Target-Specific Contrast Agents for Magnetic Resonance Microscopy

by

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S.B., Physics and S.B., Brain and Cognitive Sciences, MIT, 1999

Submitted to the Harvard-MIT Division of Health Sciences and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Health Sciences and Technology at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Abstract

High-resolution ex vivo magnetic resonance microscopy (MRM) can be used to delineate prominent architectonic features in the human brain, but increased contrast is required to visualize more subtle distinctions. The goal of this thesis is to employ target-specific MR contrast agents to regionally alter relaxation rates, resulting in increased contrast in ex vivo MRM of the human brain, with the aim of providing richer information about cyto- and/or myelo-architectonics than is currently achievable.

To accomplish this goal, a traditional optical myelin stain, luxol fast blue (LFB) MBSN with a paramagnetic copper core, has been introduced as a white-matter-selective MR contrast agent in ex vivo brain tissue. The solution relaxivity of LFB was measured at high (4.7 Tesla) and ultra-high (14 Tesla) field strengths. A methodology was developed for staining large tissue samples, enabling MR imaging. Longitudinal (R1) and transverse (R2) relaxation rates in LFB-stained tissue increased proportionally with myelination at both field strengths. R1 changes produced larger contrast-to-noise ratios (CNR), per unit time, on T1-weighted images between the deeper, more myelinated cortical layers (IV-VI) and adjacent, superficial layers (I-III) at both field strengths. Specifically, CNR for LFB-treated samples increased by 229±13 per cent at 4.7T and 269±25 per cent at 14T when compared to controls. Also, additional cortical layers (IVca, IVd, and Va) became resolvable in 14T-MR images after en bloc staining with LFB. After imaging was completed, the LFB-stained sample was prepared for light microscopy. Both macroscopic and microscopic distributions of LFB were found to mimic those of traditional histological preparations.

Next, the LFB-MR method was employed to investigate microstructure in X-linked adrenoleukodystrophy (ALD), a confluent demyelinating disorder characterized by accumulation of abnormal lipids. LFB-MR revealed an additional zone, unseen in formalin preparations and best visualized in T2*-weighted images, which produced four-fold increases in contrast-to-noise ratio. Immunohistological analysis identified a corresponding area of perivascular macrophages, and ultrastructural examination suggested LFB particulates bound to lipids within these macrophages. We thus conclude that LFB-MR is able to detect the actively demyelinating edge in cerebral ALD.

The results presented in this thesis suggest target-specific contrast agents will 1) enable more detailed MR images, permitting the construction of better MR atlases and advancing the field of MR histopathology, and 2) guide the design of future in vivo contrast agents.
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I shall end with the appropriate words of Dr. Martin Luther King:

“Free at last, free at last,
    Thank God Almighty, I’m free at last.”
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Chapter 1

Introduction

“To achieve understanding it is necessary not to see many things, but to look hard at what you do see.” - Giorgio Morandi

Since the time of Alkmaion, c. 500 BC, the brain has been valued as more than mere “bone marrow” and esteemed as the “organ of the mind.” [35]. Exploring the substructure of this organ has brought neuroscientists closer to understanding its function, as the two are inextricably linked. For example, the six layers of the cerebral cortex differ in cellular density and composition as well as in function: layers 2 and 3 are reserved for corticocortical connections, layers 4 and 5 for thalamic input, and layer 6 for output to the thalamus [104].

The thickness, prominence, and composition of the six layers of neocortex serve as one metric for dividing the brain into cytoarchitecturally uniform areas [24]. The epitome of this categorization involves the transition between primary and secondary visual cortex, marked by the abrupt ending of prominent layer IVb [104]. The average cortical thickness is 2.5 millimeters, varying between the very thin (< 2 mm) postcentral gyrus (somatosensory cortex) to the large (> 4 mm) precentral gyrus (motor cortex), home of the gargantuan Betz cells [36]. Brodmann defined 46 areas that are the most widely-used parcellation of neocortex, but not without criticism. Other maps of the brain have been laboriously constructed from two-dimensional histological slices, stained with dyes to reveal regional
distributions of neuronal density [149], