Tsc2} gene. The Tuberous Sclerosis Complex (TSC) includes both \textit{Tsc1} and \textit{Tsc2} which inhibit mTORC1 (mammalian target of rapamycin complex) and activate mTORC2 signaling. Therefore, deletion of either gene activates mTORC1 and inhibits mTORC2 signaling. When \textit{Tsc2} is conditionally knocked out there is a decrease in the number and the maturation of oligodendrocytes, which maybe contribute to the decrease in myelination (7). \textit{Rictor} is targeted in the second model which is required for mTORC2 signaling and embryonic development. Since \textit{Rictor} is directly related to mTORC2 signaling, by targeting \textit{Rictor}, (in comparison to \textit{Tsc2}), the model is more specific to determine contributions if mTORC2 signaling. While myelination decreases in this model, it is not as drastic as with the conditional knockout of \textit{Tsc2} suggesting that mTORC2 is not completely responsible for changes
associated with \(Tsc2\) (11–13). The third model targets \(Pten\) (phosphatase and tensin homologue deleted on chromosome 10), which affects the PI3K pathway upstream of \(Tsc2\) and \(Rictor\). Deletion of \(Pten\) is known to be characteristic of high-grade tumors since it negatively regulates the PI3K pathway resulting in cell proliferation. In oligodendrocytes and OPCs, deletion of \(Pten\) overactivates \(Akt\) and downstream \(mTOR\) and has been shown to result in thicker myelin (14). Between the three conditional knockout models, we can interrogate changes in myelin from changes in the PI3K pathway as a whole (\(Pten\)), after \(Akt\) signaling (\(Tsc2\)), and at a key endpoint protein of the pathway (\(Rictor\)). This provides a rich data set for understanding myelin development and imaging capabilities.

### 2.3 FUNDAMENTALS OF NUCLEAR MAGNETIC RESONANCE (15)

Magnetic resonance imaging (MRI) relies on the basic principles of nuclear magnetic resonance (NMR). Nuclei that have unpaired protons, such as \(^1\text{H}\), exhibit spin and act as magnetic dipoles. While the spins are normally randomly-oriented, when placed in an external magnetic field (\(\vec{B}_0\)) in the \(z\)-axis, these spins align with \(\vec{B}_0\) either parallel or anti-parallel. There is a small excess of spins aligned parallel and thus there is a net magnetic moment (\(\vec{\mu}\)). Due to the net torque on \(\vec{\mu}\) from the magnetic field, we can describe the motion of \(\vec{\mu}\) as

\[
\frac{d\vec{\mu}}{dt} = \gamma \vec{\mu} \times \vec{B}_0
\]

[2.1]

which describes a precession of \(\vec{\mu}\) about \(\vec{B}_0\) with an angular frequency (\(\omega_0\)) of

\[
\omega_0 = \gamma B_0
\]

[2.2]
where $\gamma$ is the gyromagnetic ratio, which is $\sim 2.68 \times 10^8$ rad/s/T or $\gamma/2\pi \sim 42.6$ MHz/T for $^1$H protons and $B_0$ is in units of Tesla [T]. We call $\omega_0$ the Larmor precession frequency, which is dependent on the external magnetic field strength ($B_0$) shown in Eq. 2.2.

Instead of describing individual spins ($\vec{\mu}$), we can describe a sum of protons as a magnetization vector, $\vec{M}$. As before, we can describe the equation of motion of $\vec{M}$ due to an external magnetic field ($\vec{B}_{\text{ext}}$) as

$$\frac{d\vec{M}}{dt} = \gamma \vec{M} \times \vec{B}_{\text{ext}} \tag{2.3}$$

When $\vec{B}_{\text{ext}}$ is a constant field ($\vec{B}_0$), $\vec{M}$ is in equilibrium ($M_0$) and precessing at $\omega_0$. To obtain signal, we need to perturb the spins by applying another magnetic field ($\vec{B}_1$), orthogonal to $\vec{B}_0$ at the Larmor frequency. Therefore, now $\vec{B}_{\text{ext}} = \vec{B}_0 \hat{z} + \vec{B}_1 \hat{x}$ and we can write Eq. 2.3 in terms of each axis as

$$\frac{dM_x}{dt} = (\omega_0 - \omega)M_y$$

$$\frac{dM_y}{dt} = -(\omega_0 - \omega)M_x + \omega_1 M_z \tag{2.4}$$

$$\frac{dM_z}{dt} = -\omega_1 M_y$$

where $\omega_1 = \gamma B_1$ and $\omega$ is the angular frequency of the rotating reference frame. The angle that $\vec{M}$ is tipped from the $z$-axis ($\theta$) is dependent of the strength and duration of $\vec{B}_1$ for a constant amplitude, rectangular pulse as

$$\theta = \gamma B_1 t = \omega_1 t. \tag{2.5}$$

Assuming $\omega = \omega_0$ and $M_z(0) = M_0$ we can solve Eq. 2.4 as,
2.3.1 Relaxation (15)

Spins aligned with the main, static magnetic field are at a state of minimum energy; after magnetization is flipped to the transverse plane, the spins now have higher energy. In order to return to equilibrium, spins interact with the local environment and transfer energy to the surrounding lattice. The return to equilibrium, or relaxation, can be described as,

$$\frac{dM_z}{dt} = \frac{1}{T_1}(M_0 - M_z)$$ \hspace{1cm} [2.7]

where \( T_1 \) is the spin-lattice relaxation time. Transverse magnetization decreases in time due to this longitudinal recovery, but it also decreases due to interactions between spins. Some spins may precess at frequencies slightly higher or lower than Larmor frequency, which changes the phase of the spins. When these spins are summed, phase coherence is lost and the magnetization vector decreases. This process can be described as below

$$\frac{dM_\perp}{dt} = -\frac{1}{T_2}(M_\perp)$$ \hspace{1cm} [2.8]

where \( M_\perp \) is the magnetization in the transverse \((x,y)\) plane and \( T_2 \) is the spin-spin relaxation time. The \( T_2 \) is affected by the motion of spins. For example, spins that are moving around more (such as free water) have an averaging out effect in phase and a slower decrease in transverse magnetization.

As before, we can solve Eqs. 2.7 and 2.8 to describe relaxation in time.
We see that $M_\perp$ decays exponentially with time constant $T_2$ and $M_z$ recovers back to equilibrium exponentially with time constant $T_1$. Relaxation time constants are characteristic to different tissue types and used to create a variety of contrasts as described below.

\[
M_\perp(t) = M_\perp(0) \exp \left( -\frac{t}{T_2} \right) \\
M_z(t) = M_0 + [M_z(0) - M_0] \exp \left( -\frac{t}{T_1} \right)
\]

2.4 CURRENT WHITE MATTER MRI TECHNIQUES

Magnetic Resonance Imaging is a very valuable tool in brain imaging, providing contrast between gray and white matter. While qualitative techniques can detect gross changes to tissue, such as MS lesions, these techniques are nonspecific to underlying pathology and white matter health. Information obtained from more advanced quantitative techniques can probe more specific changes to tissue microstructure and provide more detailed information about the disease, its progression and/or drug treatment efficacy (16).

As described above, myelin is mainly a lipid structure, which has a very short-lived signal from lipids ($< 1$ ms), making direct imaging of myelin difficult. Therefore, most current techniques can only indirectly investigate myelin and axon integrity based on myelin water or interactions between water and myelin. Current myelin imaging approaches include qualitative $T_1$-weighted (spin-lattice relaxation) and $T_2$-weighted (spin-spin relaxation) imaging, as well as quantitative measures of MR Spectroscopy (MRS), Diffusion Tensor Imaging (DTI), Magnetization Transfer Imaging (MTI), and Multi-Exponential $T_2$ imaging (MET$_2$) (4). $T_2$-weighted images provide highly sensitive contrast based on spin-spin relaxation ($T_2$) differences. In normal myelinated white matter, the water in axons and between myelin sheaths has shorter $T_1$
and $T_2$ values compared to gray matter. This results in hypointense white matter structures on $T_2$-weighted images and hyperintense signal in $T_1$-weighted images compared to gray matter (17).

Beyond qualitative imaging, quantitative imaging techniques such as MRS and DTI provide more specific, microscopic information. MR Spectroscopy provides the ability to detect signal from metabolites in the brain, which provides very particular information about pathology and metabolism, providing insight into disease mechanisms (4). Diffusion Tensor Imaging is capable of detecting changes in the direction and anisotropy of water diffusion. In the brain, axons are highly directional, creating a strong diffusion anisotropy with water diffusing axially (along the axon) much more than radially. In axon degeneration or demyelination, axon integrity is lost and DTI can detect this as a change in the diffusion anisotropy (18,19). While MRS and DTI are sensitive to myelin, $\text{MET}_2$ and MTI have been investigated as more specific biomarkers of myelin.

2.4.1 Multi-Exponential $T_2$

$\text{MET}_2$ imaging is based on the spin echo pulse sequence seen in Fig 2.4 (20,21).

![Multi-exponential $T_2$ experiment pulse diagram](image)

**Figure 2.4. Multi-exponential $T_2$ experiment pulse diagram.** A 90-degree excitation pulse is followed by a train of 180-degree pulses to sample the $T_2$ decay.

In a spin echo experiment, signal is rotated from alignment with $B_0$ to the transverse plane by a 90-degree excitation pulse. After excitation, signal dephases exponentially in the transverse...
plane at the rate $R_2^* = 1/T_2^*$; therefore, at a time, $T_E/2$ after the 90-degree pulse, a 180-degree refocusing pulse is applied. The refocusing pulse flips the spins and they begin to rephase, increasing transverse signal exponentially. Since spins rephase at the same rate as dephasing, after a time $T_E/2$ after the 180-degree pulse, spins are in phase, forming the peak spin echo signal (i.e., Echo 1, 2, and N in Fig 2.4). MET$_2$ data is acquired by repeating 180-degree refocusing pulses every $T_E$ numerous times ($N$ times in Fig 2.4). This samples the signal, which decays exponentially with time constant $T_2$, from echo to echo (See Eq. 2.9). Each echo time provides a qualitative $T_2$-weighted image, described above. After $N$ echoes are acquired, the remaining time in the defined $T_R$ is elapsed allowing for $T_1$ recovery (See Eq. 2.9) before acquiring another train of echo signals.

Through non-negative least squares (NNLS) analysis (described below), we can fit MET$_2$ data to obtain a $T_2$ spectrum. In the central nervous system (CNS), 3 peaks are generally found in the spectrum of $T_2$ values. The $T_2$ peaks correspond to the hindrance of water motion; therefore, the shortest peak represents water trapped between myelin sheaths ($T_2 \sim 5-40$ms, depending on $B_0$), the middle peak represents intra- and extra-axonal water ($T_2 \sim 30-80$ms, depending on $B_0$), and cerebrospinal fluid ($T_2 > 1$ s) is the longest peak in vivo. The percentage of short $T_2$ signal is a quantitative measure of myelin water content, termed the Myelin Water Fraction (MWF) and correlates well with myelin content (22,23).

2.4.1.1 Non-negative Least Squares (NNLS) Analysis

Myelin water imaging (MWI) by MET$_2$ provides specific information about myelin content and microstructure in white matter (23,24). Equation 2.10 describes MET$_2$ decay signal from a spin echo experiment, where $y$ is the acquired signal, $M$ is the number of $T_2$ values to fit, typically 100
as to not bias results, $N$ is the number of echoes acquired, and $s$ is the spectral amplitude of each $T_2$ component. Typically, $T_j$ is logarithmically-spaced and ranges from a few milliseconds to 500ms to encompass the range of expected relaxation times.

$$y_i = \sum_{j=1}^{M} s_j e^{-t_i/T_j}, \quad i = 1, 2, \ldots, N$$ \hspace{1cm} [2.10]

This can be generalized into a linear system in equation 2.11,

$$y_i = \sum_{j=1}^{M} A_{ij}s_j, \quad i = 1, 2, \ldots, N$$ \hspace{1cm} [2.11]

where $A_{ij}$ represents a matrix describing exponential decay with $N$ echoes at $M$ possible $T_2$ relaxation times, respectively. Knowing $y$ and defining $A$ based on the current experimental conditions (number of acquired echoes and echo spacing ($T_E$)), the vector $s$ can be determined by minimizing the least-squares misfit in equation 2.12.

$$\sum_{i=1}^{N} \sum_{j=1}^{M} (A_{ij}s_j - y_i)^2, \quad i = 1, 2, \ldots, N$$ \hspace{1cm} [2.12]

Since noise in the measured data ($y_i$) is inherent, the misfit cannot be reduced to zero. The fitting can therefore be regularized based on some characteristic of the resulting spectrum in order to increase reproducibility in return for decreased resolution. For example, one can minimize the curvature of the spectrum, or the second order finite difference, as seen in equation 2.13.

$$\sum_{i=1}^{N} \sum_{j=1}^{M} (A_{ij}s_j - y_i)^2 + \mu \sum_{j=1}^{M-2} (s_{j+2} - 2s_{j+1} + s_j)^2, \quad i = 1, 2, \ldots, N$$ \hspace{1cm} [2.13]

$\mu$ is generally chosen in order to smooth the $T_2$ spectrum so that the chi-squared statistic of the misfit is equal to about the number of data points, so each data point is misfit by one standard deviation. If it is smaller, the fit is too accurate and will fit the noise. If the chi-squared value is
much larger, the data will not be accurately fit. Smoothing provides a continuous and physically realistic model of the spectrum (22).

After the spectrum is found, the $T_2$ peaks and the fractional components of the area under those peaks can be determined by using the spectrum derivative. From this, MWF is determined as the fractional component of $T_2$ signal below a defined $T_2$ threshold based on typical $T_2$ characteristics (dependent on $B_0$) as seen in Fig 2.5 (23).

![Figure 2.5. $T_2$ spectra from gray matter and white matter regions of interest. White matter has a short $T_2$ peak which corresponds to signal from myelin water (MWF).](image)

2.4.1.2 Extended Phase Graph Algorithm

MET$_2$ is an important characterization of neurological tissue microstructure. However, accurate measurement of MET$_2$ using a standard multi-exponential NNLS fitting depends on pure exponential decay with an assumed perfect refocusing flip angle ($B_1$) of 180 degrees. Across a large volume, exponential decay is hampered by multiple coherence pathways arising from $B_1$ non-uniformity, resulting in stimulated echoes. Stimulated echoes arise from spins that see the original 90 degree excitation pulse and subsequent refocusing pulses less than 180 degrees. Instead of being refocused, the signal can be returned to the longitudinal axis where it does not experience transverse relaxation and a later refocusing pulse can return the spin back to the transverse plane. This increases signal at that echo time since these spins did not encounter true
$T_2$ relaxation as expected. This phenomenon can be seen beginning at the second echo time with increasing effects as refocusing flip angle decreases as seen in Fig 2.6. Using NNLS to fit decay curves that exhibit impure exponential behavior, results in improper curve fitting and biased $T_2$ values as seen in Fig 2.7 (25).

**Figure 2.6. Example decay curves.** Stimulated echoes contamination from non-uniform B1 on exponential decay curves. Non-negative least squares (NNLS) $T_2$ fitting techniques expect pure exponential decay.

**Figure 2.7. Exponential fitting of decay curves.** Exponential fitting with a refocusing flip angle near perfect 175 degrees and 135 degrees. The stimulated echoes produced by the lower flip angle do not follow the exponential decay model.
One approach to avoid stimulated echo contamination is to use variable crusher gradients to remove the stimulated echo pathway. Here, each refocusing pulse has a unique pair of symmetric crusher gradients straddling the refocusing pulse with phase $\varphi_n$, where $n = 1$ to number of echoes. With this scheme, the spins in a stimulated echo pathway will accumulate phase $\varphi_1$ before being restored to the longitudinal axis. The phase is then rewound by $\varphi_2, \varphi_3 \ldots \varphi_N$ after returning to the transverse plane by a later refocusing pulse. Since $\varphi_1 \neq \varphi_2, \varphi_3, \ldots, \varphi_N$, phase will not be cancelled and the signal will be incoherent. While this technique is effective, signal is inherently thrown away and the gradient strengths needed to crush all unwanted echoes in a linearly modulated crusher scheme, especially as $B_1$ decreases, become increasing large (26,27).

Another option to remove simulated echo contamination is to use composite refocusing pulses. These are a series of pulses with varied flip angles designed to produce a net 180-degree refocusing pulse that is minimally sensitive to inhomogenous $B_0$ and $B_1$ fields (28,29). However, this technique also uses large crusher gradients to remove out-of-slice signal and has limited multi-slice capability. The pulse also greatly increases the specific absorption rate (SAR) as compared to other shaped pulses. Instead of avoiding stimulated echoes, recently, it has been shown that the Extended Phase Graph (EPG) algorithm (25) can be utilized to concurrently fit $B_1$ and multiple $T_2$s from an appropriate CPMG multiple spin echo measurement (30,31).

The EPG algorithm is an iterative method that tracks the phase and orientation of spins, known as the Magnetization Phase State Vector ($MPSV$) throughout a defined pulse sequence. Three phase states are identified: dephasing spins in the transverse plane ($F$), rephasing spins in the transverse plane ($F^*$), and spins along the longitudinal axis that maintain phase ($Z$). The $MPSV$ contains the three phase states for each echo time period, giving a column vector 3 times the number of echoes in length, since the maximum number of dephasing states that a spin can
accrue is defined by the echo train length. The initial magnetization vector \((\text{MPSV}_0)\), Eq. 2.14, describes the normalized magnetization in the transverse plane prior to relaxation \((F_i)\).

\[
\text{MPSV}_0 = \begin{bmatrix} 1 & 0 & 0 & 0 & \cdots \end{bmatrix}^T
\]  

[2.14]

Throughout the defined pulse sequence, a series of matrices are applied to the \(\text{MPSV}\) to describe changes to spin states. The rotation matrix \((R)\), Eq. 2.15, defines the effect of an applied pulse \((\alpha)\) on spin vectors. The relaxation matrix \((E)\), Eq. 2.16, defines the signal decay between refocusing pulses. The transition matrix \((T)\) in Eq. 2.17 describes the change in phase state of magnetization vectors after a refocusing pulse, whether they continue in the dephasing state \((F)\), enter the rephasing state \((F^*)\), or maintain phase in the longitudinal axis \((Z)\).

\[
R: \begin{bmatrix} F_n^+ \\ F_n^* \\ Z_n \end{bmatrix} = \begin{bmatrix} \cos^2 \left( \frac{\alpha}{2} \right) & \sin^2 \left( \frac{\alpha}{2} \right) & -i \sin \alpha \\ \sin^2 \left( \frac{\alpha}{2} \right) & \cos^2 \left( \frac{\alpha}{2} \right) & i \sin \alpha \\ -\frac{1}{2} i \sin \alpha & \frac{1}{2} i \sin \alpha & \cos \alpha \end{bmatrix} \begin{bmatrix} F_n \\ F_n^* \\ Z_n \end{bmatrix}
\]  

[2.15]

\[
E: \begin{bmatrix} F_1^+ \\ F_1^* \\ Z_1 \\ F_2^+ \\ F_2^* \\ Z_2 \\ F_3^+ \\ F_3^* \\ Z_3 \end{bmatrix} = \begin{bmatrix} E_2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & E_2 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & E_1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & E_2 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & E_1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & E_2 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & E_2 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & E_1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & E_1 \end{bmatrix} \begin{bmatrix} F_1 \\ F_1^* \\ Z_1 \\ F_2 \\ F_2^* \\ Z_2 \\ F_3 \\ F_3^* \\ Z_3 \end{bmatrix}
\]  

\[
E_1 = \exp \left( -\frac{\tau E}{2T_1} \right)
\]

\[
E_2 = \exp \left( -\frac{\tau E}{2T_2} \right)
\]  

[2.16]

\[
T: \begin{bmatrix} F_1^+ \\ F_1^* \\ Z_1 \\ F_2^+ \\ F_2^* \\ Z_2 \\ F_3^+ \\ F_3^* \\ Z_3 \end{bmatrix} = \begin{bmatrix} 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} F_1 \\ F_1^* \\ Z_1 \\ F_2 \\ F_2^* \\ Z_2 \\ F_3 \\ F_3^* \\ Z_3 \end{bmatrix}
\]  

[2.17]
The matrices are applied to the Magnetization Phase State Vector (MPSV) from right to left according to the pulse sequence, as described in Eq. 2.18, which begins as normalized transverse signal \((MPSV_0)\) shown in Eq. 2.14. The signal created by the spin echo \((M_n)\) is described by the signal in \(MPSV_n(1)\) (Eq. 2.19). This describes the spins that were rephasing and have transitioned to the dephasing state, which necessitates a period of coherence and hence a spin echo.

\[
MPSV_n = ETR(\alpha_n) \cdot E^2TR(\alpha_{n-1}) \cdots E^2TR(\alpha_1) \cdot E \cdot MPSV_0 \tag{2.18}
\]

\[
M_n = MPSV_n(1) \rightarrow Signal = F_1 (1st \ in \ phase \ state) \tag{2.19}
\]

For voxel-by-voxel \(B_1\) and \(T_2\) fitting, the EPG algorithm first utilizes the described matrix multiplication to define a basis set of exponential curves based on the full range of \(T_2\) values and a discrete number of refocusing flip angles \((N_\alpha \sim 5-10)\). The basis flip angles are linearly spaced for a range of expected \(\alpha\) (i.e., 130-180 degrees), which are appropriate assumptions for \(B_1\) in a 3D acquisition. The basis set is calculated prior to fitting any voxels to reduce computation time. A \(T_2\) spectrum is fit via NNLS for each of the basis \(\alpha\) and the sum-squared error between the experimental decay data and the fitted EPG-defined decay curves is calculated. This provides a coarse estimate of probable \(\alpha\) that voxel experienced. Then, a fine spline interpolation (<0.5 degree spacing) is applied to the residuals to find the minimum corresponding to the precise \(\alpha\) that voxel experienced. Once \(\alpha\) is determined, basis decay curves are recalculated for the specific \(\alpha\) and the \(T_2\) spectrum is fit using NNLS as described previously. An example of the fitting can be seen in Fig 2.8 in comparison to pure exponential fitting in Fig 2.7. EPG estimation allows for 3D volumetric coverage without bias in fitted \(T_2\) data due to non-uniform \(B_1\) (30,31).
Figure 2.8. EPG fitting of decay curves. Exponential fitting based on the EPG Algorithm at near perfect 180 degree refocusing pulse and much lower (135 degrees). The EPG Algorithm accurately accounts for the stimulated echoes and properly fits the decay curve unlike exponential decay fitting in Fig 2.6.

2.4.1.3 Cramer-Rao Lower Bound (CRLB) Calculations

Given data measurements, \( y_i \) with noise, \( v_i \), and \( g_i \), the estimated value of \( y_i \) based on model parameters (\( \theta_j \)), the data can be described by equation 2.20

\[
y_i = g(x_i, \theta_j) + v_i, \quad i = 1:N, \quad j = 1:J
\]  

[2.20]

Where \( N \) is the number of data points and \( J \) is the number of model parameters, \( \theta \). The added noise \( (v_i) \) is a random variable drawn from the Gaussian-distributed probability density function (PDF) with an expected mean of 0 and variance of \( \sigma^2 \).

\[
f(v_i) = \frac{1}{\sqrt{2\pi\sigma_i^2}} \exp \left( \frac{-v_i^2}{2\sigma_i^2} \right)
\]  

[2.21]

Then, \( y_i \) is also a random variable and can also be described by a normally-distributed PDF

\[
f(y_i) = \frac{1}{\sqrt{2\pi\sigma_i^2}} \exp \left( \frac{-(y_i - g(x_i, \theta))^2}{2\sigma_i^2} \right)
\]  

[2.22]
While the PDF describes the probability of certain outcomes as a function of model parameter values, the likelihood function describes the likelihood of model parameter values given observed outcomes. In other words, the likelihood function is considering the probability density function as a function of parameter space instead of the random variable $y_i$. Therefore, the likelihood function describes the most likely value of $\theta$ based on a distribution. If we assume the measurements are independent and the added noise follows a Gaussian distribution, then the likelihood function is described as

$$f(\theta; x, y) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left(-\frac{1}{2} \sum_{i=1}^{N} \left(\frac{y_i - g_i(\theta)}{\sigma}\right)^2\right)$$  \hspace{1cm} [2.23]

When the likelihood, or equivalent, log-likelihood function used for mathematical simplicity, is maximized, an optimal parameter estimator can be chosen by minimizing $\chi^2$ defined in equation 2.24.

$$\chi^2 = \sum_{i=1}^{N} \left(\frac{y_i - g_i(x,\theta)}{\sigma_i}\right)^2$$  \hspace{1cm} [2.24]

To minimize $\chi^2$, the function can be differentiated and set to zero to solve for optimal parameters. However, if we want to statistically evaluate the estimated parameters, one can consider the $\chi^2$ function with respect to each parameter. The curvature of these functions inversely describes the uncertainty of the model parameter. Therefore, we can take the second order partial derivative of $\chi^2$ with respect to each parameter to determine as shown in equations 2.25.
\[
\frac{\partial^2 \chi}{\partial \theta_j \partial \theta_k} = \frac{-2}{\sigma^2} \sum_{i=1}^{N} \left[ -\frac{\partial g(x_i, \theta)}{\partial \theta_k} \frac{\partial g(x_i, \theta)}{\partial \theta_j} + (y_i - g(x_i, \theta)) \frac{\partial^2 g(x_i, \theta)}{\partial \theta_j \partial \theta_k} \right] \tag{2.25}
\]

Since this the left hand term in eq. 2.25 is a random function, we need to consider its expected value. For an unbiased estimator, \( E[y_i - g(x_i, \theta)] = 0 \), therefore, the second term on the right hand side equals zero and we are left with,

\[
E \left[ \frac{\partial^2 \chi}{\partial \theta_j \partial \theta_k} \right] = \frac{-2}{\sigma^2} \sum_{i=1}^{N} \left[ -\frac{\partial g(x_i, \theta)}{\partial \theta_k} \frac{\partial g(x_i, \theta)}{\partial \theta_j} \right] = J^T J \tag{2.26}
\]

where \( J \) is the Jacobian matrix which is an \( N \times N \) matrix of partial derivative of \( g \) with respect to each fitted parameter, \( \theta \). The Fisher Information Matrix (FIM, \( F_{jk} \)) is then defined as the expected value of curvature matrix. Additionally, if we do not assume identically distributed noise, \( \sigma^2 \) is not constant for all \( N \) as shown in equation 2.27,

\[
F_{jk} = \sum_{i=1}^{N} \frac{1}{\sigma_{y_i}^2} \frac{\partial g(x_i)}{\partial \theta_j} \frac{\partial g(x_i)}{\partial \theta_k} = J^T \Sigma_y^{-1} J \tag{2.27}
\]

where \( j \) and \( k = 1:M \), the number of fitted parameters and \( \Sigma_y \) is the uncertainty of the observed data, an \( N \times N \) matrix of variances and covariances. The FIM describes the amount of information that is known about each parameter based on the curvature of the \( \chi^2 \) statistic of fit. The parameter covariance matrix (\( \Sigma_{\theta} \)) is the inverse of the curvature, or Fisher information matrix, and the Cramer-Rao lower bound of variance for each parameter is defined as

\[
\sigma_{\theta_j}^2 \geq [F^{-1}]_{jj} = \left[ (J^T \Sigma_y^{-1} J)^{-1} \right]_{jj} \tag{2.28}
\]
where $\theta_j$ is the estimated model parameter. The CRLB defines the maximum precision possible based on experimental conditions (32,33).

2.4.2 Magnetization Transfer

Magnetization transfer, first described by Wolff and Balaban (Wolff and Balaban, 1989), is a mechanism that describes the exchange of magnetization between the free water protons ($^1$H) and protons bound to macromolecules. To describe this exchange, a two-pool model is used as seen in Fig 2.9 below,

\[ \text{Free protons} \quad \text{Bound protons} \]

$k_{fb}$ is the first order rate constant of $^1$H from the free to the bound, macromolecular pool and $k_{bf}$ is rate constant in the opposite direction. This exchange occurs via dipolar interactions or chemical exchange when free $^1$H are near macromolecular $^1$H. As discussed above, myelin is a large component of white matter and mainly consists of $^1$H bound to macromolecules; therefore, the magnetization transfer contrast is much greater in normal white matter than gray matter. However, other structures, like cell membranes, also contain bounded $^1$H, so magnetization transfer contrast is not specific to myelin.

Figure 2.9. Two pool model of exchange. Free protons represent those water protons moving in space, whereas macromolecular protons are those attached and motionally restricted.
Since the motion of bound $^1$H is restricted, these nuclei will be greatly influenced by their local magnetic field, causing a loss in phase coherence and a very short $T_2$ (< 1ms). Conversely, with freely moving $^1$H, local field fluctuations average out leading to a much longer $T_2$ (> 10 ms). As $T_2$ is inversely related to spectral width, macromolecular protons exhibit a broad frequency spectrum, while free water protons have a narrow bandwidth centered at Larmor frequency ($\Delta = \omega_o - \omega$) as seen below in Fig 2.10.

![Figure 2.10. Frequency spectrum of bound protons and liquid, free protons.](image)

The short $T_2$ of macromolecular $^1$H prevents direct imaging with standard MRI sequences, but they can be investigated through the MT effect on the free water. By selectively affecting one of the proton pools, subsequent magnetization exchange between the two pools will alter the observed free water signal based on the amount of bound proton content. Since free water has a relatively narrow bandwidth, if an off-resonance pulse is applied prior to an imaging sequence, the macromolecular pool is partially saturated, while the free water pool remains mainly unaffected. The saturated magnetization will transfer energy to the free pool based on the rate of exchange between the two pools and the resulting image will have decreased signal. By
comparing this saturated image signal ($M_{SAT}$) to a typically acquired image signal ($M_0$), a Magnetization Transfer Ratio ($MTR$) can be calculated as,

$$MTR = \frac{M_0 - M_{SAT}}{M_0}$$  \[2.26\]

where $MTR$ will be lowest in regions of high bound, macromolecular content. However, this ratio is only semi-quantitative and varies among scanners and protocol choices (34–36).

2.4.2.1 Quantitative Magnetization Transfer

Another option is to perform a quantitative Magnetization Transfer (qMT) experiment. Several methods have been developed (37–39) but in this work, the selective inversion recovery (SIR) technique described by Gochberg and Gore is used and described by the pulse sequence shown in Fig 2.11. This technique is based on a normal inversion recovery (IR) preparation followed by a Fast Spin Echo (FSE) readout; however the shortest inversion times ($T_I$) used are in the millisecond range, which is much shorter than typical IR sequences.

![Selective Inversion Recovery pulse diagram](image)

**Figure 2.11. Selective Inversion Recovery pulse diagram.** A 180-degree inversion pulse is followed by a Fast Spin Echo readout to acquire all lines of k-space.

The inversion pulse is much longer than the $T_2$ of bound $^1$H but much shorter than the $T_2$ of free $^1$H and utilizes a low power pulse to selectively invert the free water pool, while bound spins are
Magnetization transfer occurs between the two pools via dipolar cross-relaxation or chemical exchange and can be modeled by replacing $1/T_1$ in Eq. 2.7 with a matrix and adding a coupling term to the Bloch equations commonly known as the Bloch-McConnell equations (40–42) as seen below in Eqs. 2.27 and 2.28

$$\frac{dM_z}{dt} = L_1 (M_z(t) - M_0)$$  \hspace{1cm} [2.27]

$$L_1 = \begin{pmatrix} -(R_{1f} + k_{fb}) & k_{bf} \\ k_{fb} & -(R_{1b} + k_{bf}) \end{pmatrix}$$  \hspace{1cm} [2.28]

where, as before, $f$ and $b$ represent the free and bound pools, respectively, the equilibrium magnetization in both pools is $M_0 = [M_{0f}; M_{0b}], R_1$ is the longitudinal relaxation rate of each pool without MT, and $M_z(t)$ is the longitudinal magnetization at time $t$. From these equations, it is clear that in the case of no exchange ($k = 0$) only the first term remains and relaxation is only dependent on one pool as in Eq. 2.7. To describe acquired $M_z(t)$, Eq. 2.27 can be solved as before giving Eq. 2.29

$$M_z(t) = [M_z(0) - M_0] \exp(-L_1 t) + M_0$$  \hspace{1cm} [2.29]

where $L_1$ is the matrix in Eq. 2.28. While this describes inversion recovery data with full recovery ($T_R \gg T_1$), here full recovery is not reached and there is a finite delay time ($T_d$) between the last echo and subsequent inversion pulse. However, at the end of signal acquisition, both pools (free and bound) have no longitudinal magnetization ($M_z = 0$). The free pool is saturated after a 90-degree pulse followed by a train of 180-degree pulses and the bound pool is drawn to the free pool via the MT effect. Therefore, the initial condition, $M_z(0) = M_z(t = T_d)$ is the solution of Eq. 2.29 with $t = 0$ after acquisition readout, so $M_z(t = 0) = 0$ as shown in Eq 2.30
\[ M_z(T_d) = R[M_0 - M_0 \exp(-L_1T_d)] \]  \[ 2.30 \]

In Eq. 2.30, \( M_0 \) is dependent on the efficiency of the inversion pulse described by \( R = [S_f, S_b] \) where \( S_f \) and \( S_b \) describe the inversion and saturation effects of the inversion pulse on the free and bound pools, respectively. Based on mass balance between the two pools, \( k_{fb}M_{0f} = k_{bf}M_{0b} \). Therefore, from Eqs 2.28 and 2.30, there are 7 unknowns: \( R_{1f}, R_{1b}, k_{mf}, M_{0f}, M_{0b}, S_f, \) and \( S_b \). It has been shown in prior studies (37) that the fitting has little dependence on \( S_b \) and \( R_{1b} \). \( S_b \) has been estimated based on a 1-ms hard inversion pulse, a bound pool with Gaussian linewidth, and bound \( T_2 = 10-20 \) µs, providing \( S_b = 0.83 \) (37). With little dependence on \( R_{1b} \), we can set it equal to 1s for fitting. From this, the remaining 5 parameters are estimated and BPF = \( \frac{M_{ob}}{M_{0f} + M_{ob}} \).

2.5 REFERENCES


CHAPTER 3

MULTI-EXPONENTIAL T2 MYELIN WATER IMAGING IN EX-VIVO RODENT BRAIN

3.1 INTRODUCTION

Magnetic Resonance Imaging (MRI) offers the ability to non-destructively evaluate normal and diseased white matter across whole rodent brain. Through conventional MRI measures, image contrast has been shown to be sensitive to various white matter pathologies, such as demyelination (1–3), axonal damage (4–6), and edema (7,8); however, these approaches generally lack specificity. In order to relate changes in MRI contrast to specific changes in white matter microstructure, more advanced quantitative techniques have been developed. In particular, multi-exponential analysis of transverse relaxation (MET2) offers a relatively specific approach to myelin water imaging (MWI).

MET2 analysis of the water proton magnetic resonance signal from white matter provides a $T_2$ spectrum which is typically comprised of two signal components. The faster-relaxing signal component has been shown to primarily result from the water that is trapped between the lipid bilayers which comprise myelin (9,10), while the slower-relaxing component is primarily derived from the remainder of the water in both intra- and extra-axonal spaces. The myelin water fraction (MWF) is defined as the fraction of fast-relaxing component compared to the total water signal. Despite that this approach was first demonstrated in humans two decades ago (11), there is a paucity of reports of MWI in small animals. Gareau et al. demonstrated MWI in guinea pig brain (12), and a few papers have presented MWI in rat spinal cord (13–15), but to our knowledge MWI by MET2 analysis has not been effectively applied in rat or mouse brain. Does
and Gore used MET$_2$ analysis in studies of rat brain *in vivo* but did not find a reproducible myelin water signal from cerebral white matter, which was attributed to limitations of spatial resolution and signal-to-noise ratio (SNR) (16).

If resolution and SNR are the limiting factors, then using higher static magnetic field strengths ($B_0$) and imaging excised and chemically fixed brains, which permits long scan times, may provide the necessary resolution and SNR for effective MWI in rodent brains *ex vivo*. The SNR efficiency when imaging excised tissues can also be increased by loading the tissue with paramagnetic contrast agent to reduce longitudinal relaxation time ($T_1$) (17–19). However, both increased $B_0$ and use of paramagnetic agents will reduce $T_2$, making the MET$_2$ analysis more difficult, statistically, and so the benefit of either of these approaches for MWI is unclear.

In addition to SNR limitations, conventional multiple spin echo imaging necessary for MET$_2$ analysis assumes near perfect $B_1$ refocusing and gradient spoiling of stimulated echoes (20), which limits coverage of multi-slice or 3D acquisitions to highly uniform regions of the transmit radiofrequency (RF) coil. The extended phase graph (EPG) algorithm (21) can be used to account for $B_1^+$ variation in $T_2$ measurements (22,23), thereby alleviating this restriction. In short, the algorithm fits the effective refocusing pulse flip angle along with $T_2$, making $T_2$ estimates roughly independent of $B_1^+$.

Presented here is an evaluation of MWI at high and ultra-high field in rodent brain. We first determine the effect of gadolinium (Gd), (in the form of Gd-DTPA) on $T_1$ and $T_2$, of rat cerebral white matter at $B_0 = 7$ T and 15.2 T. Next, we consider the influence of choice of regularization technique for fitting MET$_2$ data. Then, we assess the benefits of higher field strengths and/or the addition of Gd. Then, we evaluate the reproducibility of MWF across animals and across
different concentrations of Gd loading. Finally, we evaluate the impact of scan parameters, namely echo time and receiver bandwidth, on MWF.

3.2 MATERIALS AND METHODS

3.2.1 Tissue Preparation

Animal studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Twelve adult female Sprague-Dawley rats were anesthetized with isoflurane and sacrificed via transcardial perfusion. The perfusion consisted of 1X phosphate-buffered saline (PBS) wash followed by 2.5% glutaraldehyde + 2% paraformaldehyde (modified Karnovsky solution). Following perfusion, brains were quickly removed from skull and immersed in the fixative solution for 1 week. Brains were then washed with 1X PBS + 0.01% sodium azide, changing wash 4-5 times over 1 week to remove excess fixative. In seven of eleven cases, Gd-DTPA (Magnevist; Berlex, Montville, NJ) at concentrations of 0.25 mM (2), 0.5 mM (3), or 1.0 mM (2) were included in the perfusate, immersion and wash solutions. The time course of fixation and loading tissues with Gd-DTPA was not optimized, but was consistent with previous studies (18,19) and no obvious signs of non-uniform Gd distribution were observed. Studies on larger brains may require longer preparation periods to achieve a uniform distribution of Gd.

3.2.2 Magnetic Resonance Imaging

Imaging was performed on two systems: i) a 7-T 16-cm horizontal bore Agilent/Varian (Santa Clara, CA) DirectDrive scanner, using a 38-mm diameter Litz quadrature coil (Doty Scientific, Columbia, SC) for transmission and reception, and ii) a 15.2-T 11-cm horizontal bore Bruker
(Rheinstetten, Germany) BioSpec scanner, using a 35-mm diameter Bruker quadrature volume coil for transmission and reception.

First, longitudinal and transverse relaxation times ($T_1$ and $T_2$) were measured in one brain with 0.5 mM and one brain without Gd at both 7 T and 15.2 T. For these measurements, low-resolution imaging allowed relatively rapid scans with high SNR, minimal echo spacing, and long repetition times ($T_R$). $T_1$ was measured using a slice-selective fast spin echo sequence with minimum echo time ($T_E$) and 8 logarithmically-spaced $T_R$ values ranging from minimum $T_R$ to $>3 \times$ the expected $T_1$. Slice thickness and in-plane voxel dimensions were each 500 µm. $T_2$ was measured using a 3D multiple spin-echo sequence with non-selective excitation and refocusing pulses, 160 µs and 100 µs in duration, respectively. Each refocusing pulse was surrounded by 428 µs duration 6 G/cm amplitude crusher gradients, phase-encoding gradients were rewound after each echo, and a two-part (+X/-X) phase cycling scheme was used. With these constraints, secondary echoes that were not excited by the initial excitation pulse were removed, and the observed echo magnitudes could be computed with the extended phase graph (EPG) algorithm (Hennig, 1991). Sampling of transverse signal decay was achieved with $T_E = 3.75$ ms, number of echoes ($N_E$) = 60, and receiver bandwidth ($BW$) = 50 kHz. Repetition times were set to be $\approx 3 \times T_1$ for each case. Images were encoded in a $64 \times 64 \times 64$ matrix over a $3.2 \times 3.2 \times 3.2$ cm$^3$ field of view (FOV) resulting in 500 µm isotropic resolution.

Based on analysis of these low resolution scans, high resolution (250 µm isotropic resolution) 3D multiple spin echo imaging was implemented in three brains without Gd and in three brains loaded with Gd = 0.25 mM, 0.5 mM, and 1.0 mM, respectively. In each case, scan parameters were predicted to be optimal for MWF estimation (see 2.3 Data Analysis) for a 4-hr scan time as seen in Table 3.1. These scans were encoded with a matrix size of $128 \times 64 \times 64$
over a $3.2 \times 1.6 \times 1.6$ cm$^3$ FOV. High-resolution data were zero-padded to $256 \times 128 \times 128$ resulting in images reconstructed to 125 µm isotropic resolution.

Table 3.1. **Scan Parameters used at 7T and 15.2T** Parameters were found based on predicted maximum MWF SNR (See 3.2.3 Data Analysis) for a 4-hr scan duration and defined concentration of Gadolinium ([Gd]). Optimal repetition times ($T_R$), echo times ($T_E$), number of echoes ($N_E$), number of excitations ($N_{EX}$), and receiver bandwidths ($BW$) are shown.

<table>
<thead>
<tr>
<th>[Gd] (mM)</th>
<th>$T_R$ (s)</th>
<th>$T_E$ (ms)</th>
<th>$N_E$</th>
<th>$N_{EX}$</th>
<th>$BW$ (kHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.76</td>
<td>7.2</td>
<td>37</td>
<td>2</td>
<td>29.76</td>
</tr>
<tr>
<td>0.25</td>
<td>0.88</td>
<td>8.6</td>
<td>24</td>
<td>4</td>
<td>20.98</td>
</tr>
<tr>
<td>0.5</td>
<td>0.88</td>
<td>9.1</td>
<td>24</td>
<td>4</td>
<td>19.39</td>
</tr>
<tr>
<td>1.0</td>
<td>0.59</td>
<td>11.5</td>
<td>15</td>
<td>6</td>
<td>14.22</td>
</tr>
<tr>
<td>15.2T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.76</td>
<td>5.5</td>
<td>36</td>
<td>2</td>
<td>42.61</td>
</tr>
<tr>
<td>0.25</td>
<td>0.88</td>
<td>6.4</td>
<td>26</td>
<td>4</td>
<td>32.89</td>
</tr>
<tr>
<td>0.5</td>
<td>0.88</td>
<td>6.9</td>
<td>24</td>
<td>4</td>
<td>29.09</td>
</tr>
<tr>
<td>1.0</td>
<td>0.59</td>
<td>8.6</td>
<td>16</td>
<td>6</td>
<td>20.98</td>
</tr>
</tbody>
</table>

To assess sensitivity of MWF to $T_E$, we also acquired 2D MET$_2$ data from an EPG-compatible sagittal single slice acquisition. To maintain the same refocusing pulse through the slice, the 180-degree hermite pulse bandwidth was 3.75X the 90-degree Gaussian pulse
bandwidth. At both 7T and 15.2T, we acquired 6 scans at 6 different \( T_E \) equally space between 4.5/4.94ms and 15/18ms at 7/15.2T with minimum receiver bandwidth per \( T_E \). For each scan, \( T_E \)* \( N_E = 3.9*T_{2,\text{IE}} \), \( T_R \) provided 82% signal recovery, and \( NEX = \) max multiple of 2 for scan time ~ 20 minutes. This was performed in four brains loaded with Gd = 0, 0.25 mM, 0.5 mM, and 1.0 mM resulting in 36 MET\(_2\) scans per brain per field strength. Scans were encoded with a matrix size of 128 \( \times \) 64 over a 3.2 \( \times \) 1.6 cm\(^2\) FOV with 0.75mm slice thickness. High-resolution data were zero-padded to 256 \( \times \) 128 resulting in images reconstructed to 125 \( \mu \)m in-plane resolution.

3.2.3 Data Analysis
All data analysis was performed using MATLAB R2013b (The Mathworks, Natick MA) and MET\(_2\) analyses were performed using the freely available Multi Exponential Relaxation Analysis (MERA) toolbox (http://www.vuiis.vanderbilt.edu/~doesmd/MERA/MERA_Toolbox.html). Prior to Fourier reconstruction, k-space data for all images were apodized using a 2D or 3D Tukey window with a 0.25 taper-to-window ratio. The low-resolution variable-\( T_R \) image magnitudes from a region of interest comprising the corpus callosum were fitted to a 3-parameter mono-exponential function to estimate \( T_1 \), with and without contrast agent. The longitudinal relaxivity of Gd was then computed as \( r_1 = (1/T_{1\text{Gd}}-1/T_{1\text{noGd}})/0.5\text{mM} \) and assumed to be the same for all white matter water.

For MET\(_2\) analysis of the low-resolution images, the \( N_E \) echo magnitudes from each image voxel were fitted to a linear combination of signals described by 2 gaussian-shaped components in the log\(-T_2\) domain and a refocusing pulse flip angle (\( \theta \)), as outlined in Appendix A. Thus, for each voxel, five independent parameters were fitted: \( \theta, f_S, T_{2S}, f_L, T_{2L} \), where subscripts S and L

35
indicate the short-lived and long-lived components, respectively. In cases where $T_{2S}$ fell within a certain domain, $T_{2\text{myelin,min}} \leq T_{2S} \leq T_{2\text{myelin,max}}$, this signal component was defined as a myelin water signal, with signal fraction and $T_2$ defined as $f_M$ and $T_{2M}$, respectively. Similarly, $f_{IE}$ and $T_{2IE}$ were defined for intra- and extra-axonal spaces from signals components with $T_{2\text{IE,min}} \leq T_{2L} \leq T_{2\text{IE,max}}$. Defining myelin and intra-/extra-axonal water signals this way automatically excluded spurious second components when the signal was effectively mono-exponential, without having to do both one- and two-component fits. Domain boundaries ($T_{2M/IE,min/max}$) were determined from representative measured spectra—see 3. Results. Finally, transverse relaxivity was computed as $r_2 = (1/T_{2L,Gd}-1/T_{2L,\text{noGd}})/0.5$ mM. In principle, one could measure relaxivity of the short- and long-$T_2$ signals independently, but the precision of $T_2$ estimates for the long-$T_2$ component is much greater than that of the short $T_2$ (24,25) and a previous study suggests that loading the tissue with Gd affects both components approximately equally (26). Consequently, the same $r_2$ value was used for both short- and long-$T_2$ signals.

Given these relaxation and relaxivity measures, a predictive two-component signal model for multiple spin echo measurements was defined as

$$S_n = \left[ f_M \exp\left(-nT_{E} \left(\frac{1}{T_{2\text{M, noGd}}} + r_2 C_{Gd}\right)\right) + f_{IE} \exp\left(-nT_{E} \left(\frac{1}{T_{2\text{IE, noGd}}} + r_2 C_{Gd}\right)\right) \right]$$

$$\times \left[ 1 - \exp\left(-T_R - N_E \times T_E \left(\frac{1}{T_{1,\text{noGd}}} + r_1 C_{Gd}\right)\right) \right],$$

[3.1]

where $n$ indicates echo number, $C_{Gd}$ is the concentration of Gd in the tissue preparation solutions, and all other terms are as previously defined. With this signal model and the known relationship between image noise variance and receiver bandwidth, the Cramér-Rao lower bounds (CRLB) of
variance of $f_M$ were formulated as a function of $T_E$, $N_E$, $T_R$, $C_{Gd}$, image matrix size, number of averaged excitations ($N_{EX}$), and total scan time ($T_{scan}$). Details of this relationship are omitted here, but follow a similar prior analysis (25) with the addition of a $T_R$ and $C_{Gd}$ to the signal equation. Using CRLB calculations, optimal values (for minimum coefficient of variation of $f_M$) of $T_E$, $N_E$, and $T_R$ were computed assuming a $128 \times 64 \times 64$ acquisition matrix, $N_{EX}$ equal to a multiple of 2 (to provide averaging and the add/subtract phase-cycle), and a range of total scan times and $C_{Gd}$ values. From these CRLB calculations, the predicted myelin water SNR was defined as $f_M/\sigma_M$, where $\sigma_M$ is the square root of the CRLB of variance of $f_M$, and myelin water SNR efficiency was defined as $f_M/\sigma_M/\sqrt{T_{scan}}$, where $T_{scan}$ is the total scan duration.

Since there is no standard for $T_2$-spectral fitting, the MET$_2$ analysis of high-resolution multiple spin echo data were fit using 4 different methods to explore options. $T_2$ spectra were estimated using the multiple-gauss fitting along with three variations of a conventional linear inverse approach (27). Compared to low-resolution data, there was one key difference in the multiple-gauss fitting. As some previous literature has found that 3 $T_2$ components may be necessary to describe transverse relaxation in white matter (28,29), multiple-gauss fitting was implemented with 3 components, although spectral analysis reduced the results to five independent parameters, ($\theta, f_M, T_{2M}, f_{IE}, T_{2IE}$), as described above. Again, this permitted identical analysis of all voxels did not require repeated fitting with varied number of components. For comparison, one brain was also analyzed using only 2-gaussian components. For the three linear inverse methods, data were fit by non-negative least-squares (NNLS; (30)) to the sum of signals from 100 logarithmically spaced $T_2$ components, as defined by the EPG algorithm and spanning $T_e/2$ to 500 ms in the $T_2$ domain, similar to previous work (23). In two variations the spectra were regularized using a minimum curvature constraint with either regularization adjusted for
each voxel via the generalized cross validation (GCV) method (31) or a conservative regularization held constant across all voxels. In the third variation, the spectra were not regularized.

3.3 RESULTS

From the low resolution scans, Fig 3.1 shows the voxels exhibiting $\geq 5\% f_M$ and the average of their $T_2$ spectra, with and without Gd, and at both 7 T and 15.2 T. The corresponding measured $T_{2M}$ and $T_{2IE}$ values are shown in Table 3.2 and are generally consistent with literature (18,26,32,33) although there are no directly comparable studies. Likewise, the measured relaxivities, both $r_1$ and $r_2$ (measured from the long-$T_2$ signals) are consistent with previous literature in value and dependence on $B_0$ (18,34). It is also apparent from Table 3.2 that transverse relaxivity measured from the myelin water component did not yield expected results, with values of $\approx 120$ s$^{-1}$/mM at 7 T but close to 66 s$^{-1}$/mM at 15.2 T, thereby supporting the use of long-$T_2$ signals only to estimate $r_2$. 
Figure 3.1. Mean $T_2$ spectra (left) of voxels with $>5\%$ Myelin Water Fraction ($f_M$) (right). At 7T and 15.2T doping with Gadolinium (Gd) (dashed) lowers $T_2$ as compared to undoped tissue (solid) as expected. Myelin water cutoff values were found from the $T_2$ with the lowest amplitude between the $T_{2M}$ and $T_{2IE}$ peaks.

Table 3.2. Longitudinal and transverse relaxation times ($T_1$ & $T_2$) and corresponding relaxivity values ($r_1$ & $r_2$). Two-component $T_1$ and $T_2$ peaks and relaxivity measures were found as explained in Data Analysis at 7T and 15.2T for brains loaded with 0mM and 0.5mM of Gadolinium ($C_{\text{Gd}}$) from voxels with $>5\%$ myelin water fraction ($f_M$) (see Fig 3.1).

<table>
<thead>
<tr>
<th>$C_{\text{Gd}}$ (mM)</th>
<th>0</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_1$ (ms)</td>
<td>$T_2$ (ms)</td>
</tr>
<tr>
<td>7 T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelin Water</td>
<td>13.8 ± 0.5</td>
<td>7.55 ± 0.5</td>
</tr>
<tr>
<td>I/E Water</td>
<td>1498 ± 27</td>
<td>59.3 ± 0.8</td>
</tr>
<tr>
<td>15.2 T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelin Water</td>
<td>10.7 ± 0.6</td>
<td>7.9 ± 0.4</td>
</tr>
<tr>
<td>I/E Water</td>
<td>1498 ± 27</td>
<td>59.3 ± 0.8</td>
</tr>
</tbody>
</table>
Fig 3.2 shows a plot of predicted MWF SNR efficiency for high resolution imaging as a function of $C_{\text{Gd}}$ at both 7 T and 15.2 T. The results predict that both increasing $B_0$ and loading tissue with Gd offer some advantage for MET$_2$ analysis in excised and fixed rat brain. CRLB calculations predict maximum SNR efficiency at $C_{\text{Gd}} = 0.2$ and 0.3 mM at 7 T and 15.2 T, respectively, with a net benefit compared to no Gd up to $C_{\text{Gd}} \approx 0.5-0.75$ mM. While obtaining highest possible SNR is desirable, scan duration may be the limiting factor. Therefore, as shown before, Table 3.1 displays scan parameters required to obtain maximal predicted $f_M$ SNR given a 4hr scan time and $C_{\text{Gd}} = 0, 0.25, 0.5$, and 1.0mM. A less intuitive observation from Table 3.1 is that optimal $T_E$ increases with increasing $C_{\text{Gd}}$—one might expect the opposite, since $T_2$s are reduced. However, as the ratio of long- to short-$T_2$ values becomes smaller (i.e., the two signal components are relaxing at a more similar rate) it is predicted from simulation and CRLB calculations that increased SNR to separate them in fitting, obtained through the lower receiver bandwidth afforded by the increased $T_E$ is beneficial (25).

Figure 3.2. Myelin Water Fraction SNR Efficiency versus concentration of Gd ($C_{\text{Gd}}$) doping at 7T and 15.2T. Curves are based on relaxivity measures (see Table 3.2) and Cramér-Rao lower bounds (CRLB) of variance calculations of myelin water fraction ($f_M$). For each $C_{\text{Gd}}$, the maximum SNR efficiency is found for any scan duration.
The comparison of different MET\textsubscript{2} analysis methods is presented in Figs 3.3 – 3.5. Fig 3.3 shows $f_M$ maps derived from (left) 2- and (right) 3-gaussian component fitting as well as corresponding example spectra from a voxel in the internal capsules. The $f_M$ maps are similar or identical for most of the voxels in the corpus callosum and external capsules, but show noticeably greater $f_M$ values in the internal capsules and much of the sub-cortical gray matter. The cause of this effect is apparent from the spectra. The 3-component spectrum shows a typical myelin water signal ($T_2 \approx 10$ ms), an intermediate $T_2$ component ($T_2 \approx 40$ ms) and a small longer-lived component ($T_2 \approx 100$ ms). Fitting this same signal with only two components results in the shorter-lived component capturing both myelin water and some intermediate $T_2$ signals. For voxels adequately described by two components, all fitted components with $T_2$ in the aforementioned myelin $T_2$ domain were summed to define the myelin water signal. The only cost to using 3-component fitting was computation time, but fitting every voxel with three components was still much faster than fitting each voxel separately with 1-, 2-, and 3-components then using a statistical test to select the most appropriate fit.

![Figure 3.3. 2-Gaussian versus 3-Gaussian component fitting.](image)

Figure 3.3. 2-Gaussian versus 3-Gaussian component fitting. Increasing the number of Gaussian components on the log-$T_2$ domain to 3, greatly reduced $f_M$ in the internal capsules and voxels near borders. The difference in the two spectra from a representative voxel (right) displays the decrease in $f_M$ as the error of the fit decreased.
Of course, the need to define any number of components can be effectively removed by using a conventional linear inverse approach (27). Fig 3.4 shows representative $f_M$ maps derived from the multiple gauss fitting and three linear inverse approaches with different spectral regularizations (GCV-adjusted minimum curvature, constant minimum curvature, or unregularized). Qualitatively, all four maps are similar, so one could argue that any one of these approaches is reasonable. The map from unregularized spectra, in particular, shows greater $f_M$ values, while the multiple gauss fitting produced the smoothest maps. Fig 3.5 quantitatively displays $f_M$ and standard deviation from an ROI of the corpus callosum from each brain in Fig 3.4. Generally, we see expected results, with an increase in $f_M$ and standard deviation from multiple gaussian fitting to unregularized fitting at both 7T and 15.2T. Additionally, we see similar results from $C_{Gd} = 0$ and 0.5mM at each field strength. The average root mean square error across the different analysis methods were all <0.25% of maximum signal amplitude. With no gold-standard, absolute accuracy of the different fitting methods cannot be evaluated, so spatially-invariant minimum curvature spectral regularization was used for all subsequent MET analysis because it was relatively fast and yielded similar results to other regularized spectral methods.
Figure 3.4. Myelin Water Fraction ($f_M$) maps at 7T and 15.2T doped with 0 and 0.5mM Gd and analyzed using 4 different MET$_2$ data fitting techniques. MET$_2$ data was collected using optimal scan parameters (see 2. Materials and Methods) at 7T (top) and 15.2T (bottom). $f_M$ was deemed as the fraction of signal with a peak $T_2 < T_{2M}$ (see Fig 3.1) after MET$_2$ analysis based on four techniques: i) three Gaussian-components in the log-$T_2$ domain ii) Non-Negative Least Squares (NNLS) regularized with a minimizing curvature constraint with regularizing parameter adjusted for each voxel by generalized cross-validation, iii) NNLS regularized with a constant regularizing parameter for all voxels, and iv) NNLS without regularization.
Figure 3.5. Myelin Water Fraction ($f_M$) differences from choice of MET$_2$ analysis at 7T and 15.2T with 0 and 0.5mM Gd. $f_M$ and standard deviation at (left) 7T and (right) 15.2T from MET$_2$ analysis based on four techniques: i) three Gaussian-components in the log-$T_2$ domain ii) Non-Negative Least Squares (NNLS) regularized with a minimizing curvature constraint with regularizing parameter adjusted for each voxel by generalized cross-validation, iii) NNLS regularized with a constant regularizing parameter for all voxels, and iv) NNLS without regularization with (dark gray) 0mM and (light gray) 0.5mM Gd.

High resolution $f_M$ maps obtained using the predicted optimal 4-hour scan protocols for the case of $C_{Gd} = 0$ and 0.5 mM (See 2.2 Magnetic Resonance Imaging and Table 3.1), and at both 7 T and 15.2 T, are shown in Fig 3.6. Across scan protocols (with and without Gd at both field strengths), $f_M$ maps display qualitatively similar results in white matter and expected $f_M \approx 0$ in gray matter. The $f_M$ is $\sim 10$-15% in most white matter, consistent with similar observation in human brain values (11,23). The concurrently fitted $B_1^+$ maps in Fig 3.7 from a $C_{Gd} = 0$ mM brain (results were similar at $C_{Gd} = 0.5$ mM) are generally smooth and primarily varying in the axial direction, as expected for $B_1^+$ variation that is primarily induced by the geometry of the RF coil. Some differences in $B_1^+$ between white and gray matter regions are apparent, (particularly in the axial view of the images collected at 15.2 T), although these differences are small and had little impact on the fitted $T_2$ spectra. Fig 3.8 displays mean $f_M$ and standard deviation from three repeated scans for several regions of interest in the sagittal and axial slices. Mean $f_M$ is similar in
each region between scan protocols, but variations between 7T and 15.2T are generally larger than differences between \( C_{\text{Gd}} \) at the same field strength. This is consistent with previous works comparing MWF at 1.5T and 3.0T (35,36), although it is not entirely clear why MWF increases with \( B_0 \).

**Figure 3.6.** Myelin Water Fraction (\( f_{\text{M}} \)) maps at 7T and 15.2T with 0 and 0.5mM Gd. MET\(_2\) data was collected using optimal scan parameters (see 2. Materials and Methods) at 7T and 15.2T with \( C_{\text{Gd}} = 0\) mM (top) and 0.5mM (bottom). \( f_{\text{M}} \) was deemed the fraction of signal with a peak \( T_2 \leq T_{2\text{M}} \) (see Fig 3.1) after MET\(_2\) analysis using NNLS and a defined regularization parameter (see 3.2.3 Data Analysis).
Figure 3.7. \( B_1^+ \) maps from 7T and 15.2T. \( B_1^+ \) is found from the Extended Phase Graph (EPG) algorithm (see 3.2.3 Data Analysis). \( B_1^+ \) at 15.2T varies more in the axial direction than at 7T and \( B_1^+ \) in the central axial plane at both fields is uniformly \( \sim 170-180^\circ \), as expected.

Figure 3.8. Region of Interest (ROI) \( f_M \) values across scans. Similar ROIs were drawn in each region of the brain for scans at 7T and 15.2T with \( C_{\text{Gd}} = 0 \) and 0.5mM. Three ROIs in the sagittal slice were the middle corpus callosum (Mid CC), Splenium (Spl), and Genu. Three ROIs in the axial slice were the CC, external capsule (EC), and internal capsule (IC).

Reproducibility of \( f_M \) maps between animals is demonstrated in Fig 3.9 and 3.10. Fig 3.9 displays maps from approximately the same axial slice in 3 different rat brains (a, b, and c), all with \( C_{\text{Gd}} = 0 \) mM acquired at 7T and 15.2T. Qualitatively, all maps look similar between field
strength and across brains. Fig 3.9 also displays quantitative measures of $f_M$ and standard deviation in a corpus callosum ROI in each brain at both field strengths. We see no significant differences between brains, but higher $f_M$ at 15.2T compared to 7T as discussed above. Similarly, Fig 3.10 displays $f_M$ maps of 4 rat brains with $C_{Gd}=0$ mM, 0.25 mM, 0.5 mM, and 1.0 mM, each acquired with predicted optimal 4-hr protocols, respectively at 15.2T. All maps in Fig 3.10 exhibit comparable results, which displays consistency over various $C_{Gd}$. Fig 3.10 also shows quantitative differences in $f_M$ from a corpus callosum ROI of each slice. We see no significant differences from $C_{Gd}=0$ mM – 1mM, but there might be a slight increase as $C_{Gd}$ increases.

**Figure 3.9.** $f_M$ maps of three different rat brains (A–C) with $C_{Gd}=0$ mM at 7T (left) and 15.2T (right). Maps display strong similarities between 7T and 15.2T for each brain. Based on similar axial slices, all brains (A–C) show comparable $f_M$ maps. Bar graph displays $f_M$ and standard deviation in the corpus callosum of each brain at 7T and 15.2T with no significant differences between brains, but an increase with field strength.
Figure 3.10. $f_M$ maps of four rat brains with $C_{\text{Gd}}$=0mM, 0.25mM, 0.5mM, and 1.0mM at 15.2T obtained from predicted optimal protocols for each $C_{\text{Gd}}$. Based on similar axial slices, all brains show comparable $f_M$ maps. Bar graph displays $f_M$ and standard deviation in the corpus callosum of each brain with no significant differences between $C_{\text{Gd}}$ but a slight increase may be due to acquisition parameters used ($T_E$).

Finally, we assessed the optimal parameter predictions from CRLB calculations used here and proposed by Dula et al (25). Fig 3.11 displays (top) $f_M$ and (bottom) standard deviation across a corpus callosum ROI from 2D MET$_2$ sagittal scans at 7T and 15.2T. Data was acquired at 6 different $T_E$ with $C_{\text{Gd}}$ = 0, 0.25, 0.5, and 1.0mM (See 3.2.2 Magnetic Resonance Imaging). Contrary to CRLB predictions, for both 7T and 15.2T, we see the best agreement in $f_M$ across $C_{\text{Gd}}$ at the shortest $T_E$. As $T_E$ increases, $f_M$ tends to increase, likely due to statistically more challenging data fitting, especially with $C_{\text{Gd}}$ = 0.5 or 1.0mM. This could explain the slightly higher $f_M$ with increasing $C_{\text{Gd}}$ in Fig 3.10 due to longer $T_E$ used for predicted optimal SNR (see Table 3.1). 7T data suggest potentially some benefit in using slightly longer than minimum $T_E$ (7.6ms) affording lower bandwidth to increase SNR and lower standard deviation.
Figure 3.11. Mean and standard deviation of \( f_M \) at 7T and 15.2T. Results are from an ROI from the corpus callosum on a sagittal slice from 2D MET2. Data shown from four different \( C_{Gd} \).

Fig 3.12 displays mean +/- standard deviation of image SNR from 7T and 15.2T at each echo time. First, we see that image SNR at the shortest echo time (minimal \( T_2 \)-weighting) at 15.2T is ~2.23X image SNR at 7T at all \( C_{Gd} \), as we would expect (15.2÷7 = 2.17). Next, we can see that image SNR is > 500 in all cases at 15.2T, so this explains minimal benefit to increasing \( T_E \) since SNR is already high at minimum \( T_E \). In contrast at 7T, image SNR with \( C_{Gd} = 0 \)mM is ~270 at minimum \( T_E \). In combination with Fig 3.11, this shows that as \( B_0 \) decreases and \( T_2 \) increases, there may be some benefit to slightly longer \( T_E \) to increase SNR, especially without or with minimal \( C_{Gd} \). But in opposition to the CRLB predictions, at both field strengths, shorter \( T_E \) (< \( T_{2,M} \)) is likely the best choice for consistent \( f_M \).
3.4 DISCUSSION

The results demonstrate feasibility of whole brain MWF mapping in excised and chemically fixed rat brains. The primary conclusions based on the experimental data, are i) increased $B_0$, up to at least 15.2 T, provides increased SNR efficiency with little effect on $f_M$, and ii) loading tissue with up to $\approx 1$ mM Gd improves or modestly decreases $f_M$ precision. While Fig 3.11, especially at 15.2T, does not agree with predictions from the two-pool model, this is not surprising since tissue is much more complicated than bi-exponential water components. As noted above, another factor that may be limiting the accuracy of the two-pool model is the assumption that both pools are affected equally by the addition of contrast agent. In the least, the model and experimental data support the idea that MWI of ex vivo rodent brains can be extended to ultra-high field and will tolerate the use of some Gd, with little or no cost in $f_M$ precision with $T_E < T_{2,M}$. These observations are important because both increased $B_0$ and the use of Gd have been shown previously to benefit other ex vivo brain imaging protocols (17,37,38). For example, preparing
monkey brains slices with 1 mM Gd was previously recommended for optimizing diffusion weighted imaging 4.7T (18).

Between the four protocols demonstrated in Figs 3.4 and 3.5, \( f_M \) maps are similar but not identical, suggesting that comparing \( f_M \) values should be restricted to data acquired at the same \( B_0 \) and loaded with the same concentration of Gd. Similarly, because MET\(_2\) data analysis is an inherently ill-posed inverse problem, the choice of fitting technique will impact results. The linear inverse approach has the advantage of not requiring any a priori knowledge about the number of distinct \( T_2 \) components; however, one must still decide on the type and extent of spectral regularization, and how to extract a finite number of signal components from the fitted spectrum. Fitting multiple gaussian shaped components requires a priori determination of the number of components and the width of these components, presenting the potential for a poor fit. However, as noted here, there is little cost in over-estimating the number of components somewhat, and this approach brings two other advantages: a reduction in the degrees of freedom of the problem, resulting in greater fitted parameter precision, and the ability to accurately account for overlapping spectral components. While Fig 3.4 demonstrates somewhat smoother \( f_M \) maps from multiple gaussian fitting and minimum curvature with voxel-wise adjustment of regularization, these techniques are time-consuming and may not be appropriate for whole brain applications without increased computer power. For example, to estimate 7,355 non-zero voxels in one axial slice implemented using MATLAB and run on a computer with 3.0GHz AMD Phenom processor with 16Gb RAM, average processing times were 1.85, 3.24, 0.070, and 0.036 sec/voxel for multiple-gaussian, GCV-adjusted minimum curvature, minimum curvature with constant regularization weighting, and unregularized fit, respectively. Therefore, in the current case, constant regularization weighting provides consistency with commonly used methods
which employ some spectral regularization while obtaining large-scale results in a time-efficient manner. Ultimately, the important point is that any number of MET$_2$ analysis routines may be used to produce similar results.

One aspect of the MET$_2$ analysis that is demonstrated to be critical for whole brain studies is the inclusion of B$_1^+$ as a free parameter. For a single axial slice, it is likely that one can calibrate effective refocusing to be 170°-180° across the entire slice, as we see from central axial slices in Fig 3.7. At this range of flip angles the refocusing is > 99% efficient ($\sin^2(170°/2) = 0.992$), so spoiling the non-refocused signal will increase apparent transverse relaxation rate only modestly by $-\log\{\sin^2(\theta/2)\}/T_E < 1$ s$^{-1}$ for $T_E \geq 7.5$ ms (39). However, for flip angles of 140°, 150°, and 160°, (as can be seen at the axial extremes of the rat brains in Fig 3.6) the corresponding apparent rate increases with $T_E = 7.5$ ms are a much larger 16.6, 9.2, and 4.1 s$^{-1}$, respectively. Without B$_1^+$ fitting as part of the MET$_2$ analysis, these rate increases will shift the $T_2$ spectrum to the left, reducing the ratio of long- to short-$T_2$ values and, therefore, result in noisier $f_M$ maps. Additionally, as $\theta$ deviates further from 180°, greater crusher gradient areas are required to effectively remove non-spin-echo signals. For example, dephasing transverse magnetization by $2\pi$ radians across a 250-µm thick slice using a 1-ms crusher gradient requires approximately 100 mT/m gradient amplitude, so a multiple spin echo sequence using linearly modulated crusher gradients (40) requires $N_E/2 \times 100$ mT/m peak gradient amplitude. Typical gradient systems available on small animal MRI systems have a maximum amplitude of 400 – 1000 mT/m, making proper spoiling impractical to implement. B$_1^+$ fitting can account for spatial variations in flip angle and reduce effects from these confounding factors in MET$_2$ data.

While the methods described here were based on rat brain model predictions, they were also tested on an ex vivo mouse brain as demonstrated in Fig 3.13. The voxel size was decreased
to 150 µm isotropic resolution and scan time was increased to 6 hours to increase SNR. The brain was doped with 1 mM of Gd, because it was prepared for a separate study including diffusion weighted imaging. Using calculated parameters from our model of $T_R/T_E/N_E/BW/N_{EX} = 520 \text{ ms}/5.8 \text{ ms}/18/38.5 \text{ kHz}/6$ and fit using the NNLS fitting with a constant minimum curvature regularization as above, $f_M$ maps were obtained in mouse brain at 15.2 T. Maps display comparable results to those from rat brain (Fig 3.5), with $f_M \sim 10$-15% in most white matter, demonstrating the ability to extend MWI to ex vivo mouse brain.

![Mouse $f_M$ maps collected at 15.2T.](image)

**Figure 3.13. Mouse $f_M$ maps collected at 15.2T.** (Left to right) Images are from the axial, sagittal and coronal planes with respect to the magnet, respectively. Data was collected using a 6 hour protocol at 150µm isotropic resolution and tissue was loaded with 1mM of Gd.

### 3.5 CONCLUSIONS

Using a two-pool model of transverse relaxation in white matter as well as measurement relaxation and Gd relaxivity characteristics in rat brain at 7 T and 15.2 T, predicted optimal approaches for MWI in ex vivo rodent brains is presented. While the results do not fully support the simple two-pool model, the results demonstrate that both increasing $B_0$ and the loading of tissue with Gd provides improved SNR efficiency with minimal effect on MWF. Whole brain high-resolution (250 µm isotropic) MWF maps were demonstrated with 4hr scan times, and results were roughly consistent across a range of Gd concentrations and between 7 T and 15.2 T. Mouse brain MWI was demonstrated at 150 µm isotropic resolution in a 6-hr scan with similar results. Additionally, we show that it is not critical to optimize protocols, but $T_E < T_{2,M}$ should be used.
3.6 APPENDIX

MULTIPLE GAUSSIAN FITTING

Fitting the multiple spin echo data to a $T_2$ spectrum and an effective refocusing pulse flip angle, $\theta$, was performed as follows. First, the linear inverse solutions followed the method described by Prasloski et al (23) and briefly restated here. Let column vector $d$ define the $N_E$ spin echo signals; let column vector $s$ define the full fitted $T_2$ spectrum, $N_T$ elements long; and let $A(\theta)$ be the system matrix that defines a normalized signal amplitude at every echo time, $t_i$, $i = 1$ to $N_E$, for every time constant $T_{2,j}$, $j = 1$ to $N_T$, and refocusing flip angle $\theta$. This $A$ matrix can be computed using the EPG algorithm (21). The signal equation is then

$$d = A(\theta)s$$  \hspace{1cm} [A1]

and $s$ can be estimated by NNLS,

$$s = \arg \min_s \| A(\theta)s - d \|, \quad s \geq 0$$  \hspace{1cm} [A2]

given $d$ and $A(\theta)$. Since $\theta$ is unknown, it must be estimated as $\hat{\theta}$; to do so, a system matrix $A(\theta_k)$ was computed for each of $k = 1$ to $N_\theta$, where the range of $\theta$ values spanned the expected flip angles. For the present work, $N_\theta = 10$ and $\theta$ was uniformly spaced between $130^\circ$ and $180^\circ$. For linear inverse solutions, the sum square errors, $e_k$, were computed as

$$e_k = \| A(\theta_k)s_k - d \|, \quad k = 1 \text{ to } N_\theta$$  \hspace{1cm} [A3]
A spline interpolation was then used to compute $\theta$ as the flip angle that minimizes the fit error, and the final spectral estimate, $s$, was computed from Eq [A2] with system matrix $A(\theta)$.

Extending this framework to multiple gaussian component fitting was done by decomposing the spectrum $s$ into a linear combination of $N_G$ gaussian shaped spectral components, then employing a separable least squares approach (Golub and Pereyra, 2003). That is, let

$$s = G(\alpha)f$$

where $G(\alpha)$ is a $N_T \times N_G$ matrix, each column being a gaussian shaped function in log-$T_2$ space, centered at $T_{2,l} = \alpha_l$ for $l = 1$ to $N_G$, and $f$ is a column vector of $N_G$ component amplitudes. Therefore,

$$d = A(\theta)G(\alpha)f$$

The component $T_2$ values are estimated by non-linear regression

$$\hat{\alpha} = \arg \min_\alpha \| A(\theta)G(\hat{\alpha})f(\hat{\alpha}) - d \|$$

where

$$f(\alpha) = \arg \min_f \| A(\theta)G(\alpha)f - d \|, \quad f \geq 0.$$ 

As above, $\theta$ is estimated by repeating the process over a range of flip angles.

3.7 REFERENCES


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CHAPTER 4

DEVELOPMENT OF HISTOLOGY ANALYSIS METHODS

4.1 INTRODUCTION

Histology is the gold standard for validation of MRI parameters; however, there is no standard technique to quantitatively analyze electron microscopy images to obtain robust measures of features such as axon diameter and myelin thickness. This is exhibited by numerous groups utilizing different, independently written in-house code to analyze microscopy images (1–4). Generally, images can be segmented manually, semi-automatically or fully-automatically. Manual segmentation provides flexibility for variations in images but is time-consuming and user-dependent. Conversely, fully automatic segmentation requires no user-input but must be very robust to adapt to variability between image illumination, structure, etc. Instead, semi-automatic segmentation decreases user-dependency but provides some user-control to ensure proper segmentation.

Segmentation algorithms are based on one or a combination of a few different techniques: intensity thresholding, feature detection, morphological filtering, region accumulation, and deformable model fitting. Intensity thresholding assumes objects of interest are significantly different from the background. These are the simplest methods and remain a predominantly used segmentation technique. Feature detection utilizes a unique property such as a characteristic shape of the objects of interest to pick it out. Morphological filtering is generally
used as a pre- or post-processing step and uses operators such as erosion, dilation, etc. to edit the geometric properties of the image. Region accumulation begins at a seed point and depicts neighborhood regions with similar properties. Finally, deformable model fitting, such as snakes, minimizes the deformation energy based on a priori image information. Here, we use several of these techniques to analyze electron microscopy images via manual and semi-automatic methods to obtain a robust segmentation method (5).

4.2 MEASURING AXON DIAMETER (d)

4.2.1 Manual Measurement

We used a binary image defined manual global threshold, defined by the nadir of the image histogram as described below (See 4.4.1 Global Threshold). To measure axon diameter (d), we manually drew perpendicular line across the major and minor axes of the axon as shown by the blue lines in Fig 4.1. The axon diameter for each axon was the mean of these two measurements.

Figure 4.1. Manual axon diameter measurements. Two lines are manually draw across the major and minor axes of the axon (blue lines).
This method was very user dependent in the following ways: 1) the user determined which axes were the major and minor axes, which is even more difficult in irregularly-shaped axons, 2) measurements were dependent on how perpendicular the user drew lines, and 3) this method was time-intensive on the user.

4.2.2 Region-growing Method

To decrease user-dependency from manual measurements of \( d \), we implemented a region-growing technique (6). The user selected a seed point within each axon on the threshold image and pixels \( = 1 \) inside axons but not deemed by the operator to be myelin were manually set \( = 0 \). After this, from the seed voxel the region growing algorithm \( (\text{region\_growing}) \) grew. Each pixel was compared to the region mean intensity and grown until it reached a pixel with a difference larger than 0.1 as shown in Fig 4.2. The diameter of each axon \( (d) \) was then computed from its corresponding area \( (a) \) as \( d_i = 2\sqrt{a_i/\pi} \), where \( i = 1: \text{number of axons} \). However, while this method was much more automatic and less user-dependent, it was still very time-dependent for the user. Since the region-growing algorithm can continue growing through pixel gaps, the user needed to ensure that every myelin edge from the threshold image was sealed to prevent a leak of the region-growing area which is very tedious and user-time-intensive.
Figure 4.2. Region growing axon diameter measurements. Axon region is grown from a seed point in each axon, with colors displaying axon diameter ($d$).

4.2.3 Snakes (Active contour model) Method

To decrease the sensitivity of the region-growing algorithm, we implemented the snakes algorithm – Snake2D (7–9). Similar to region-growing, the input was a threshold image, the user selected seen points in each axon and pixels labeled $= 1$ but not deemed by the operator to be myelin were manually set $= 0$. However, now it was not necessary for the user to seal each myelin boundary. This is due to the character of the snakes model. Snakes or active contour models use a contour ($x(s,t)$; $s =$ space (curve), $t =$ time (iteration)) that deforms to image features by minimizing the potential energy of the curve. Potential energy ($E_{snake}$) is defined in as,

$$E_{\text{snake}} = \oint E_{\text{internal}} (x) ds + \oint E_{\text{external}} (x) ds + \oint E_{\text{image}} (x) ds \quad [4.1]$$

where $E_{\text{internal}}$ defines the tension and stiffness of the contour line, $E_{\text{external}}$ controls attraction and repulsion forces and $E_{\text{image}}$ is defined as,
\[ E_{\text{image}} = w_{\text{line}} E_{\text{line}} + w_{\text{edge}} E_{\text{edge}} + w_{\text{term}} E_{\text{term}} \quad [4.2] \]

where \( E_{\text{line}} \), \( E_{\text{edge}} \), and \( E_{\text{term}} \) attracts the contour to light or dark regions, image edges, or image terminations (corners), respectively with weighting terms \( w_{\text{line}} \), \( w_{\text{edge}} \), and \( w_{\text{term}} \).

In our case, we want to guide the curves to the axon/myelin edge so we use a strong edge energy weighting \( W_{\text{edge}} = 3 \), with some weighting to guide the contour to myelin, light regions \( W_{\text{line}} = 0.2 \) and no terminal (corners) weighting \( W_{\text{term}} = 0 \). By controlling the tension and stiffness in \( E_{\text{internal}} \) \( \alpha = 0.075 \) and \( \beta = 0.1 \), respectively) we create a contour that can conform to the irregular shapes of axons but not escape breaks in the myelin.

After we run snakes on each axon, we can again compute the diameter, \( d \) for each axon by \( d_i = 2 \sqrt{a_i / \pi} \) where \( i = 1 \): number of axons. Fig 4.3 displays the results from \textit{Snakes}, showing good agreement with axon edges and the final contours. The colors represent the axon diameter calculated for each axon.

\[ \text{Figure 4.3. Snakes axon diameter measurements.} \quad \text{Axon region is grown using snakes algorithm from a seed point in each axon, with colors displaying axon diameter (d).} \]
4.2.4 Comparison of Methods

Next, we wanted to compare the sensitivity of $d$ to various measurement techniques-- manual, region-growing, and snakes (active contour) methods. It is of note that the manual measurements were performed on only half of the data ($n = 2135$ axons), so we limited comparisons with manual measures to these data. First, we compare $d$ from manual versus region-growing in Fig 4.4 and see a strong 1:1 relationship between measures with Pearson’s $\rho = 0.97$ and mean-squared error (MSE) = 0.007.

![Figure 4.4. Comparison of manual and region-growing axon diameter techniques.](image)

Each point represents a single axon with axon diameter ($d$).

Next, we compare $d$ from manual to snakes as show in Fig 4.5 and again see strong correlation with $\rho = 0.97$ and MSE = 0.006.
Figure 4.5. Comparison of manual and snakes axon diameter techniques. Each point represents a single axon with axon diameter ($d$).

Finally, we compare region-growing to snakes methods and see that these two techniques are extremely similar, as hoped, with $\rho = 0.99$ and $\text{MSE} = 0.03$ and almost all points lying on the line of unity (Fig 4.6). Therefore, we can conclude that snakes method is the best technique due to user-independence in measurements and decreases in user time-demand. We also see that axon diameter is a relatively insensitive measurement, with all three techniques providing similar results.

Figure 4.6. Comparison of region-growing and snakes axon diameter techniques. Each point represents a single axon with axon diameter ($d$).

4.3 MEASURING MYELIN THICKNESS ($\Delta$)

4.3.1 Manual Measurement
We used a binary image defined by the nadir of the image histogram (See 4.4.1 Global Threshold). To measure myelin thickness ($\Delta$), we manually drew perpendicular line across the myelin thickness at the major and minor axes of the axon as shown by the pink lines in Fig 4.7. The myelin thickness for each axon was the mean of these two measurements. However, this technique suffers from the same negatives as manual measurements of axon diameter—user-dependency and time intensive.

![Figure 4.7. Manual myelin thickness measurements](image)

**Figure 4.7. Manual myelin thickness measurements.** Two lines are manually draw across the myelin (pink lines) at the major and minor axes of the axon (blue lines in Fig 4.1).

4.3.2 Radial Sampling

To increase reliability of $\Delta$ measures, we wanted an automatic approach that would radially sample the myelin thickness around the axon. Because the Otsu threshold resulted in uneven segmentation of the myelin (10), the myelin of each axon was independently segmented on the original histology image. The final boundary from the snakes axon segmentation was used as the starting contour (blue stars). Each contour was grown outward along lines normal to its tangent ($\sim 0.06\mu m$ between lines) until reaching a pixel intensity greater than the average intensity of pixels from the initial contour (green stars) displayed in Fig 4.8.
Figure 4.8. Radial sampling myelin thickness measurements. Lines are automatically grown radially perpendicular to the perimeter of the axon area with ~0.06µm between lines.

In order to reject normal lines that grew into the myelin of adjacent axons, a line was discarded if 1) its terminal point matched the location of an axon boundary defined above (red stars), or 2) length was greater than 3 times the median absolute deviation (magenta stars) (11). The median of the remaining lines defined the myelin thickness for that axon, Δ. Finally, myelin area for each axon was determined by uniformly growing the initial contour of each axon to a uniform thickness, Δ, as shown in Fig 4.9. The resulting binary image provided a measure of myelin volume fraction, \( f_{M, Hist} = \frac{a_{\text{pixels}=1}}{a_{\text{im}}} \) for each image, where \( a_{\text{pixels}=1} \) is the area of myelin pixels and \( a_{\text{im}} \) is the image area.
Figure 4.9. Segmented myelin image from radial sampling. The sum of pixels = 1 provides a myelin volume fraction, $f_{MHIST}$.

While the technique performs well most of the time, we must still perform quality assurance. Fig 4.10 displays an example of how the technique fails when the myelin cannot be adequately sampled, in this case due to being surrounded by other axons (blue arrows). To overcome this, we acquire 2 manual measurements on the original image and use the mean value.

Figure 4.10. Breakdown in segmented myelin image from radial sampling. Blue arrows point to axons where radial sampling did not perform well and must be manually assessed.
4.3.3 Comparison of Methods

Next, we wanted to compare the sensitivity of Δ measurements to the measurement techniques—manual and radial sampling methods. The manual Δ measurements were performed on almost all of the data (n = 3447 axons). First, we compare Δ from manual versus radial sampling in Fig 4.11 below and see a strong linear relationship between measures with Pearson’s ρ = 0.68 and root mean-squared error (RMSE) = 0.040. We can see that we are consistently getting larger myelin thickness measures from our radial grow technique. This is not surprising since we expected that we were underestimating myelin using our global thresholding technique. (See 4.4.1 Global Threshold).

![Figure 4.11. Myelin thickness from manual and radial sampling techniques. Each point represents one axon.](image)

However, it is difficult to assess how “correct” our measures of myelin thickness are. To assess our technique we compared the results to a technique developed by Begin et al. for analyzing coherent anti-Stokes Raman scattering (CARS) microscopy images (12). Fig 4.12 shows and original EM image along with the segmented myelin image from the CARS technique and our radial growing technique. Qualitatively, the segmented images look similar. It is of note that the
CARS histology technique is not able to assess axons that do not lie fully in the image, which is important for our application. Additionally, the CARS technique smooths the axon perimeter more than the radial technique.

Figure 4.12. Comparison between CARS histology method and radial sampling for obtaining segmented myelin images.

If we quantitatively compare the two measures, we see a near 1:1 line of best fit and data points lying around the line of unity shown in Fig 4.13. There is a strong linear correlation (Pearson’s $\rho = 0.71$) and a much lower root mean-squared error (RMSE) = 0.019 than the comparison to manual measurements.
From these analyses, we see that measures of $\Delta$ are much less robust than measures of axon diameter, due to its small size and blurry axon/myelin boundaries. However, if we use an automated technique, we can reduce the user-dependent error and assess each axon consistently.

4.4 MEASURING MYELIN VOLUME FRACTION ($f_{\text{M,HIST}}$)

4.4.1 Global Threshold

First, we wanted to assess the capability of a global threshold for segmentation of all electron microscopy images. To do this, a threshold was applied to each image based on the nadir of the histogram between the two peaks of myelin and non-myelin pixels. Since the myelin is stained, it is dark in the image and captured in the first signal peak as displayed below in Fig 4.14.
In the binary image, all pixels falling below the threshold = 1 and are considered myelin, while all pixels above the threshold = 0 and are considered non-myelin. From these binary images the $f_{M,HIST}$ was calculated as the number of pixels = 1 over the total number of image pixels. While this technique was a good start, it did not work consistently and needed manual correction often.

We also assessed other global thresholding techniques: Manual (as described above), Otsu (multithresh- 1 value; (10)), Reddi (reddi; (13)), Kittler (kittler) and Kittler 3-class (kittlerMinimimErrorThresholding; (14)), Otsu 3-class (multithresh- 2 values; (10)) and non-uniform-illumination-corrected Otsu (local Otsu- adaptivethreshold – described below). To qualitatively assess how different methods compared, I implemented a MATLAB GUI (Threshold_segmentation) shown in Fig 4.15. The GUI allows you choose an input image and displays the histogram of pixels in that image. You can then toggle between different threshold techniques. The GUI will display the threshold on the histogram and in value form below, the segmented image, and $f_{M,HIST}$ calculated. There is also a toggle for a built in bias field correction method (15) and the ability to remove stray pixels from the threshold image based on user-defined minimum number of connected pixels (bwareaopen). A screenshot of the GUI is displayed below using the Kittler 3-class technique.
While there are many techniques for global thresholding, in the end no global technique was consistent for all different images. Some images displayed non-uniform illumination, which made it difficult to find a global threshold value. Others (especially images from diseased mouse brain) had very little myelin, which created a nearly indiscernible peak from myelin tissue, which also created a challenge to find the correct threshold value globally.

4.4.2 Local Threshold
As opposed to global thresholding, local thresholding allows for correction from non-uniform illumination across the image. We used a local correction followed by 2-class Otsu method as displayed in Fig 4.16 below (adaptivethreshold; (10)). This approach calculates the mean pixel intensity using an averaging filter (fspecial(‘average’, pixelsize)) across the user-defined pixel window region resulting in a smooth mean intensity image (mIM). We then subtract mIM from
the image to get an inverted uniform image. It is important that the pixel window region is large enough to contain image features and some background pixels but retain local changes in the image (pixel region = 500, displayed on the raw image). The mean subtraction allows for correction of background gradients in the image, exemplified in Fig 4.16. Then, a 2-class global Otsu threshold is applied resulting in a binary image (myelin=1, non-myelin=0). In Fig 4.17 the comparison between the global Otsu method and the locally-corrected Otsu method is displayed, with more uniform segmentation using the local method.

**Figure 4.16. Demonstration of local Otsu threshold segmentation.** A mean intensity value is defined for each pixel from a moving window average. The image intensity is subtracted from mean background intensity and global Otsu is used to segment myelin with a uniform background.

4.4.3 Radial Growing

While the local threshold performed more consistently than global techniques, we only used the local segmentation as a first pass for estimating myelin volume fraction. Overall, all threshold techniques cannot distinguish between myelin and other dark, non-myelin features such as nuclei or other membranous structures. Instead, to obtain a more accurate value for \( f_{M,HIST} \), we decided to use myelin measurements to create a segmented myelin image as described in section 4.3.2 to obtain a segmented myelin image.
4.4.4 Comparison of Methods

We assessed $f_{M,HIST}$ obtained from manual global threshold, local threshold and radial grow techniques. Figure 4.17 displays one example image using global, local and radial grow techniques. While all images qualitatively look similar, global threshold appears to non-uniformly depict myelin, with areas of myelin dropout. As expected, the local threshold more uniformly represents myelin but as stated above, non-myelin pixels are also incorporated into the segmentation. The radial grow technique displays a similar but much cleaner segmentation as it avoids the need for distinguishing myelin from non-myelin and is calculated on an axon-by-axon basis.

![Figure 4.17. Demonstration of threshold segmentation methods.](image)

Figure 4.17. Demonstration of threshold segmentation methods. Global threshold suffers from non-uniform illumination which is corrected using a local threshold. However, only the radial grow technique can consistently segment pure myelin.

Next, we quantitatively compared the three segmentation techniques image-by-image between control and 2 models of hypomyelination and 1 model of hypermyelination shown in Fig 4.18.
Figure 4.18. Comparison of $f_{M,HIST}$ from different threshold segmentation methods. Global threshold underestimates $f_{M,HIST}$. Local threshold overestimates $f_{M,HIST}$ due to non-myelin objects, especially in low $f_{M,HIST}$ images. Radial grow provides the most robust method for $f_{M,HIST}$.

From these comparisons, we see that — 1) local threshold and region grow provides higher $f_{M,Hist}$ compared to global threshold in almost all cases, 2) global threshold and region grow are well correlated, and 3) local threshold and region grow are nearly identical except for cases where myelin content is very low. Observation #3 is demonstrated in Fig 4.19 below. While global threshold cannot distinguish all myelin pixels leading to lower $f_{M,HIST}$, local threshold finds extraneous pixels, leading to much higher $f_{M,HIST}$. Overall, radial grow technique provided the most consistent results using the positives from global and local threshold without the drawbacks.
Figure 4.19. Comparison of $f_{M,HIST}$ from different threshold segmentation methods in hypomyelination model. Global threshold underestimates $f_{M,HIST}$, local threshold overestimates $f_{M,HIST}$ due to non-myelin objects, and radial grow provides the most robust method for $f_{M,HIST}$.

4.5 REFERENCES


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5.1 INTRODUCTION

There is a long-standing effort to develop MRI methods that are not just sensitive to myelin but report on changes in myelin with specificity. Recent interest in using MRI to measure the g-ratio (1–3) has raised the aims of myelin imaging a step further, beyond specificity to accuracy. That is, an ideal method for g-ratio imaging includes more than just a correlative measure of myelin content, but an absolute measure of myelin volume fraction (MVF). To date, two myelin imaging techniques have been particularly well studied: myelin water imaging (MWI) via multi-exponential $T_2$ (MET$_2$) analysis (4) and quantitative magnetization transfer (qMT) imaging (5). Both techniques have been shown to provide correlative measures of myelin content (6–9), but exactly how each relates to MVF remains unclear.

In the case of MWI, white matter is modeled as being comprised of two micro-anatomically separated water compartments with different transverse relaxation time constants ($T_2$): 1) water trapped between the lipid bilayers of myelin (myelin water, $T_2 = 5$ - 40 ms, depending on static field strength, $B_0$), and 2) water in both the intra- and extra-axonal spaces (i/e water, $T_2 = 30$-100 ms, depending on $B_0$). Given sufficient signal-to-noise ratio (SNR), multiple spin-echo amplitudes can be fitted to a model that distinguishes these water pools based on $T_2$, and the myelin water fraction (MWF) is typically reported as a measure of relative myelin content (4,10,11).
Measures of MWF have been shown to correlate with optical density in luxol fast blue stained sections of cadaver brain from MS patients (6) and with direct measures of myelin cross sectional area in electron microscopy of control and injured rat nerve (7,8). Also, Laule et al., used literature values of the composition of white matter to predict MWFs that were in close agreement with their observed values (12). However, none of these studies attempted to explicitly estimate and/or validate values of MVFs from MWF measures. The relationship between MWF and MVF depends on the relative water proton densities in the myelin and non-myelin compartments, but may also depend on the rate at which water exchanges between these compartments (13). Studies in rat spinal cord have indicated that variations in MWFs between different white matter tracts may be due to differences in water exchange rates, mediated by variations in axon diameter and myelin thickness (14,15). This effect has been postulated to exist in brain (16,17), but it remains unclear to what extent it effects observed MWF values.

Similar to MWI, the qMT method is based on a two-pool model of protons in white matter, but instead of two anatomically separated pools they are two pools of different molecular origins, water protons and protons bound to macromolecules. Although the bound proton signal is not typically measured directly, the exchange of magnetization between the bound and water protons results in contrast that depends on bound proton concentration (18,19). Thus, given an appropriate series of images with different MT contrast, the ratio of bound protons to total protons, or bound proton fraction (BPF), can be estimated. Note that, unlike the two-pool model used for MWI, this two-pool model: i) incorporates no anatomical information (both water and bound protons pools are assumed to be well mixed from one anatomical compartment, meaning that myelin is not explicitly part of the model), and ii) is predicated on the exchange of magnetization between the two pools (while the MWI model assumes no exchange of
magnetization between the two water pools) (5,20). The lack of anatomy in the model presents a problem in relating BPF to MVF because bound protons will exist in both myelin and non-myelin regions of the tissue, and there is no reason to believe that all bound protons exchange magnetization with water at the same rate. As in MWI, this raises the question of whether geometric characteristics of axons/myelin contribute to the measured BPF.

Similar to literature on MWF, measures of BPF (or similar/related quantities) have been demonstrated to linearly correlate with MVF as measured by histology in both human cadaver brain (9) and rodent brain and nerve (8,21–23). Stikov et al. have recently used such a linear correlation to estimate MVF from BPF (2), but otherwise, there has been limited effort in explicitly estimating MVF from estimates from qMT measures.

Using literature information on the composition of white matter, this study proposes analytical expressions for computing estimates of MVF from MET₂ and qMT data. These approaches are applied with high resolution 3D MRI protocols to excised and fixed mouse brains from control mice and three mouse models of abnormal myelination. MRI results are quantitatively evaluated with transmission electron microscopy.

5.2 THEORY

To derive myelin volume measures from MRI, a model of white matter tissue that uses volumes, not just populations, of the different proton pools is presented in Fig 5.1. The model includes four proton pools, with volumes of bound and water protons in the myelin (\(V_{B,M}\) and \(V_{W,M}\), respectively) and non-myelin (\(V_{B,NM}\) and \(V_{W,NM}\), respectively). The model assumes exchange of longitudinal magnetization between the bound and water protons, enabling qMT analysis, but no exchange of water or magnetization between myelin and non-myelin compartments.
The MVF ($f_M$) by definition is

\[
    f_M = \frac{V_{B,M} + V_{W,M}}{V_{B,NM} + V_{W,NM} + V_{B,M} + V_{W,M}}. \tag{5.1}
\]

Using the simplifying assumption that molar concentration of protons is equal in all four compartments (see 5.7 Appendix), magnetization fractions are equal to volume fractions, which permits BPF measured by qMT to be expressed in terms of compartment volume fractions,

\[
    BPF = \frac{V_{B,NM} + V_{B,M}}{V_{B,NM} + V_{W,NM} + V_{B,M} + V_{W,M}}. \tag{5.3}
\]

Similarly, the myelin water fraction (MWF) measured by MET\textsubscript{2} is

\[
    MWF = \frac{V_{W,M}}{V_{W,NM} + V_{W,M}}. \tag{5.4}
\]

From previous literature (see 5.7 Appendix), the volume fraction of water in myelin ($\Phi_{W,M}$) is estimated as
\[ \Phi_{w,m} = \frac{V_{w,m}}{V_{b,m} + V_{w,m}} = 0.475 \text{ ml H}_2\text{O/ml tissue}, \quad [5.5] \]

and for non-myelin is

\[ \Phi_{w,nm} = \frac{V_{w,nm}}{V_{b,nm} + V_{w,nm}} = 0.859 \text{ ml H}_2\text{O/ml tissue} \quad [5.6] \]

Combining Eqs 5.1, and 5.4-5.6, MWF can be written in terms of \( f_m \)

\[ \text{MWF} = \frac{\Phi_{w,m} \times f_m}{\Phi_{w,nm} \times (1 - f_m) + \Phi_{w,m} f_m}. \quad [5.7] \]

which can then be solved to write \( f_m \) as a function of MWF,

\[ f_{m,T2} = \frac{MWF \times \Phi_{w,nm}}{MWF \times (\Phi_{w,nm} - \Phi_{w,m}) + \Phi_{w,m}}, \quad [5.8] \]

with the additional “T2” subscript indicating that this is myelin volume fraction as estimated by MET\(_2\) analysis.

For qMT analysis, there is an additional unknown: the volume fraction of the non-myelin bound proton pool, defined here as \( \beta \).

\[ \beta = \frac{V_{b,nm}}{V_{b,nm} + V_{w,nm} + V_{b,m} + V_{w,m}}. \quad [5.9] \]

Assuming that \( \beta \) were known, and from Eq 5.3,

\[ [BPF - \beta] = \frac{V_{b,m}}{V_{b,nm} + V_{w,nm} + V_{b,m} + V_{w,m}}, \quad [5.10] \]

then combining Eqs 5.5 and 5.10, results in
The sum of Eqs 5.10 and 5.11 is the myelin volume fraction,

$$f_{M,MT} = \left(1 + \frac{\Phi_{W,M}}{1 - \Phi_{W,M}}\right) \times [BPF - \beta],$$

with the “MT” subscript indicating that this is myelin volume fraction as estimated by qMT analysis.

5.3 MATERIALS AND METHODS

5.3.1 Tissue Preparation

The Vanderbilt University Institutional Animal Care and Use Committee approved animal studies. Fifteen adult mice were anesthetized with isoflurane and sacrificed via transcardial perfusion. The perfusion consisted of 1X phosphate-buffered saline (PBS) wash followed by 2.5% glutaraldehyde + 2% paraformaldehyde (modified Karnovsky solution). Following perfusion, brains were quickly removed from skull and immersed in the fixative solution for 1 week. Brains were then washed with 1X PBS + 0.01% sodium azide, changing wash 4-5 times over 1 week to remove excess fixative. In all cases, 1.0 mM Gd-DTPA (Magnevist; Berlex, Montville, NJ) was included in the perfusate, immersion and wash solutions, resulting in relatively uniform distribution of Gd-DTPA throughout the brain.

This study used control animals (n=6) along with two previously described models of hypomyelination and one of hypermyelination (n = 3 for each model). All models utilized the *Olig2-Cre* driver to conditionally target proteins involved in PI3K/Akt signaling in
oligodendrocyte precursor cells. In the first model, the Tsc2 gene is deleted (Tsc2 CKO—conditional knockout) and exhibits extreme loss of myelin (24). The second model targets Rictor (Rictor CKO), a key component of the mTORC2 complex, and also displays hypomyelination, but less severe than the Tsc2 model and similar to the Rictor Emx1-Cre model shown previously (25). The third model results from the deletion of Pten (Pten CKO) leading to activation of the PI3K/Akt signaling pathway and subsequent hypermyelination (26).

5.3.2 Magnetic Resonance Imaging

All imaging was performed on a 15.2-T 11-cm horizontal bore Bruker (Rh reinstetten, Germany) BioSpec scanner, using a 35-mm diameter Bruker quadrature volume coil for transmission and reception. To provide a signal-free background and prevent tissue dehydration, brains were placed in am MR-compatible tube filled with perfluoropolyether liquid (Fomblin, Solvay Solexis, Thorofarem NJ, USA). Both MET2 and qMT scans were encoded with a matrix size of 128 × 96 × 72 over a 1.92 × 1.44 × 1.08 cm³ FOV, providing 150 µm isotropic resolution.

For MET2 imaging, a 3D multiple spin-echo sequence was used with non-selective excitation and refocusing pulses, 160 µs and 100 µs in duration, respectively. Each refocusing pulse was surrounded by 428 µs duration 6 G/cm amplitude crusher gradients, phase-encoding gradients were rewound after each echo, and a two-part (+X/-X) phase cycling scheme was used. With these constraints, secondary echoes that were not excited by the initial excitation pulse were removed, and the observed echo magnitudes could be computed with the extended phase graph (EPG) algorithm (27–29). Scan parameters were: repetition time (TR) = 520 ms, echo time (TE) = 5.8 ms, number of echoes (NE) = 18, receiver bandwidth (BW) = 38.5 kHz, and number of excitations (NEX) = 6. Total scan time was ≈ 6 hr.
For qMT imaging, a 3D selective inversion-recovery prepared fast spin echo sequence (20) was used with 8 collected echoes, 5-ms echo spacing and centric phase encoding. To ensure that the longitudinal magnetization ($M_z$) recovered from $M_z = 0$ at the start of every pre-delay period, 8 additional refocusing pulses, also with 5-ms echo spacing, followed the 8th echo. A 1-ms hard pulse was used to selectively invert the free water magnetization, while macromolecular spins were mostly unaffected. The sequence was repeated $N_I = 15$ times with inversion times ($T_i$) log-spaced from 3.5 to 2000 ms. A constant predelay ($T_d$) of 590 ms resulted in a scan time of ~3.5hr.

5.3.3 Data Analysis

All data analysis was performed using MATLAB R2015a (The Mathworks, Natick MA) and MET$_2$ analyses were performed using the freely available Multi Exponential Relaxation Analysis (MERA) toolbox (30). Prior to Fourier reconstruction, k-space data for all images were apodized using a 3D Tukey window with a 0.25 taper-to-window ratio and zero-padded 2×, resulting in 75µm nominal isotropic resolution.

For each voxel, the MET$_2$ analysis used a separable non-linear approach. The $T_2$ spectrum was estimated by linear inverse (31) using a non-negative least-squares fit (32) of the $N_E$ echo magnitudes to the sum of 100 EPG-defined signals with $T_2$ values logarithmically spaced between $T_E/2$ as 500 ms. The linear model was augmented with minimum curvature constraint weighted at a constant and conservative level across all voxels ($\mu = 0.002$). This linear inverse was repeated to find the refocusing pulse flip angle ($\theta$) by non-linear regression, similar to previous work (28,33). From all spectra, the myelin water fraction (MWF) was defined as the
fraction of signal with $T_2 < 17$ ms, based on previous work (see Chapter 3) and long $T_2$ component was defined as the $T_2$ from the largest fractional component $> 17$ms.

For qMT analysis, the $N_i$ image magnitudes were fitted voxel-wise to the Bloch-McConnell equations describing longitudinal relaxation and magnetization transfer between water and macromolecular protons (20,34). The five fitted model parameters were: $M_{0f}$, $M_{0b}$, $k_{mf}$, $R_{1f}$, and $S_f$, where $M_{0f/b}$ are the equilibrium magnetizations of the free and bound pools, respectively, $k_{bf}$ is the rate constant of magnetization transfer from the bound to free water pool, $R_{1f}$ is the longitudinal relaxation rate of the free water pool, and $S_f$ is the efficiency of the inversion pulse on the free water pool. The corresponding $R_{1b}$ and $S_b$ values were constrained to $R_{1b} = 1$s and $S_b = 0.83$ in accord with prior studies (20). The bound proton fraction (BPF) was then defined as $\text{BPF} = M_{0b} / (M_{0b} + M_{0f})$.

After MET$_2$ and qMT analysis, all parameter maps of a given model were co-registered in order to define closely comparable regions of interest (ROI) in each brain. For each mouse model, the first spin echo image of one brain was arbitrarily defined as the reference and the corresponding image from each other brain was registered to the reference using a rigid affine registration followed by a non-rigid deformable demons registration (35). The resulting deformation fields of this registration were then applied to parameter maps. Four ROIs were drawn corresponding to the four regions extracted for histology (below): 3 in the corpus callosum in the mid-sagittal slice (genu (GCC), mid-body (MidCC), and splenium (SCC)) and the other in the anterior commissure (AC). In addition, a cortical gray matter (GM) ROI was drawn in the sagittal slice for comparison. Each ROI was $\sim 12.5$ voxels/ROI ($\sim 450 \times 450$ µm or $202.5$ mm$^2$), which is similar to histologic ROIs and the ROI for each region was held constant.
across brains. Mean ROI values from different parameter maps and values derived from histology were compared using two-sample t-tests ($\alpha = 0.05$) and Pearson’s linear correlation.

5.3.4 Microscopy
For each brain, after MRI, a 1-2 mm thick sagittal section of tissue was cut from the left hemisphere beginning at the mid-brain. Subsequently, 4 regions of white matter were cut from the slice: the genu, mid-body, and splenium of the corpus callosum and the anterior commissure. Tissue samples were then processed for Transmission Electron Microscopy (TEM) in the Vanderbilt Cell Imaging Shared Resource-Research Electron Microscopy facility. Samples were placed in 1% osmium tetroxide in cacodylate buffer for 12 hours and dehydrated in graded ethanol. Tissue was then embedded in epoxy resin and thick sections (0.5-1 µm) were collected and stained with 1% toluidine blue. Subsequently, ultra-thin sections (~ 500 x 500 x 0.07 µm, see Fig 5.2b) were cut and collected on 300-mesh copper grids. Copper grids were stained at room temperature with 2% uranyl acetate (aqueous) for 15 minutes and then with lead citrate for 10 minutes.

Ultra-thin sections were imaged on the Philips/FEI Tecnai T12 electron microscope (FEI Company, Hillsboro, OR) at various magnifications and pictures were acquired with a side-mounted AMT CCD camera. For quantification of myelinated axon microstructure, 6-12 15,000× images were collected (~300 axons) per ROI per animal. Each image was analyzed semi-automatically to derive myelin volume fraction ($f_{M,HIST}$), axon volume fraction ($f_{A,HIST}$), as well as per axon measures of diameter ($d_i$) and myelin thickness ($\Delta_i$) (subscript “$i$” indicating the $i^{th}$ axon).
The histology pipeline was implemented using MATLAB 2015a (The Mathworks, Natick MA) and is shown in Fig 5.2. First, a local Otsu threshold was applied to each image, resulting in a binary image (myelin=1, non-myelin=0). In this binary image, the operator manually identified an intra-axonal point of each myelinated axon and corrected the labeling of pixels that were deemed to be erroneously identified as myelin. Beginning from each manually identified seed point, an active contour algorithm (36) was used to segment each individual axon. The resulting image (Fig 5.2d) provided per axon measures of area and diameter ($a_i$ and $d_i = 2\sqrt[4]{a_i/\pi}$, respectively, for the $i^{th}$ axon) and total axon volume fraction. (Note that only axons lying fully within the image frame were included in the per-axon measures, but all axon area contributed to the axon volume fraction.)
Figure 5.2. Histology flow chart. a) MRI $T_2$-weighted image with box drawn for ROI analysis. b) Histology thick slice where the red box signifies the location of ultra-thin sections. c) Transmission Electron Microscopy (TEM) 15,000X image. d) Output of region-growing algorithm with colorbar denoting axon diameter ($d$), and the fraction of area from all axons = $f_{A,HIST}$. e) For each axon, normal lines are grown from seeds from the region-growing axon edge (blue stars) until the image value is less than the mean image value in seeds to obtain a measures of myelin thickness (green stars). The measurement is disregarded if i) the end point lands in another axon space (red stars) or ii) if the line is > 3 times median absolute deviation (magenta stars). A median myelin thickness is calculated for each axon. f) Myelin is then grown using the median thickness from the region-growing axon boundary to obtain a segmented myelin image, with the fraction of area from all myelin = $f_{M,HIST}$.

Because the Otsu threshold resulted in uneven segmentation of the myelin, the myelin of each myelinated axon was independently segmented on the original histology image using the final boundary of the axon segmentation as the starting contour. Each contour was grown outward along lines normal to its tangent (lines spaced ~0.06 µm apart along the starting contour) until reaching a pixel intensity greater than the average intensity of pixels from the initial contour (Fig 5.2e). In order to reject normal lines that grew into the myelin of adjacent axons, a line was discarded if 1) its terminal point matched the location of an axon boundary defined above, or 2) its length was more than 3 times the median absolute deviation (37). The ends of the remaining
normal lines created a contour defining the outer myelin boundary, and the median length of these lines defined the myelin thickness for that axon, $\Delta i$. Finally, myelin area for each axon was determined by uniformly growing the initial contour of each axon to a uniform thickness $= \Delta i$, as shown in Fig 5.2f. The resulting binary image provided a measure of myelin volume fraction, $f_{M,HIST}$, for each image. Across all images per mouse and brain region, mean values $\bar{f}_{M,HIST}$, $\bar{f}_{A,HIST}$, $\bar{d}$, and $\bar{\Delta}$ were calculated.

5.4 RESULTS

Representative TEM histology in Fig 5.3 demonstrates the abnormal myelination characteristics expected from these mouse models of tuberous sclerosis. The Rictor CKO and especially the Tsc2 CKO mice exhibited loss of myelinated axons, consistent with previous literature (24,25). Similarly consistent with literature (26), the myelin in Pten CKO mice was noticeably thicker than in control mice. These variations in both myelin content and myelin thickness made these a useful combination of models for evaluating the specificity of MRI methods for reporting on myelin volume fraction.
Figure 5.3. Representative histology images. Representative 15,000X (left) TEM Histology images and (right) segmented myelin images from (top-bottom) control, Rictor CKO, Tsc2 CKO, and Pten CKO mice with scale bar = 1 µm.

A summary of the detailed quantitative evaluation of the TEM histology is presented in Fig 5.4. The mean ± SD (across animals) of three metrics (\(f_{M,HIST}\), \(f_{A,HIST}\), and \(\Delta\)) are shown for each of the four white matter tracts and all four different mouse models. As expected from Fig 5.3, \(f_{M,HIST}\) is significantly reduced in both Rictor and Tsc2 CKO compared to controls, and slightly higher in the SCC and AC regions of the Pten mice. Measures of \(f_{A,HIST}\) were similar between control, Rictor, and Pten CKO mice, but dramatically reduced in Tsc2 CKO mice,
(although it is worth noting that the \( Tsc2 \) histology did exhibit a large fraction of non-myelinated axons, but due to their small size are difficult to quantify accurately). The control and \( Rictor \) CKO mice showed similar myelin thickness, while \( \Delta \) was generally greater in Tsc2 CKO \( Pten \) CKO mice.

![Bar chart showing mean myelin volume fraction, mean axon volume fraction, and mean myelin thickness across different regions of interest for control, Rictor CKO, Tsc2 CKO, and Pten CKO mice.](image)

**Figure 5.4. Region of interest (ROI) histology analysis.** (top-bottom) mean myelin volume fraction \( (f_{M,HIST}) \), mean axon volume fraction \( (f_{A,HIST}) \), and mean myelin thickness \( (\Delta) \) from (left-right) mid-body of corpus callosum (MidCC), genu of corpus callosum (GCC), splenium of corpus callosum (SCC), anterior commissure (AC) and cortical gray matter (GM) in control, \( Rictor \) CKO, \( Tsc2 \) CKO, and \( Pten \) CKO mice. Dots above bars represent significant differences of (.) = \( p < 0.05 \), (..) = \( p < 0.01 \), (…) = \( p < 0.001 \).

Representative MRI data from control and the three mouse models are shown in Fig 5.5—sagittal slices from a \( T_2 \)-weighted image \( (T_E = 5.8 \text{ ms}) \), long \( T_2 \) component value, MWF, and BPF parameter maps. As expected from histology (Fig 5.3 and 5.4), white matter regions are generally invisible in \( Tsc2 \) CKO mice, reduced in contrast in \( Rictor \) CKO mice, and enhanced in...
contrast in Pten CKO mice. A detailed summary of MRI measures is presented in Fig 5.6, which shows mean values (±SD across animals) of the long $T_2$, MWF, and BPF for controls, Rictor, Tsc2, and Pten CKO mice in the 4 white matter ROIs and the cortical gray matter ROI. Statistically significant differences in $T_2$, MWF, and BPF were found between controls and both models of hypomyelination (Rictor and Tsc2 CKO), consistent with the loss of myelin observed by histology. Between the Rictor and Tsc2 CKO mice, differences in all measures were consistent with less myelin in the Tsc2 CKO mice, but only reached statistical significance for BPF in 3 of 4 ROIs and in 1 for MWF. Similarly, the Pten mice, expected to exhibit hypermyelination, had generally increased MWF and BPF and decreased $T_2$, although the BPF and $T_2$ differences were only statistically significant in the AC.

Figure 5.5. Representative MRI images. (left-right) $T_2$-weighted, long $T_2$ component, myelin water fraction (MWF), and bound pool fraction (BPF) parameter maps from (top-bottom) control, Rictor CKO, Tsc2 CKO, and Pten CKO mice.
Figure 5.6. Region of interest (ROI) MRI analysis. (top-bottom) long $T_2$ component, myelin water fraction (MWF), andbound pool fraction (BPF) from (left-right) mid-body of corpus callosum (MidCC), genu of corpus callosum (GCC), splenium of corpus callosum (SCC), anterior commissure (AC) and cortical gray matter (GM) in control, Rictor CKO, Tsc2 CKO, and Pten CKO mice. Dots above bars represent significant differences of (.) = $p < 0.05$, (..) = $p < 0.01$, (…) = $p < 0.001$.

For the purpose of this study, more important than detecting differences in myelin between animals or brain regions, is the accuracy with which MRI can measure myelin content and how these measures are sensitive to myelin thickness. To this end, Fig 5.7 displays scatter plots of (top) MWF and BPF vs $f_{M,HIST}$. As expected from previous literature (6–9,22,23) both MWF and BPF show strong linear correlation with histological measures of MVF ($r = 0.81$, 0.84, respectively); however, neither measure is accurate (i.e., lines of best fit do not lie near line of unity). The offset in BPF ($\beta$) is also displayed visually and equals 0.086. Also shown in Fig 5.7 (bottom) are the two calibrated MRI measures of MVF, $f_{M,T2}$ and $f_{M,MT}$, vs $f_{M,HIST}$, which demonstrate the potential for both MET$_2$ and qMT to provide accurate, not just correlative ($r =$
0.82, 0.84 respectively), measures of MVF, with lines of best fit lying near the line of unity. Overall, $f_{M,T2}$ slightly underestimates $f_{M,HIST}$ and there is a noticeable variation between animal models, which may be due to the sensitivity of MET$_2$ to variations in myelin thickness. In contrast, the $f_{M,MT}$ estimates $f_{M,HIST}$ more accurately, although this is a somewhat misleading observation because the $f_{M,MT}$ data were calibrated at $f_{M,HIST} = 0$.

Figure 5.7. MRI versus histology scatter plots. (a,b) MWF and BPF versus $f_{M,HIST}$ with line of best fit and line of unity (gray, dashed). $r = 0.81$ and 0.84, respectively. Mean standard errors from MWF and BPF are 0.007 and 0.004, respectively. (c,d) $f_{M,T2}$ and $f_{M,MT}$ versus $f_{M,HIST}$ with line of best fit and line of unity (gray, dashed). $r = 0.82$ and 0.84, respectively.
5.5 DISCUSSION

For the purpose of establishing MRI methods for measuring MVF, this paper presents MRI data and quantitative histology acquired in excised and fixed mouse brains with normal and abnormal myelination. One of the current goals of the authors’ lab is to develop robust MRI assays for routine use in rodent brain studies, and to that end the findings here demonstrate that both MET<sub>2</sub> and qMT methods have the potential to provide both specific and accurate whole brain maps of MVF.

Looking beyond excised tissues to in vivo imaging of animals or humans, there are a number of important factors to consider. Previous studies have investigated the effects of chemical fixation on qMT in post-mortem human tissues (38) and found differences in absolute values but similar predictive value of myelin content. Similarly, previous MET<sub>2</sub> studies have shown a close correspondence in the T<sub>2</sub> spectrum between fixed and in vivo human white matter (6) and between fixed (14) and in vivo (39) rat spinal cord. However, there is a noticeable absence of in vivo MET<sub>2</sub> data from rat or mouse brain in the literature and even evidence suggesting it cannot be measured (40). This limit may be due to SNR at the resolution necessary for rodent brain imaging, or it may reflect the consequences of more rapid water exchange between myelin and non-myelin compartments in these particular tissues at physiological temperatures in rats and mice. In addition to the tissues being excised and fixed, this study also utilized the addition of Gd-DTPA to boost SNR efficiency (41). To assess the effect of the Gd-DTPA on the MET<sub>2</sub> and qMT data, independent scans were run on a control brain that was prepared without the added Gd-DTPA. These scans required a longer T<sub>R</sub> but were otherwise identical and resulted in similar (< 10% different, not shown) measures of MWF and BPF to those found in the Gd-DTPA loaded brains. Although this was not a thorough investigation, it indicated that loading the tissue with 1
mM Gd-DTPA had no more than small effects on the quantitative myelin measures, and presumably little effect on their relationships to myelin content.

The observations that MWF and BPF correlate strongly with histological measures of myelin content is not novel, having been demonstrated in several previous studies (6–9,22,23). However, it is worth noting that with the exception of a recent study of the corpus callosum in cuprizone-fed mice (23), previous studies that involved brain tissue, as opposed to peripheral nerve, made independent measures of myelin content by optical densitometry of luxol-fast-blue stained histology. Optical densitometry is a practical approach for measuring myelin content over large sections of histology, but it presents challenges with calibration. That is, relating a densitometry measure to MVF itself requires calibration and is sensitive to the extent and spatial uniformity of staining. The Thiessen study included measures akin to MVF and found a strong correlation with BPF, but their MRI measurements did not include MWF. Thus, the present study, which involved direct visualization of myelin and quantitative analysis of approximately 500 TEM images, is unique in providing objective measures of MVF in brain to compare with both MWF and BPF. The TEM analysis also provided measures of myelin thickness, which cannot be extracted from large field optical densitometry studies.

Of course, all histology suffers from changes in tissue during the embedding process, and small-field-of-view TEM presents the potential for sampling bias. In this study, total tissue shrinkage < 10% is expected (42,43), and errors in MVF result only from the difference in shrinkage between myelin and non-myelin regions. If we postulate that the extent of shrinkage in different compartments is proportional to water density, then the effect of tissue shrinkage on MVF may be just a few percent—small compared to the variance in $f_{M,HIST}$. The area of ultra-thin sections is on the order of our MRI ROIs (See Fig 5.2a,b) but for high-resolution analysis, we are
limited to sampling ~1% of the area. However, by one-way ANOVA across all control sections, we see that variance between brains and ROIs is significantly higher than within a region, suggesting there were minimal effects from sampling bias. Histology also relies on an accurate analysis technique, which has no standard and is quite challenging. While our method generally performed well, in cases of poorly defined myelin to non-myelin boundaries we chose to err on the side of slightly overestimating myelin.

In terms of evaluating and comparing the two MRI methods studied here—MET$_2$ and qMT—the most obvious difference is that the calculation of $f_{M,T2}$ required a priori information about the volume fractions of water in myelin and non-myelin compartments of white matter, while $f_{M,MT}$ required the same and a calibration value of BPF when MVF = 0, i.e., $\beta$ in Eqs [5.9]-[5.12]. Here, $\beta$ was estimated from the linear regression of BPF vs $f_{M,HIST}$ (Fig 5.7b), which makes the evaluation of $f_{M,MT}$ vs $f_{M,HIST}$ somewhat circular. Ideally, the value of $\beta$ measured here will be applicable to future MRI studies of myelin in excised and fixed mouse brains at 15.2T, but how effectively one can generalize the intercept of BPF and $f_{M,HIST}$ is unclear. There is evidence that BPF changes between fresh and fixed tissue (38); it may also differ between in vivo and ex vivo tissue states, and perhaps between different white matter tracts and/or species. Moreover, even within a given type/state of tissue, the literature values of BPF vary widely, which is likely due to the wide variety of qMT methods in use.

Nonetheless, it may be possible to generalize the offset term, $\beta$, by normalizing white matter BPF values to those from cortical gray matter, which contains relatively little myelin. For example, in this study, the relative amplitude of $\beta$ and BPF in cortical gray matter is 0.72, and similar ratios, 0.76 and 0.77, can be drawn from the data in two previous studies, Janve et al. (Janve et al. 2013) and Thiessen et al. (23), respectively. The Thiessen et al. study also involved
fixed excised mouse brains but a very different qMT protocol, suggesting that a standard $\beta$ might work for these samples, independently of the qMT protocol. From rat brain in vivo (22), the results were somewhat different with a ratio of 0.62, but this is to be expected given a previous study that showed a greater relative effect of chemical fixation on BPF for normal appearing white matter and multiple sclerosis lesions (38). Nonetheless, this meta-analysis suggests potential for this approach to estimating $\beta$ without histology driven calibration.

Unlike $f_{M,MT}$, $f_{M,T2}$ does not require an offset term because MWF goes to 0 in the absence of myelin. However, previous studies in rat spinal cord (14,15) and rat optic nerve (44) have shown that MWF may be affected by inter-compartmental water exchange. Thus, smaller axons with thinner myelin may result in lower MWF relative to the MVF. This phenomenon may be responsible for the systematic deviation between animal models from the overall linear fit in Fig 5.7c. That is, all data points from the Pten CKO mice, which exhibited generally thicker myelin, fall above the linear fit line, while 11 of 12 points from the Rictor CKO mice, which exhibited generally thinner myelin, fall below. This additional variance between models may also reflect differences in water densities in myelin and non-myelin regions ($\Phi_{W,M}$ and $\Phi_{W,NM}$), but the effect is the same—MWF is not simply a function of MVF. That said, the effect in this study is relatively small. It may be that in vivo and/or at lower magnetic fields (where transverse relaxation rates are slower and, therefore, more sensitive to the rate of water exchange) this effect is more significant and more problematic. While one might speculate that BPF should also be sensitive to the thickness of myelin, since macromolecular protons from deep within myelin may not exchange as effectively with the bulk intra-/extra-axonal water, the effect is not apparent in Fig 5.7d, which agrees with previous simulation studies (45).
Beyond MET$_2$ and qMT, there are a number of other MRI methods that aim to image myelin content, which raises the question of why the present study involved only MET$_2$ and qMT. Similar to MET$_2$, one can image myelin with multi-exponential $T_2^*$ which permits use of spoiled gradient echo rather than spin echo acquisitions at the cost of introducing a new unknown (the additional dephasing of transverse magnetization). While this approach shows promise (46–48) it is not at the point of providing accurate measures of MVF and, in the authors’ experience, is especially challenging at the high magnetic fields used for small animal imaging. Other approaches that use steady state gradient echo are thus far uninterpretable at practical SNR, even for animal imaging (49).

Exploiting $T_1$ differences between myelin and non-myelin water to selectively excite only myelin water has been used in nerve (50) and white matter (40), but again, not for accurate MVF measures because additional unknowns come into play ($T_1$s and water exchange rates). Directly measuring multi-exponential $T_1$ has been proposed (51) but this is complicated not just by water exchange rates but also magnetization transfer. In fact, the qMT method used here is effectively a bi-exponential measurement of longitudinal relaxation. Because qMT already requires calibration, if the fast relaxing component is due to a combination of MT and multi-exponential $T_1$ (MET$_1$), there is no additional cost in specificity. However, attempting to interpret a bi-exponential recovery of longitudinal relaxation as being specifically a measure of myelin and non-myelin water pools will likely be inaccurate.

Finally, a few simpler approaches have also been proposed, such as a mono-exponential $T_1$ measurement (52) and measures of the proton density (53). These and similar approaches are essentially a sensitive but not specific measure of myelin, since numerous factors beyond MVF can alter their measurement. That said, from a practical perspective for human imaging, being
fast, reproducible, and quantitative measure has obvious advantages. For white matter imaging, the simplest model and, correspondingly, the greatest potential specificity comes from the proton density measurement. For example, in this study, integrating the $T_2$ spectrum and normalizing the amplitude to that of a cortical gray matter voxel results in a measure that correlates strongly with $f_{M,HIST}$ (Fig 5.8). An interesting feature of this method is that it will be independent of water exchange between myelin and non-myelin compartments. Indeed, variance in $f_{M,T2}$ between animal models seen above and attributed to water exchange (Fig 5.7c) is not apparent in Fig 5.8. In fact, considering a model where MVF is the only determinant of proton density variation in white matter, then a full $T_2$ spectrum with amplitude calibrated to a known water concentration provides enough information to estimate both MVF and water exchange rates, which may in-turn offer an alternative approach to measuring myelin thickness.

![Figure 5.8. Normalized water content scatter plot.](image)

Normalized water content versus $f_{M,HIST}$ in control, Rictor CKO, Tsc2 CKO, and Pten CKO mice, ($r = -0.74$).

5.6 CONCLUSION

Here, we assess MRI measures of myelin in control and 3 different models of white matter disease in mouse brain. Both MWF (from MET$_2$) and BPF (from qMT) show strong correlations
to quantitative histology. Using a volumetric model of white matter, MWF and BPF were converted to absolute measures of MVF, and displayed strong agreement with histologic myelin volume fraction. Using MET\textsubscript{2}, MVF measures were derived independently from histology but may be affected by inter-compartmental water exchange depending on myelin microstructure. Using qMT provides a somewhat more accurate estimate of MVF that exhibits less dependence on other microstructure characteristics, but requires calibration, here using histology. This work also provides promise for quantification of g-ratio from MRI where obtaining absolute measures of MVF is important.

ACKNOWLEDGMENTS

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5.7 APPENDIX

The relationship between equilibrium magnetization \((M_{0,x})\) and volume \((V_x)\) for compartment \(x\), is determined by the molar concentration of protons in each compartment, \(C_x\),

\[
\frac{M_{0,x}}{V_x} = kC_x, \tag{5.A1}
\]
where $k$ is a constant converting mol $^1$H to magnetization, $C_x = \text{molar concentration of pool } x$ (mol $^1$H / ml), and $V_x = \text{volume of pool } x$ (ml). Further, the concentration, $C_x$, can be expressed as

$$C_x = \rho_x / \zeta_x,$$  \[5.A2\]

where $\rho_x = \text{mass density of } x$ (g/ml) and $\zeta_x = \text{molar mass of } ^1\text{H in } x$ (g/mol $^1$H).

For both water compartments, knowing $\rho_w = 1$ g$_{\text{H}_2\text{O}}$ / ml$_{\text{H}_2\text{O}}$, $\zeta_w$ is

$$\zeta_w = \frac{18.02 \text{ g}}{1 \text{ mol } \text{H}_2\text{O}} \times \frac{1 \text{ mol } ^1\text{H}}{2 \text{ mol } ^1\text{H}} = 9.01 \frac{\text{g}}{\text{mol } ^1\text{H}},$$  \[5.A3\]

and using $\rho_w$ and $\zeta_w$ in Eq. 5.A2, $C_w$ is

$$C_w = \frac{1 \text{ g } \text{H}_2\text{O}}{1 \text{ ml } \text{H}_2\text{O}} \times \frac{1 \text{ mol } ^1\text{H}}{9.01 \text{ g } \text{H}_2\text{O}} = 0.111 \frac{\text{mol } ^1\text{H}}{\text{ml } \text{H}_2\text{O}}.$$  \[5.A4\]

For the bound proton compartments, $C_{B,M}$ and $C_{B,NM}$, were considered to be the concentration of methylene protons in myelin and non-myelin compartments, respectively. These values were estimated by first estimating average values of $\zeta_{B,M}$, $\zeta_{B,NM}$, $\rho_{B,M}$ and $\rho_{B,NM}$. The average $\zeta$, for each compartment was computed as the weighted average of molar proton masses of each of the most abundant lipids and proteins as,

$$\zeta_{B,x} = \sum_{i=1}^{n} w_{i,x} \times \zeta_i,$$  \[5.A5\]

where, $x$ indicates either myelin or non-myelin, and $n$ is the number of different molecules.

For example, cholesterol, [C$_{27}$H$_{46}$O], has 45 methylene protons and a molar mass of 386.65 g/mol, making the molar proton (methylene) mass of cholesterol $\zeta_{\text{cholesterol}} = 8.59$ g/mol $^1$H. In myelin and non-myelin, the dry mass fraction of cholesterol was estimated as, $w_{\text{cholesterol,M}}$
= 0.216 and \( w_{\text{cholesterol,NM}} = 0.108 \), respectively. Similarly, \( \zeta, w_{\text{M}}, \) and \( w_{\text{NM}} \) were estimated for all constituent molecules as shown in Table 5.1 (54,55).

<table>
<thead>
<tr>
<th>Constituent</th>
<th>( \zeta_i ) (g/mol (^1\text{H}))</th>
<th>( w_{\text{M}} )</th>
<th>( w_{\text{NM}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>0.80</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.59</td>
<td>0.216</td>
<td>0.108</td>
</tr>
<tr>
<td>Ganglioside</td>
<td>13.94</td>
<td>0.208</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>9.75</td>
<td>0.160</td>
<td>0.095</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>9.18</td>
<td>0.080</td>
<td>0.108</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>10.51</td>
<td>0.068</td>
<td>0.043</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>9.19</td>
<td>0.068</td>
<td>0.077</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td>0.20</td>
<td>0.57</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>20.64</td>
<td>0.075</td>
<td>0.214</td>
</tr>
<tr>
<td>Proteolipid protein</td>
<td>18.19</td>
<td>0.125</td>
<td>0.356</td>
</tr>
</tbody>
</table>

To calculate \( \rho_{B,M} \) and \( \rho_{B,NM} \), first, water mass fractions of myelin and non-myelin tissue (\( w_{W,M} \) and \( w_{W,NM} \)) were converted to volume fractions (\( \Phi_{W,M} \) and \( \Phi_{W,NM} \)) using literature values of \( \rho_{W}, \rho_{M}, \) and \( \rho_{NM} \) (12) as,

\[
\Phi_{W,x} = w_{W,x} \times \frac{\rho_x}{\rho_W} \tag{5.A6}
\]

where \( x \) indicates either myelin or non-myelin.

Using \( \rho_{M}, \rho_{NM}, \Phi_{W,M}, \) and \( \Phi_{W,NM}, \) the mass densities of bound protons in myelin and non-myelin tissues (\( \rho_{B,M} \) and \( \rho_{B,NM} \), respectively) were determined from,

\[
\rho_{B,x} = \frac{\rho_x - \left( \Phi_{W,x} \times \rho_{W,x} \right)}{1 - \Phi_{W,x}} \tag{5.A7}
\]

where \( x \) indicates either myelin or non-myelin. Then, with \( \zeta_{B,M}, \zeta_{B,NM}, \rho_{B,M} \) and \( \rho_{B,NM} \), and Eq. 5.A2, \( C_{B,M} \) and \( C_{B,NM} \) are,
\[ C_{B,M} = \frac{\rho_{B,M}}{\zeta_{B,M}} = 0.095 \frac{\text{mol} \ ^1\text{H}}{\text{mL bound}} \]

\[ C_{B,NM} = \frac{\rho_{B,NM}}{\zeta_{B,NM}} = 0.090 \frac{\text{mol} \ ^1\text{H}}{\text{mL bound}} \]  

Thus, the molar concentrations of \(^1\text{H}\) in the macromolecule compartments of myelin and non-myelin (0.095, and 0.090 \(^1\text{H} / \text{ml}, \text{respectively}) are estimated to be 15-20\% lower than that of free water. For simplicity of modeling, we treat all four compartment molar proton concentrations as equal, making magnetization \(M_{0,x}\) (and, therefore, signal) directly proportional to volume \(V_x\) (Eq [5.A1]).

5.8 REFERENCES


6.1 INTRODUCTION

Chapter 5 displayed the ability of MET₂ and qMT to assess myelin content in normal and abnormal mature mice. However, it would also be beneficial to use these techniques during development to monitor myelination. Based on previous studies, myelin content and the estimated rate of myelin production in rats is shown in Fig 6.1. From this figure, it can be seen that myelin formation begins around postnatal days 10-12 (P10-P12). Around P20, ~15% of total myelin content exists, which is also the peak rate of myelin formation. While myelin production continues through maturation, the rate of myelin production greatly declines by P60, when rats are considered to be mature (1,2).

![Figure 6.1. The accumulation of myelin plotted as a function of the logarithm of postnatal age. The circles (o) represent actual yields of myelin in mg/brain (left ordinate). The rate of accumulation is plotted as a smooth curve, with the maximum of 2.1 mg myelin day⁻¹brain⁻¹ (right ordinate) occurring at 20 days (1).](image-url)
Diffusion-weighted imaging (DWI) and diffusion tensor imaging (DTI) techniques have been used to study various normal and abnormal models of myelin development (3–5); however diffusion parameters are not a specific measure of myelin content. This is exemplified by groups observing changes in diffusion parameters prior to postnatal day 10, the start of myelin formation (6,7). Since diffusion is sensitive to axonal development in addition to myelination, DTI provides valuable information about development, but is not specific to myelin development. On the other hand, relaxation measures (MWF and BPF) may provide a more specific predictor of myelin, but have yet to be evaluated. In the present study, control mice at P10, P20, P30 and P60 are evaluated using MET2 and qMT methods to obtain MWF and BPF and myelin volume measures, \( f_{M,T2} \) and \( f_{M,MT} \).

### 6.2 MATERIALS AND METHODS

#### 6.2.1 Tissue Preparation

The Vanderbilt University Institutional Animal Care and Use Committee approved animal studies. Twenty-seven control mice (P10 \((n=3)\), P20 \((n=9)\), P30 \((n=9)\), P60 \((n=6)\)) were anesthetized with isoflurane and sacrificed via transcardial perfusion. The perfusion consisted of 1X phosphate-buffered saline (PBS) wash followed by 2.5% glutaraldehyde + 2% paraformaldehyde (modified Karnovsky solution). Following perfusion, brains were quickly removed from skull and immersed in the fixative solution for 1 week. Brains were then washed with 1X PBS + 0.01% sodium azide, changing wash 4-5 times over 1 week to remove excess fixative. In all cases, 1.0 mM Gd-DTPA (Magnevist; Berlex, Montville, NJ) was included in the perfusate, immersion and wash solutions, resulting in relatively uniform distribution of Gd-DTPA throughout the brain.
6.2.2 Magnetic Resonance Imaging

All imaging was performed on a 15.2-T 11-cm horizontal bore Bruker (Rheinstetten, Germany) BioSpec scanner, using a 35-mm diameter Bruker quadrature volume coil for transmission and reception. Both MET\textsubscript{2} and qMT scans were encoded with a matrix size of $128 \times 96 \times 72$ over a $1.92 \times 1.44 \times 1.08$ cm$^3$ FOV, providing 150 µm isotropic resolution.

For MET\textsubscript{2} imaging, a 3D multiple spin-echo sequence was used with non-selective excitation and refocusing pulses, 160 µs and 100 µs in duration, respectively. Each refocusing pulse was surrounded by 428 µs duration 6 G/cm amplitude crusher gradients, phase-encoding gradients were rewound after each echo, and a two-part (+X/-X) phase cycling scheme was used. With these constraints, secondary echoes that were not excited by the initial excitation pulse were removed, and the observed echo magnitudes could be computed with the extended phase graph (EPG) algorithm (8–10). Scan parameters were: repetition time ($T_R$) = 520 ms, echo time ($T_E$) = 4.5 ms, number of echoes ($N_E$) = 24, receiver bandwidth ($BW$) = 75 kHz, and number of excitations ($N_{EX}$) = 8. Total scan time was $\approx$ 8 hr.

For qMT imaging, a 3D selective inversion-recovery prepared fast spin echo sequence (11) was used with 8 collected echoes, 5-ms echo spacing and centric phase encoding. To ensure that the longitudinal magnetization ($M_z$) recovered from $M_z = 0$ at the start of every pre-delay period, 8 additional refocusing pulses, also with 5-ms echo spacing, followed the 8\textsuperscript{th} echo. A 1-ms hard pulse was used to selectively invert the free water magnetization, while macromolecular spins were mostly unaffected. The sequence was repeated $N_I = 15$ times with inversion times ($T_I$) log-spaced from 3.5 to 2000 ms. A constant predelay ($T_d$) of 590 ms resulted in a scan time of $\sim$ 3.5 hr.
6.2.3 Data Analysis

All data analysis was performed using MATLAB R2015a (The Mathworks, Natick MA) and MET\textsubscript{2} analyses were performed using the freely available Multi Exponential Relaxation Analysis (MERA) toolbox (12). Prior to Fourier reconstruction, k-space data for all images were apodized using a 3D Tukey window with a 0.25 taper-to-window ratio and zero-padded 2\times, resulting in 75 \mu m nominal isotropic resolution.

For each voxel, the MET\textsubscript{2} analysis used a separable non-linear approach. The $T_2$ spectrum was estimated by linear inverse (13) using a non-negative least-squares fit (14) of the $N_E$ echo magnitudes to the sum of 100 EPG-defined signals with $T_2$ values logarithmically spaced between $T_E/2$ as 500 ms. The linear model was augmented with minimum curvature constraint weighted at a constant and conservative level across all voxels ($\mu = 0.002$). This linear inverse was repeated to find the refocusing pulse flip angle ($\theta$) by non-linear regression, similar to previous work (9,10). From all spectra, the myelin water fraction (MWF) was defined as the fraction of signal with $T_2 < 17$ ms.

For qMT analysis, the $N_I$ image magnitudes were fitted voxel-wise to the Bloch-McConnell equations describing longitudinal relaxation and magnetization transfer between water and macromolecular protons (11,15). The five fitted model parameters were: $M_{0f}$, $M_{0b}$, $k_{mf}$, $R_{1f}$, and $S_f$, where $M_{0f,b}$ are the equilibrium magnetizations of the free and bound pools, respectively, $k_{bf}$ is the rate constant of magnetization transfer from the bound to free water pool, $R_{1f}$ is the longitudinal relaxation rate of the free water pool, and $S_f$ is the efficiency of the inversion pulse on the free water pool. The corresponding $R_{1b}$ and $S_b$ values were constrained to $R_{1b} = 1$ s and $S_b = 0.83$ in accord with prior studies (11). The bound proton fraction (BPF) was then defined as $BPF = M_{0b} / (M_{0b} + M_{0f})$. 
After MET$_2$ and qMT analysis, all parameter maps of a given model were co-registered in order to define closely comparable regions of interest (ROI) in each brain. For each mouse model, the first spin echo image of one brain was arbitrarily defined as the reference and the corresponding image from each other brain was registered to the reference using a rigid affine registration followed by a non-rigid deformable demons registration (16). The resulting deformation fields of this registration were then applied to parameter maps. Four ROIs were drawn corresponding to the four regions extracted for histology (below): 3 in the corpus callosum in the mid-sagittal slice (genu (GCC), mid-body (MidCC), and splenium (SCC)) and the other in the anterior commissure (AC). In addition, a cortical gray matter (GM) ROI was drawn in the sagittal slice for comparison. Mean ROI values from different parameter maps and values derived from histology were compared using two-sample $t$-tests ($\alpha = 0.05$) and Pearson’s linear correlation.

6.3 RESULTS AND DISCUSSION

Figure 6.2 displays representative $T_2$-weighted images ($N_E = 1$) along with parameter maps of long $T_2$ component, MWF, and BPF from P10, P20, P30 and P60 animals. There is an increase in $T_2$-weighted image contrast and long $T_2$ component between white and gray matter with development as expected with increasing myelin. Similarly, MWF and BPF display higher fractions in white matter with age.
Figure 6.2. Representative images and parameter maps. (top to bottom) $T_2$-weighted images and long $T_2$, MWF and BPF maps from (left to right) P10, P20, P30, and P60 control mice.

Fig 6.3 displays quantitative analysis of parameters in four white matter (WM) regions of interest (ROIs) – midbody, genu and splenium of the corpus callosum (MidCC, GCC, SCC, respectively) and the anterior commissure (AC) – along with one cortical gray matter (GM) ROI. As expected, there is a decrease in long $T_2$ and an increase in MWF and BPF from P10 to P60 across all WM ROIs. There are significant decreases in long $T_2$ and increases BPF between each age in all WM ROIs but one. MWF is significantly different between P20 and P30 in all ROIs and between P30 and P60 in 3 out of 4 ROIs. Additionally, there are significant changes in $T_2$ and BPF in GM through P30, while MWF is consistently near zero.
Figure 6.3. Region of Interest (ROI) MRI analysis. Mean ± standard deviation (top to bottom) long $T_2$, MWF and BPF maps. Values are shown from 4 WM regions of interest and one gray matter region from P10, P20, P30, and P60 control mice where bars represent statistically significant differences ($\alpha = 0.05$). ($\ast = p <0.05$, $\ast\ast = p <0.01$, $\ast\ast\ast = p <0.001$).

Fig 6.4 displays correlation between MWF and BPF showing strong linear correlation ($\rho = 0.85$), but the relationship is clearly not 1:1. Next, measures of myelin volume fraction (MVF) are calculated (See Chapter 5) from MWF ($f_{M,T2}$) and BPF ($f_{M,MT}$). While conversion from MWF to $f_{M,T2}$ is straightforward, calculating $f_{M,MT}$ requires a calibration factor, $\beta$. Since calibration from histology is not available, Chapter 5 proposed to use $\beta = 72\%$ of BPF in gray matter. Using that guideline, we obtain $\beta$ values shown in Table 6.1. From these, $f_{M,MT}$ is calculated and Fig 6.4 displays strong correlation between $f_{M,T2}$ and $f_{M,MT}$ ($\rho = 0.85$), with points lying around the line of unity.
**Figure 6.4. MRI scatter plots.** (left) BPF versus MWF and line of unity (gray, dashed). $\rho = 0.85$. Mean standard errors from MWF and BPF are 0.0066 and 0.0032, respectively. (right) $f_{M,MT}$ versus $f_{M,T2}$ and line of unity (gray, dashed). $\rho = 0.85$.

<table>
<thead>
<tr>
<th>Age</th>
<th>$\beta$</th>
<th>$% \beta_{P60}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P10</td>
<td>0.0574</td>
<td>66.8%</td>
</tr>
<tr>
<td>P20</td>
<td>0.0784</td>
<td>91.3%</td>
</tr>
<tr>
<td>P30</td>
<td>0.0836</td>
<td>97.3%</td>
</tr>
<tr>
<td>P60</td>
<td>0.0859</td>
<td>100%</td>
</tr>
</tbody>
</table>

Fig 6.5 shows representative $f_{M,T2}$ and $f_{M,MT}$ maps from P10, P20, P30 and P60 animals displaying good qualitative agreement. Gray matter values in $f_{M,MT}$ are closer to zero compared to BPF and remain consistent between ages. This displays the ability to detect changes specific to myelin with MRI myelin volume imaging. Previous works have largely used diffusion-weighted imaging and/or $T_1$ and $T_2$ imaging to assess development (17). While these techniques are sensitive to myelin changes, they are also sensitive to other aspects of brain growth and maturity such as gliosis and axon development. This is exemplified by a ‘pre-myelination’ state where changes in diffusion parameters precede the presence of myelin determined by myelin basic
protein and proteolipid protein histology staining (18,19). In addition, studies have also shown that unmyelinated nerves also display similar diffusion anisotropy (20). While myelin will contribute to anisotropy, this study and others demonstrates the sensitivity of DTI to changes in cytoskeletal development, but lack specificity to myelin development (21).

In fact, BPF from qMT will also be sensitive to axonal and cytoskeleton development. To calculate myelin volume fraction, $f_{M,MT}$, the contribution from non-myelin macromolecules, $\beta$, must be removed. Previous work in rodents has shown that peak brain growth is ~P7-10 with gliogenesis and increasing axon and dendritic density. By P20, the brain has reached ~90-95% of its adult size and myelin growth takes over (22). Our values in Table 6.1 reflect this expected growth. This suggests that our applied calibration found from histology in Chapter 5 is reliable. Additionally, this reiterates the specificity that only MWF, $f_{M,T2}$, and $f_{M,MT}$ can provide to myelin development.

Figure 6.5. Group mean myelin volume fraction maps. Group mean $f_{M,T2}$ and $f_{M,MT}$ maps from (left to right) P10, P20, P30, and P60 control mice.
6.4 CONCLUSIONS

Development is a critical time for future neuronal health. However, in the first 30 days of rodent life there is much growth and development beyond myelin; so, it is important to have a measure that can tease out absolute changes in myelin content. We show that BPF suffers from non-myelin contributions of axons and cytoskeleton similar to DTI. However, myelin volume imaging allows for specific monitoring of myelin during development.

6.5 REFERENCES


CHAPTER 7

A REVISED MODEL FOR ESTIMATING G-RATIO FROM MRI

7.1. INTRODUCTION

Myelin is a critical component of white matter, increasing speed of action potential conduction along axons and improving neurological function. It has been shown that there is a range of values between axon size and myelin thickness for optimal efficiency in healthy tissue (1,2). The g-ratio describes the relationship between axon size and myelin thickness, and deviations in the g-ratio are thought to be involved in abnormal development and disease (3–6). However, currently, the only way to assess properties of tissue microstructure such as axon diameter, myelin thickness, and g-ratio is through quantitative histology, such as electron microscopy. Such methods are time consuming, expensive, and destructive to the tissue. Magnetic resonance imaging (MRI) methods to measure these microstructural characteristics would be useful to more efficiently study white matter disease processes and treatments and, further, provide the potential for in vivo assessment.

Recently, it has been proposed that two quantitative MRI measures can be combined and interpreted with a geometric model of white matter to provide quantitative estimates of the g-ratio (7,8). Specifically, Stikov and colleagues have suggested that using MRI estimates of 1) myelin volume fraction (from, for example, quantitative magnetization transfer measurements), and 2) axon or fiber volume fraction (from, for example, suitable analysis of diffusion-weighted imaging) can be used to estimate the g-ratio. These estimates were termed an “aggregate g-ratio” because the method is predicated on the assumption that the g-ratio is constant for all axons within a voxel, which is known not to be the case in both peripheral nerve (1,9) and central white
matter (10,11). Here we extend their model to a more general one that makes no assumption about the distribution of g-ratio values within an imaging voxel, and we demonstrate the model in principal using quantitative evaluations of electron microscopy of the corpus callosum of control and hypomyelinated mice.

7.2 THEORY

Consider an ensemble of $N$ myelinated fibers with the radius and g-ratio of the $i^{th}$ fiber being $R_i$ and $g_i$, respectively, and axon radius being $r_i$ (hence, $g_i = r_i/R_i$, see Fig 1e). The total cross-sectional areas of fibers, axons, and myelin, are, respectively,

$$A_F = \pi \sum_{i=1}^{N} R_i^2$$
$$A_A = \pi \sum_{i=1}^{N} g_i^2 R_i^2 = \pi \sum_{i=1}^{N} r_i^2$$
$$A_M = \pi \sum_{i=1}^{N} R_i^2 \left(1 - g_i^2\right) \quad [7.1]$$

From here, the ratio of myelin to fiber areas is

$$\frac{A_M}{A_F} = \frac{\sum_{i=1}^{N} R_i^2 \left(1 - g_i^2\right)}{\sum_{i=1}^{N} R_i^2} \quad [7.2]$$

In the case that $g_i = g$ for all $i = 1$ to $N$, as assumed in the model presented by Stikov et al. (7,8), this ratio reduces to

$$\frac{A_M}{A_F} = 1 - g^2 \quad [7.3]$$

Assuming one can measure myelin volume fraction ($MVF$) and fiber volume fraction ($FVF$) with MRI, the ratio $A_M/A_F$ can be replaced with $MVF/FVF$, and the resulting MRI-measured g-ratio is then
\[ g_{\text{MRI}} = \sqrt{1 - \frac{MVF}{FVF}} \]  \hspace{1cm} [7.4]

as previously presented (7,8).

By starting with the ratio \( A_M/A_A \), a similar relationship is found, \( g_{\text{MRI}} = \sqrt{1/(1 + MVF/AVF)} \), where \( AVF \) is the axon volume fraction as measured by MRI.

However, without the simplifying assumption that \( g \) is constant for all axons, Eq [7.2] can be simply reduced to

\[
\frac{A_M}{A_F} = \frac{\sum_{i=1}^{N} R_i^2 - \sum_{i=1}^{N} g_i^2 R_i^2}{\sum_{i=1}^{N} R_i^2} = 1 - \frac{\sum_{i=1}^{N} g_i^2 R_i^2}{\sum_{i=1}^{N} R_i^2}.
\]  \hspace{1cm} [7.5]

Again, replacing the \( A_M/A_F \) with \( MVF/FVF \) and using Eq [7.4], we get

\[
g_{\text{MRI}}^2 = \frac{\sum_{i=1}^{N} g_i^2 R_i^2}{\sum_{i=1}^{N} R_i^2},
\]  \hspace{1cm} [7.6]

which shows that the squared value of the previously proposed MRI measure of \( g \)-ratio is equal to the area-weighted mean of \( g^2 \) values across the \( N \) fibers.

7.3. MATERIALS AND METHODS

7.3.1 Tissue Preparation

Animal studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Histology was acquired from control and \textit{Rictor} conditional knockout (CKO) mice, similar to a previously described mouse model of tuberous sclerosis complex (12). Six adult mice were anesthetized with isoflurane and sacrificed via transcardial perfusion of 1X phosphate-buffered saline (PBS) wash followed by 2.5% glutaraldehyde + 2% paraformaldehyde
in PBS (modified Karnovsky solution). Following perfusion, brains were quickly removed from the skull and immersed in the fixative solution for 1 week. For MRI studies not presented here, the perfusion and immersion solutions included a paramagnetic MRI contrast agent and the fixative was washed out of brains prior to imaging and subsequent histology. For histologic preparation, a 1-2 mm sagittal slice of tissue was cut from the left hemisphere beginning at the mid-brain from each of 6 brains (n=4 control and n=2 CKO). Subsequently, 2 regions of white matter from the corpus callosum (genu - GCC and midbody - MidCC) were cut from each slice. Two regions were analyzed to account for potentially different axon populations between regions of the corpus callosum (13). Tissue samples were then processed for transmission electron microscopy in the Vanderbilt Cell Imaging Shared Resource-Research Electron Microscopy facility. Thick sections (0.5 – 1 µm) were collected using a Leica Ultracut microtome (UC-7), then stained with 1% toluidine blue. Ultra-thin sections (70-80 nm) were then cut and collected on 300-mesh copper grids. Copper grids were post-section stained at room temperature with 2% uranyl acetate (aqueous) for 15 minutes and then with lead citrate for 10 minutes. Ultra-thin sections were imaged on the Philips/FEI Tecnai T12 electron microscope at 15,000X magnification. From each section, six images were acquired using a side-mounted AMT CCD camera, resulting in a total of 6 mice x 2 regions x 6 images/region/mouse = 72 images.

7.3.2 Data Analysis

The pipeline of histology analysis is summarized in Fig 7.1. Images were segmented using Otsu’s method (14) to distinguish between myelin (white) and non-myelin (black) pixels, providing an estimate of \( MVF \) and a binary image (Fig 1c). From the binary image, each myelinated axon was manually identified and its area \( A_{Ai} \) for the \( i^{th} \) axon was computed using a region growing algorithm. This value provided an estimate of axon radius \( r_i = \sqrt{A_{Ai}/\pi} \), and the
sum of all axon areas provided an estimate of \( AVF \). For each axon, the thickness of the surrounding myelin (\( \Delta_i \)) was calculated as the average of manual measurements made in two locations, and the g-ratio was estimated as \( g_i = r_i / (r_i + \Delta_i) \).

From each image, the \( MVF \) and \( AVF \) estimates were used to compute \( g_{\text{MRI}} \), as shown above. Also from each image, the \( N \ (= 40-60) \) measures of \( g \) were fitted to a gamma distribution as done previously for axon diameter distributions \((13,15)\), from which three descriptive measures were calculated: arithmetic mean (\( g_{\text{mean}} \)), the area-weighted mean (\( g_{\text{awm}} \)), the square-root of the area-weighted \( g^2 \) (\( g_{\text{awmgs}} \), following the right hand side of Eq \([7.6]\)). Calculating these descriptive measures of \( g \) from the fitted gamma distribution parameters rather than directly from the samples \( g_i, i = 1 \) to \( N \), resulted in lower variance estimates of the area-weighted measures of g-ratio.
Figure 7.1. **Histology analysis methods.** a) Demonstration of the middle corpus callosum region chosen for electron microscopy tissue preparation. b) A transmission electron microscope image is acquired from the middle region of the mouse corpus callosum. c) A threshold is applied to separate myelin and non-myelin pixels and obtain a binary myelin mask, providing a myelin volume fraction ($MVF$). d) A region growing algorithm is used to fill all axon areas, and the sum of all axon areas provides an axon volume fraction ($AVF$). Myelin thickness is measured manually in two locations per axon. e) Axon radius ($r$) is derived from the area of each axon, assuming circular geometry. f) The $g$-ratio is calculated per axon and the histogram of values is fitted to a gamma distribution, from which the following measures are computed: mean ($g_{\text{mean}}$), area-weighted mean ($g_{\text{awm}}$), and the square root of the area-weighted $g^2$ ($g_{\text{awmgs}}$). The proposed MRI measure, $g_{\text{MRI}}$, was computed from measures of $MVF$ and $AVF$.

### 7.4. RESULTS AND DISCUSSION

Figure 7.1f displays a representative histogram of the $g$-ratios obtained from one histology image. Each characterization of the $g$-ratio ($g_{\text{mean}}$, $g_{\text{awm}}$, $g_{\text{awmgs}}$, and $g_{\text{MRI}}$) is displayed on the histogram. It is apparent that $g_{\text{mean}}$ (~0.8) is significantly lower than the area-weighted means and $g_{\text{MRI}}$. This characteristic will be true in general for distributions of finite width, but will also depend on the skewness of the distribution.
Figure 7.2 displays comparisons of $g_{\text{MRI}}$ with $g_{\text{mean}}$, $g_{\text{awm}}$, and $g_{\text{awmgs}}$, respectively, from left to right. Each point represents the measurements from one image with both MidCC and GCC measures displayed (control = black, CKO = red). The hypomyelination present in the CKO mice is apparent from the generally greater $g$-ratios. Qualitatively, $g_{\text{MRI}}$ shows a reasonable correspondence with all three measures, but the comparisons between $g_{\text{MRI}}$ and the area-weighted measures are noticeably closer to the line of identity (dashed line). Quantitatively, these differences are reflected in the root mean squared difference (RMSD), between the two measures for each plot. Similar relationships are found between $g_{\text{MRI}}$ and the two area-weighted measures, $g_{\text{awm}}$ and $g_{\text{awmgs}}$, indicating that $g_{\text{MRI}}$ can be reasonably interpreted as an axon-area-weighted measure of $g$, which is somewhat easier to intuit than the square root of the axon-area-weighted $g^2$. Both area-weighted measures show a slight trend toward being underestimated by $g_{\text{MRI}}$ at lower values of $g$, which may reflect the limitations of the histology analysis. As apparent in Figs 7.1c and 7.1d, there was a tendency to overestimate $MVF$ from the thresholded image, particularly when myelinated axons were more densely packed and connected, which also corresponded to cases of lower $g$-ratios. This overestimation of $MVF$ will reduce estimates of $g_{\text{MRI}}$ but will not affect the other characterizations of $g$ which were derived from direct measures of myelin thickness.
Figure 7.2. Scatter plots of g-ratio. (left) $g_{\text{MRI}}$ versus $g_{\text{mean}}$, (middle) $g_{\text{MRI}}$ versus $g_{\text{awm}}$, and (right) $g_{\text{MRI}}$ versus $g_{\text{awmgs}}$ where black and red points signify control and CKO image measures, respectively. The root mean squared difference (RMSD) between each pair of measures is shown above each plot.

Fig 7.3 shows scatter plots of g-ratio versus axon diameter for all 6 images from the representative control and CKO brains for both regions. In all cases, the relationship between axon diameter and $g$ exhibited a curved shape seen in some previous studies (10,11), and appear to be well described by the log-linear equation proposed by Berthold et al. ($nl = C_0 + C_1 * d + C_2 * \log(d)$); where $nl$ = number of myelin lamellae and $d$ = axon diameter (blue line) (10). These observations are in contrast to a recent similar histological evaluation of the macaque corpus callosum (16) who showed only moderate linear correlations between g-ratio and axon diameter.
The histology data are also summarized in Table 7.1, which displays the mean ± standard error of the mean of the four g–ratio measures across the 6 images from each region (MidCC, GCC) for control and CKO mice. Reported \( g_{\text{mean}} \) values of control images agree with previously published values for mouse corpus callosum (5,17). It is apparent that the two area-weighted measures (\( g_{\text{awm}} \), \( g_{\text{awmgs}} \)) are nearly identical in all cases and similar to \( g_{\text{MRI}} \) values. All measures show statistically significant differences (using a two-tailed student’s t-test, \( \alpha=0.05 \)) between control and CKO mice, demonstrating that for this example, \( g_{\text{MRI}} \), which reports a significantly different value than \( g_{\text{mean}} \), is sufficient to detect differences in microstructure between the control and CKO mice. However, we note the time course of demyeliation and remyelination may not always be well captured by a scalar value, and because of the area-weighting effect, \( g_{\text{MRI}} \) in particular will be less sensitive to microstructural changes in smaller axons. For example, a previous study of microstructure in the mouse corpus callosum during and following exposure to cuprizone in the diet (5) observed periods with changes in myelin thickness and axon diameter.
that were not caputured by the mean g-ratio, and found that recovery periods involved preferential remyelination of smaller axons. Perhaps these limitations can be overcome with more sophisticated models that incorporate axon diameter distributions (such as is done with the AxCaliber method, (13,18)) and established relationships between axon diameter and g-ratio shown in Fig 7.3 (2,10)); however, the practical limits on MRI measures of g-ratio may come from the ability make robust estimates of MVF and FVF, which remains an area of active study.

Table 7.1. g-ratio values. $g_{\text{mean}}$, $g_{\text{awm}}$, $g_{\text{awmgs}}$, and $g_{\text{MRI}} \pm \text{SEM}$ from the middle and genu regions of the corpus callosum across all images for control and Rictor CKO mice.

<table>
<thead>
<tr>
<th>Region</th>
<th>$g_{\text{mean}}$</th>
<th>$g_{\text{awm}}$</th>
<th>$g_{\text{awmgs}}$</th>
<th>$g_{\text{MRI}}$</th>
</tr>
</thead>
</table>
| Control
| MidCC  | 0.815±0.003     | 0.848±0.003     | 0.850±0.003     | 0.844±0.004     |
| GCC    | 0.803±0.005     | 0.847±0.003     | 0.849±0.003     | 0.834±0.005     |
| CKO
| MidCC  | 0.845±0.006     | 0.873±0.007     | 0.875±0.007     | 0.870±0.010     |
| GCC    | 0.854±0.006     | 0.880±0.009     | 0.883±0.009     | 0.889±0.008     |

7.5 CONCLUSIONS

As quantitative MRI methods strive to provide more detailed information about underlying tissue properties, such as the g-ratio, histologic comparisons are vital to understand microstructural meaning of derived imaging measures. We have shown here that the recently proposed approach to estimate the aggregate g-ratio index with MRI will, in principal, provide a measure that is close to the axon-area-weighted measures of $g$ across all axons in a voxel. This measure will naturally be more sensitive to changes or differences in larger diameter axons and should be interpreted with this knowledge.
7.6 REFERENCES


8.1. INTRODUCTION

Myelin is a key component of healthy white matter leading to faster action potential conduction velocity along axons. Changes in myelin content and structure occur in several neurodegenerative diseases, such as Multiple Sclerosis (MS) (1) and Alzheimer’s disease (2). Additionally, abnormal axon and myelin development cause altered connectivity that may be a key factor in neuropsychiatric disorders such as Autism Spectrum Disorder (3), Schizophrenia (4), and Obsessive Compulsive Disorder (5). While measures of myelin volume fractions tell about the amount of myelin, a measure capable of relating myelin volume relative to axon volume provides more detail about pathologic changes. The g-ratio is the ratio of axon diameter to myelinated fiber diameter, and has been proposed to capture the microstructural changes of myelin thickness (6,7). Currently, the only validated technique to measure the g-ratio is electron microscopy, which allows direct visualization of myelinated axons in cross section. However, this approach is slow, expensive, and is limited to ex vivo samples and brain locations where axons can be sectioned perpendicular to their orientation.

To overcome these limitations of electron microscopy for evaluating the g-ratio, Stikov et al. proposed that a geometric model using the relationship between the myelin and axon volume fractions (MVF and AVF, respectively) to obtain an “aggregate” g-ratio, shown in Equation 8.1 (8,9).
However, each imaging voxel contains a distribution of g-ratios. Based on histology measures, West et al. displayed that this geometric equation for $g$ (Eq. 8.1) provides an area-weighted g-ratio from all axons in a voxel (10). This model has been proposed as a method to obtain g-ratio from MRI ($g_{\text{MRI}}$) using measures of myelin and axon volume fraction from MRI techniques.

Myelin content can be estimated using numerous techniques, but measures of MVF from MRI were displayed previously by West et al. and shown to correlate well with histologic measures of MVF (See Chapter 5). Briefly, myelin water fraction (MWF) from multiexponential $T_2$ (MET$_2$) data or bound pool fraction (BPF) from quantitative magnetization transfer (qMT) can be converted to volume fractions based on a volumetric four-pool model of white matter protons and literature values of water pool fractions. This technique results in two MRI measures of MVF: $f_{M,T2}$ and $f_{M,MT}$, where subscripts “T2” and “MT” describe the method used. While both measures provided promising results, $f_{M,T2}$ may be biased by effects from intercompartmental water exchange and $f_{M,MT}$ is dependent on a calibration factor to account for non-myelin bound proton contributions.

A measure of AVF can be derived from measures of axon water fraction obtained using an advanced diffusion model. There are a variety of techniques that can be used including Neurite Orientation Dispersion and Density Imaging (NODDI) (11), Tensor Fiber Density (TFD) (12), and White Matter Tract Integrity (WMTI) (13), along with numerous others. To obtain tissue-specific characteristics of white matter, each of these methods have a priori assumptions which may depend on each tissue of interest. Additionally, AVF still relies on an accurate myelin

\[
g = \frac{1}{\sqrt{1 + \frac{\text{MVF}}{\text{AVF}}}} \quad [8.1]
\]
volume fraction measure to convert axon water fraction to an axon volume fraction to use in Eq 8.1.

The present study uses previously displayed measures of MVF along with measures of AWF from WMTI and compare resulting $g_{\text{MRI}}$ estimates to g-ratio measures from quantitative electron microscopy ($g_{\text{Hist}}$). This is shown in control mouse brains along with two models of hypomyelination and one model of hypermyelination to display variations in g-ratio.

8.2. MATERIALS AND METHODS

8.2.1 Tissue Preparation

The Vanderbilt University Institutional Animal Care and Use Committee approved animal studies. 15 adult mice were anesthetized with isoflurane and sacrificed via transcardial perfusion. The perfusion consisted of 1X phosphate-buffered saline (PBS) wash followed by 2.5% glutaraldehyde + 2% paraformaldehyde (modified Karnovsky solution). Following perfusion, brains were quickly removed from skull and immersed in the fixative solution for 1 week. Brains were then washed with 1X PBS + 0.01% sodium azide, changing wash 4-5 times over 1 week to remove excess fixative. In all cases, 1.0mM Gd-DTPA (Magnevist; Berlex, Montville, NJ) was included in the perfusate, immersion and wash solutions, resulting in relatively uniform distribution of Gd-DTPA throughout the brain.

This study used control animals (n=6) along with two previously described models of hypomyelination and one of hypermyelination (n = 3 for each model). All models utilized the Olig2-Cre driver to conditionally target proteins involved in PI3K/Akt signaling in oligodendrocyte precursor cells. In the first model, the Tsc2 gene is deleted (Tsc2 CKO–conditional knockout) and exhibits extreme loss of myelin (14). The second model targets Rictor
(Rictor CKO), a key component of the mTORC2 complex, and also displays hypomyelination, but less severe than Tsc2 model (15). The third model results from the deletion of Pten (Pten CKO) leading to activation of the PI3K/Akt signaling pathway and subsequent hypermyelination (16).

2.2 Magnetic Resonance Imaging

All imaging was performed on a 15.2-T 11-cm horizontal bore Bruker (Rheinstetten, Germany) BioSpec scanner, using a 35-mm diameter Bruker quadrature volume coil for transmission and reception. Both MET$_2$ and qMT scans were encoded with a matrix size of $128 \times 96 \times 72$ over a $1.92 \times 1.44 \times 1.08$ cm$^3$ FOV, providing 150µm isotropic resolution.

For MET$_2$ imaging, a 3D multiple spin-echo sequence was used with non-selective excitation and refocusing pulses, 160 µs and 100 µs in duration, respectively. Each refocusing pulse was surrounded by 428 µs duration 6 G/cm amplitude crusher gradients, phase-encoding gradients were rewound after each echo, and a two-part (+X/-X) phase cycling scheme was used. With these constraints, secondary echoes that were not excited by the initial excitation pulse were removed, and the observed echo magnitudes could be computed with the extended phase graph (EPG) algorithm (17–19). Scan parameters were: repetition time ($T_R$) = 520ms, echo time ($T_E$) = 5.8ms, number of echoes ($N_E$) = 18, receiver bandwidth ($BW$) = 38.5kHz, and number of excitations ($N_{EX}$) = 6. Total scan time was ≈ 6 hr.

For qMT imaging, a 3D selective inversion-recovery prepared fast spin echo sequence (20) was used with 8 collected echoes, 5-ms echo spacing and centric phase encoding. To ensure that the longitudinal magnetization ($M_z$) recovered from $M_z = 0$ at the start of every pre-delay period, 8 additional refocusing pulses, also with 5-ms echo spacing, followed the 8th echo. A 1-ms hard pulse was used to selectively invert the free water magnetization, while macromolecular spins
were mostly unaffected. The sequence was repeated $N_i = 15$ times with inversion times ($T_i$) log-spaced from 3.5 to 2000 ms. A constant predelay ($T_d$) of 590 ms resulted in a scan time of $\sim 3.5\text{hr}$.

Diffusion kurtosis imaging data were acquired using a 3D diffusion-weighted fast spin-echo sequence with TR/TE/ESP = 200/19.0/7.1 ms and ETL = 4 (Beaulieu et al., 1993). For excitation and refocusing, non-selective hard pulses were implemented with durations of 125 and 250 µs, respectively. Receiver bandwidth for signal acquisition = 75 kHz. Diffusion weighting was achieved with gradient pulse duration ($\delta$) = 5 ms, diffusion time ($\Delta$) = 12 ms, prescribed b-values = 3000 and 6000 s/mm$^2$, 30 directions (Jones et al., 1999), and 2 signal averages with gradient polarity reversal to mitigate background gradient cross-terms. 5 b=0 images were collected and averaged to improve the precision of DKI fits. In all, for each brain, 125 3D volumes were collected in a total scan time of $\approx 12 \text{ hr}$.

8.2.3 Data Analysis

All data analysis was performed using MATLAB R2015a (The Mathworks, Natick MA) and MET$^2$ analyses were performed using the freely available Multi Exponential Relaxation Analysis (MERA) toolbox (21). Prior to Fourier reconstruction, k-space data for all images were apodized using a 3D Tukey window with a 0.25 taper-to-window ratio and zero-padded 2×, resulting in 75 µm nominal isotropic resolution.

For each voxel, the MET$^2$ analysis used a separable non-linear approach. The $T_2$ spectrum was estimated by linear inverse (22) using a non-negative least-squares fit (NNLS; Lawson and Hanson, 1974) of the $N_E$ echo magnitudes to the sum of 100 EPG-defined signals with $T_2$ values logarithmically spaced between $T_E/2$ as 500 ms. The linear model was augmented with minimum curvature constraint weighted at a constant and conservative level across all voxels ($\mu = 0.002$).
This linear inverse was repeated to find the refocusing pulse flip angle ($\theta$) by non-linear regression, similar to previous work (Lebel and Wilman, 2010; Prasloski et al., 2012). From all spectra, the myelin water fraction (MWF) was defined as the fraction of signal with $T_2 < 17$ ms. MWF was then converted to a myelin volume fraction ($f_{\text{M,T}2}$) as shown previously (See Chapter 5) using Eq 8.2

$$f_{\text{M,T}2} = \frac{MWF \times \Phi_{W,NM}}{MWF \times (\Phi_{W,NM} - \Phi_{W,M}) + \Phi_{W,M}},$$ \hspace{1cm} [8.2]

where $\Phi_{H2O,M}$ and $\Phi_{H2O,NM}$ are the proton pool volume fraction in myelin and non-myelin white matter, respectively.

For qMT analysis, the $N_1$ image magnitudes were fitted voxel-wise to the Bloch-McCon nell equations describing longitudinal relaxation and magnetization transfer between water and macromolecular protons (20,23). The five fitted model parameters were: $M_{0f}, M_{0m}, k_{mf}, R_{1f},$ and $S_f$, where $M_{0f/m}$ are the equilibrium magnetizations of the free and macromolecular pools, respectively, $k_{mf}$ is the rate constant of magnetization transfer from the macromolecular to free water pool, $R_{1f}$ is the longitudinal relaxation rate of the free water pool, and $S_f$ is the efficiency of the inversion pulse on the free water pool. The corresponding $R_{1m}$ and $S_m$ values were constrained to $R_{1m} = 1s$ and $S_m = 0.83$ based in accord with prior studies (20). The macromolecular proton fraction (MPF) was then defined as $MPF = M_{0m} / (M_{0m} + M_{0f})$. MPF was then converted to a myelin volume fraction ($f_{\text{MT}}$) as described previously (See Chapter 5) as shown in Eq 8.3.

$$f_{\text{MT}} = \left(1 + \frac{\Phi_{W,M}}{1 - \Phi_{W,M}}\right) \times [BPF - \beta],$$ \hspace{1cm} [8.3]

where $\beta$ is a calibration constant from the non-myelin macromolecular contribution defined here by histology = 0.086.
Diffusion Kurtosis image data were analyzed using in-house written code in MATLAB (Mathworks, Natick, MA, USA). For DKI analysis, 3D k-space data were zero-padded 2x in each direction before reconstruction. b=0 images were averaged and diffusion-weighted images with opposite gradient polarities were geometrically averaged to reduce contributions from background gradient cross-terms (24). Diffusion and kurtosis tensors were then estimated voxel-wise using a constrained linear least-squares approach (25). From these tensors, DTI indices FA, MD, AD, and RD and DKI indices MK, AK, and RK were calculated on a voxel-wise basis (26). For WMTI analysis, AWF (or \( f_{A,WMTI} \)) was estimated voxel-wise by Eq 8.4:

\[
\begin{align*}
  f_{A,WMTI} &= \frac{K_{\text{max}}}{K_{\text{max}} + 3} \\
\end{align*}
\]

[8.4]

where \( K_{\text{max}} \) is the maximum kurtosis over all directions derived from the kurtosis tensor.

After fitting, all parameter maps of a given model were co-registered in order to define closely comparable regions of interest (ROI) in each brain. For each mouse model, the first spin echo image of one brain was arbitrarily defined as the reference and then corresponding image from each other brain was registered to the reference using a rigid affine registration followed by a non-rigid deformable demons registration (27) of the 1\textsuperscript{st} spin echo images. The resulting deformation fields of this registration were then applied to parameter maps. Four ROIs were then drawn corresponding to the four regions extracted for histology (below): 3 in the corpus callosum in the mid-sagittal slice (genu (GCC), mid-body (MidCC), and splenium (SCC)) and the other in the anterior commissure (AC). In addition, a cortical gray matter (GM) ROI was drawn in the sagittal slice for comparison. Mean ROI values from different parameter maps and values derived from histology were compared using two-sample \( t \)-tests (\( \alpha = 0.05 \)) and linear correlation.
8.2.4 Microscopy

For each brain, after MRI, a 1-2 mm thick sagittal section of tissue was cut from the left hemisphere beginning at the mid-brain. Subsequently, 4 regions of white matter were cut from the slice: the genu, mid-body, and splenium of the corpus callosum and the anterior commissure. Tissue samples were then processed for Transmission Electron Microscopy (TEM) in the Vanderbilt Cell Imaging Shared Resource-Research Electron Microscopy facility. Thick sections (0.5-1 µm) were collected then stained with 1% toluidine blue. Subsequently, ultra-thin section (~ 500 x 500 x 0.07 µm) were cut and then collected on 300-mesh copper grids. Copper grids were stained at room temperature with 2% uranyl acetate (aqueous) for 15 minutes and then with lead citrate for 10 minutes.

Ultra-thin sections were imaged on the Philips/FEI Tecnai T12 electron microscope (FEI Company, Hillsboro, OR) at various magnifications and pictures were acquired with a side-mounted AMT CCD camera. For quantification of myelinated axon microstructure, 6-12 15,000x images were collected (~300 axons) per ROI per animal. Each image was analyzed semi-automatically to derive myelin volume fraction ($f_{M,HIST}$), axon volume fraction ($f_{A,HIST}$), mean axon diameter ($\bar{d}$), and mean myelin thickness ($\bar{\Delta}$).

The histology pipeline was implemented using MATLAB 2015a (The Mathworks, Natick MA) and is shown in Fig 5.2. First, a local Otsu threshold was applied to each image, resulting in a binary image (myelin=1, non-myelin=0). In this binary image, the operator manually identified an intra-axonal point of each myelinated axon and corrected the labeling of pixels that were deemed to be erroneous identified as myelin. Beginning from each manually identified seed point, an active contour algorithm (28) was used to segment each individual axon. The resulting image (Fig 5.2d) provided per axon measures of area and diameter ($a_{ax}$ and $d = 2\sqrt{a_{ax}/\pi}$,
respectively) and total axon volume fraction. (Note that only axons lying fully within the image frame were included in the per-axon measures, but all axon area contributed to the axon volume fraction.)

Because the Otsu threshold resulted in uneven segmentation of the myelin, the myelin of each myelinated axon was independently segmented on the original histology image using the final boundary of the axon segmentation as the starting contour. Each contour was grown outward along lines normal to its tangent (~0.06 µm/line) until reaching a pixel intensity greater than the average intensity of pixels from the initial contour (Fig 5.2e). In order to reject normal lines that grew into the myelin of adjacent axons, a line was discarded if 1) its terminal point matched the location of an axon boundary defined above, or 2) length was more than 3 times the median absolute deviation (29). The ends of the remaining normal lines created a contour defining the outer myelin boundary, and the median length of these lines defined the myelin thickness for that axon, Δ. Finally, myelin area for each axon was determined by uniformly growing the initial contour of each axon to a uniform thickness, Δ as shown in Fig 5.2f. The resulting binary image provided a measure of myelin volume fraction, \( f_{M,HIST} \), for each image. \( g_{HIST} \) was calculated per image using \( f_{A,HIST} \) and \( f_{M,HIST} \) in Eq 8.1. Across all images per mouse model and brain region, mean values of \( \overline{f_{M,HIST}}, \overline{f_{A,HIST}}, \overline{d}, \) and \( \overline{\Delta} \), and \( g_{HIST} \) were calculated.

8.3. RESULTS

Fig 8.1 displays \( g_{HIST} \) against \( f_{M,HIST} \). We see expected significant decreases in \( g_{HIST} \) for Rictor CKO and increases in \( g_{HIST} \) for Pten CKO mice compared to control mice in agreement with decreases and increases in \( f_{M,HIST} \), respectively. However, in the Tsc2 CKO model there is bulk
loss of myelination while the remaining myelinated axons maintain $g_{\text{HIST}}$ measures similar to controls.

Figure 8.1. $g$-ratio versus myelin volume fraction. Scatter plot of $g_{\text{HIST}}$ versus $f_{M,\text{HIST}}$ from control, Rictor CKO, Tsc2 CKO, and Pten CKO mice

Figure 8.2a shows MRI measures of myelin volume fraction ($f_{M,T2}$ and $f_{M,MT}$) versus histologic myelin volume fraction, $f_{M,\text{HIST}}$. $f_{M,T2}$ and $f_{M,MT}$ display strong correlation ($\rho = 0.83$ and 0.84, respectively) as shown previously (See Chapter 5). Using both measures of myelin volume fraction, axon volume fraction ($f_{A,\text{WMTI}}$) is calculated as $\text{AWF}^*(1 - f_{M,T2} \text{ or } f_{M,MT})$. Fig 8.2b displays $f_{A,\text{WMTI}}$ versus $f_{A,\text{HIST}}$ showing strong correlation ($\rho = 0.60$ and 0.58, respectively). There is little difference in $f_{A,\text{WMTI}}$ between control, Rictor CKO, and Pten CKO mice. Red lines display correlations considering all data except Tsc2 CKO mice, with very weak ($f_{M,T2}$) and non-significant ($f_{M,MT}$) correlation. While $f_{M,T2}$ and $f_{M,MT}$ lie near the line of unity, $f_{A,\text{WMTI}}$ overestimates $f_{A,\text{HIST}}$ in all cases, especially in the Tsc2 CKO mice. This is because $f_{A,\text{HIST}}$ is
specific to myelinated axons while $f_{A,WMTI}$ provides an area-weighted measure of all myelinated and unmyelinated axons.

Next, the MRI measures for MVF and AVF are used to calculate $g_{T2}$ and $g_{MT}$ ($MVF = f_{M,T2}$ or $f_{M,MT}$ and AVF = $f_{A,WMTI} = AWF * [1 - f_{M,T2} or f_{M,MT}]$) from Eq 8.1. There is good correlations to $g_{HIST}$ ($\rho = 0.64$ and 0.57, respectively) shown in Fig 8.2c. As before, both $g_{T2}$ and $g_{MT}$ slightly overestimate g-ratio compared to $g_{HIST}$ due to the unmyelinated axon contribution from $f_{A,WMTI}$. $g_{T2}$ displays slightly higher RMSE (0.094) compared to $g_{MT}$ (RMSE=0.085). However, $g_{T2}$ lies tighter to the line of best fit but with slope > 1, which may be due to intercompartmental water exchange effects, decreasing $f_{M,T2}$. In accord with $g_{HIST}$, both $g_{T2}$ and $g_{MT}$ depict a significant increase between control and Rictor CKO mice but only $g_{T2}$ displays a significant decrease from controls in Pten CKO.
Figure 8.2. MRI versus histology scatter plots. a) \(f_{M,T2}\) and \(f_{M,MT}\) versus \(f_{M,HIST}\), b) \(f_{A,WMTI}\) using \(f_{M,T2}\) and \(f_{M,MT}\) versus \(f_{A,HIST}\), c) \(g_{T2}\) and \(g_{MT}\) versus \(g_{HIST}\) from control, Rictor CKO, Tsc2 CKO, and Pten CKO mice.

Fig 8.3 displays \(g_{T2}\) and \(g_{MT}\) values from white matter overlaid on \(T_2\)-weighted images \((T_E = 5.8 ms)\) from control, Rictor CKO, Tsc2 CKO, and Pten CKO brains. The two maps display similar values across the corpus callosum with expected changes in CKO models similar to Fig 8.2c.
8.4. DISCUSSION

$f_{M,T2}$ or $f_{M,MT}$ reports on changes in myelination, which could be due to either axonal loss or demyelination. $f_{A,WMTI}$ can detect bulk axon loss but the state of myelination is otherwise unknown. Using both measures, an estimate of g-ratio can be calculated which describes the relationship between axon size and myelin thickness in each voxel. This allows for a better understanding of changes on a microstructural level to discriminate between axon loss and demyelination.

However, to accurately assess pathology, we need to fully understand what MRI measures of MVF and AVF are providing. If we consider the case of Tsc2 CKO mice, Fig 8.1 displays that although much myelin is lost, the histology measures show that the remaining myelinated axons display $g_{HIST}$ similar to control mice (~0.75 - 0.83). $f_{M,T2}$ and $f_{M,MT}$ reflect the drastic reduction in myelin similar to $f_{M,HIST}$. However, as shown in Fig 8.2b, $f_{AWMTI}$ (or any...
axon water fraction measure from MRI) reports only a small decrease in axons compared to $f_{A,HIST}$ in Tsc2 CKO mice. Therefore, $g_{MRI}$ reports the area-weighted g-ratio of all axons, not just myelinated axons. From this, we cannot distinguish if the remaining myelinated axons are healthy. This is exemplified by the diagram in Fig 8.4. Here, there are 3 diseased voxels, that all began as a normal, control tissue voxel. In voxel 1, half of the axons were lost while the remaining axons remain normally myelinated. In voxel 2, there is no axon loss, but all myelin becomes thinner. And in voxel 3, again there is no axon loss, but half of the axons become dysmyelinated while the others remain normally myelinated. Using our MRI measures of MVF and AVF to calculate $g_{MRI}$, we would be able to detect all diseased voxels from normal tissue. We could assess differences between voxels 1 and 2/3 due to both axon and myelin loss in voxel 1. However, we cannot discriminate between voxels 2 and 3 since our measure of AWF will remain the same. In many cases, it may be enough to distinguish disease from normal (which should be feasible), but it is important when developing a new metric to thoroughly assess its merits and limitations.

![Diagram](image)

**Figure 8.4. Diagram of g-ratio imaging limitations.** a) $f_{M,T2}$ and $f_{M,MT}$ versus $f_{M,HIST}$. b) $f_{A,WMTI}$ using $f_{M,T2}$ and $f_{M,MT}$ versus $f_{A,HIST}$. c) $g_{T2}$ and $g_{MT}$ versus $g_{HIST}$ from control, Rictor CKO, Tsc2 CKO, and Pten CKO mice.
In contrast to Fig 8.3b, previous work has displayed changes in AWF from WMTI due to decreases in myelin fraction. This results from an increase in extra-axonal water fraction since myelin is considered to be ‘silent’ (30,31); However, here, we are normalizing AWF by including the myelin fraction, so we are only sensitive to changes in the intra-axonal fraction. It is apparent from Fig 8.3b that $f_{A,WMTI}$ is relatively consistent between control, *Rictor* and *Pten* CKO mice which is consistent with $f_{A,HIST}$ measures across models displayed previously (See Chapter 5). This is not surprising since these models do not display large axonal loss. A limitation of the WMTI model is the assumption of highly aligned white matter tracts (13). While this is not a problem in the corpus callosum, it will not be translatable to all white matter tracts. Besides the WMTI model, there are numerous other diffusion models that can be used to estimate the axon water fraction as used previously (8,32) but many of the same issues remain regardless of the model used. We attempted to apply the NODDI model to our data, but the resulting NODDI parameters were highly sensitive to *a priori* assumptions of intrinsic diffusivity, which is unknown in *ex vivo* tissue.

However, it may be possible to assess myelin thickness without the need for diffusion models. It has been shown that MET$_2$ estimates of myelin volume fraction may be sensitive to the thickness of the myelin (33–35, Ch 5) As shown in Dula et al., we can use the sensitivity of MET$_2$ to exchange and the robustness of qMT to probe myelin thickness. Using our measures of myelin volume fraction ($f_{M,T2}$ and $f_{M,MT}$) we can compare the measures one-to-one. Fig 8.6a displays the ratio of $f_{M,T2}$ to $f_{M,MT}$ versus mean myelin thickness ($\Delta$). While these measures are a bit noisy, we see the expected trend with ratios closer to 1 as $\Delta$ increases. We can also compare $f_{M,T2}$ to the recently proposed measure of myelin volume from proton density measures (36).
While we did not measure proton density directly, the total water content \( (M_0) \) is the sum of the \( T_2 \) spectrum for each voxel. We can normalize \( M_0 \) in our white matter ROIs to \( M_0 \) in gray matter to obtain normalized water content (NWC) as displayed previously (See Chapter 5). Next, since we do not have a pure water reference marker, we can assume that when myelin volume = 0, the ratio of WM/GM should = 1. Therefore we can subtract an offset \( (\delta \sim 0.11) \). Then, our estimate of \( f_{M,PD} \) will simply be 1-[NWC-\(\delta\)]. Again in Fig 8.6b we can display the ratio of \( f_{M,T2} \) to \( f_{M,PD} \) versus mean myelin thickness \( (\Delta) \), since total proton density should not be affected by exchange. We see slightly noisier but similar results. However, with the addition of a reference water marker (which would likely increase precision), this technique would allow for assessment of myelin thickness using only MET\(_2\).

![Figure 8.5. Myelin volume fraction ratio versus myelin thickness.](image)

Figure 8.5. Myelin volume fraction ratio versus myelin thickness. a) \( f_{M,T2} / f_{M,MT} \) versus \( \bar{\Delta} \), b) \( f_{M,T2} / f_{M,PD} \) versus \( \bar{\Delta} \), from control, Rictor CKO, Tsc2 CKO, and Pten CKO mice

Beyond myelin thickness, to assess possible differences in myelin ultrastructure (i.e., changes in myelin wrapping), we acquired TEM images at 350,000X from 3 axons in the splenium region from a control animal and one animal from each CKO model. Figures 8.6a,b display an example image with a profile line (blue) drawn across the myelin. Fig 8c displays the line profile
intensities (blue) and after smoothing with a 12-pt moving average (red line). The peaks (red circles) correspond to the middle of each myelin water layer. The width of each layer is calculated by finding the peaks (green circles) and troughs (black circles) of the derivative of the smooth line profile to determine where the layer of myelin water begins and ends, respectively (Fig 8c). We applied the same process to the inverted profile line to determine the size of myelin lipid layers.

Figure 8d displays the mean +/- SEM thickness of myelin water and lipid layers. From the image in Fig 8b, we cannot distinguish between major dense lines and intraperiod lines, but this is consistent with data from similar aged (P60) rats with similar periodicity measures (~10nm) (37). Tsc2 CKO and Pten CKO mice appear to have thinner water and lipid layers, but as seen before (Fig 5.4) thicker overall myelin thickness (Δ) compared to control and Rictor CKO mice. From simulations, we know that MWF will be less affected by intercompartmental water exchange as myelin layer packing density and/or overall myelin thickness increases. These changes in micro- and ultra-structure in Tsc2 CKO and Pten CKO mice could explain the lack of underestimation of f_{M,T2} in Fig 8.5 as compared to control and Rictor CKO data points. While our MRI measures show there might be a minor exchange effect, in this ex vivo application, water diffusion is much slower and exchange effects are not as drastic as previously exhibited in spinal cord or peripheral nerve (33,34,38).
Figure 8.6. Myelin ultrastructure analysis a) 67,000X TEM image of a control axon. b) 350,000X TEM image of myelin layers and line profile. c) (blue) myelin intesity along line profile, (red) smoothed line profile with (red, circle) myelin water peaks and (black and green circles) peaks and troughs of the derivative of smooth line profile, respectively. Myelin water and lipid layers are denoted. d,) myelin (black, dahed) water and (blue) lipid thicknesses, across mouse models with (*, +) denoting significant differences to control and Rictor CKO.

Beyond the sensitivites of MVF and AVF shown here, it is also important to remember that $g_{\text{MRI}}$ is inherently an area-weighted measure. Therefore, during diseases targeting either large (amyotrophic lateral sclerosis (37)) or small axons (cuprizone model of demyelination (30,38,39) or changes during development where large axons are typically myelinated first (40,41), the observed $g_{\text{MRI}}$ will more heavily reflect changes in the larger axons. In addition, the g-ratio is not constant across axon size, with larger axons generally having higher g-ratios (42); hence, if the distribution of axons in tissue changes, a different $g_{\text{MRI}}$ may be detected.
8.5. CONCLUSIONS

Imaging g-ratio with MRI, necessitates accurate measures of both myelin volume and axon volume fraction. Using recent work to obtain MRI myelin volume fractions, we display good correlations between histologic and MRI g-ratios across multiple mouse models. However, we also highlight some sensitivities and possible limitations to the method—a) $g_{MRI}$ provides an axon-area-weighted g-ratio, b) axon water fraction from models of diffusion are sensitive to all axons, c) myelin volume fractions from MET$_2$ may be affected by intercompartmental water exchange and those from qMT require calibration. As shown here, while these can be limitations, they may also be opportunities to gain more information about the tissue such as myelin thickness. This study shows the potential and promise for imaging myelin microstructure and the g-ratio non-invasively using MRI.

8.6 REFERENCES


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CHAPTER 9

CONCLUSIONS AND FUTURE DIRECTIONS

9.1 CONCLUSIONS

Overall, the work in this dissertation aimed to advance the ability to quantitatively assess myelin and white matter microstructure in rodent brains. Before this work, myelin imaging in rodent brain was limited to magnetization transfer techniques and there were only a few studies using quantitative histology for validation. I furthered the field of white matter imaging by filling these holes.

Applying MET$_2$ to obtain MWF maps in rodent brains had never been shown due to SNR and resolution requirements. Therefore, I assessed optimal parameters to acquire and fit MET$_2$ data. We saw that use of optimal parameters was not critical at high field strengths with and without gadolinium. Additionally, we displayed the ability to obtain MWF maps in rodent brains for the first time. Next, in order to quantitatively evaluate our MRI techniques, we needed methods to accurately analyze electron microscopy images. Therefore, I developed analysis code to reproducibly calculate myelin volume fraction, axon volume fraction, axon diameter, myelin thickness, and g-ratio from electron microscopy images to compare to MRI.

Then, I displayed similar results as previous work using current myelin imaging parameters (MWF and BPF) compared to histology in adult mice with normal myelination and multiple models of white matter disease. I used a volumetric model of white matter to extend our
capabilities to myelin volume imaging and validated it against quantitative histology. This provides the ability to obtain histologic-like measures of myelin which can be utilized in models like g-ratio imaging. I also applied myelin volume imaging to normally developing mouse brains and displayed the ability to specifically investigate myelin development.

Finally, I used quantitative histology to evaluate the proposed geometric g-ratio model. I found that it provides an area-weighted g-ratio, which is an important consideration to know when the technique is applied in various disease models. Lastly, I applied g-ratio imaging using our method for myelin volume imaging and axon water fraction from diffusion imaging in adult mouse brains with normal myelination and multiple models of white matter disease. We were able to assess the capability and sensitivity of g-ratio imaging with comparison to quantitative histology. This provides promise for the technique along with an outline of possible limitations.

9.2 FUTURE DIRECTIONS

There are numerous future directions to be taken from the work in this dissertation. First, it is of interest to compare our myelin volume imaging metrics to recently proposed myelin volume from proton density measures (1). While we have attempted to do this using gray matter as a reference, we have started including a water reference to allow comparisons. This technique is of great interest because it is a straightforward measure and more easily translatable to clinical use. However, similar to BPF, this technique will still be affected by non-myelin bound protons.

A second direction of interest is extending g-ratio imaging to compare male and female brains during adolescence and fully matured adults. Previous work has displayed sexual dimorphism of myelin density during adolescence. While white matter area increases in males,
myelin content decreases leading to a higher g-ratio. It is predicted that these changes are due to larger increases in axon size in males, which may be due to testosterone levels (2–4). Using all of the techniques validated in this dissertation, we have the tools necessary to investigate this hypothesis and have begun a study with adolescent and adult male and female rats.

Finally, in addition to assessing disease, it would be useful to also evaluate possible disease treatments. This work provides validation to use MRI to obtain histologic information of myelin volume and g-ratio across whole rodent brains. Using MRI to understand what treatments are doing can aid in and speed up the drug development pipeline in preclinical studies. Additionally, MRI techniques can be translated to patients to assess the disease and treatment in the clinic. Overall, the work in this dissertation furthers the ability of white matter imaging to specifically understand myelin development, disease, and recovery.

9.3 REFERENCES


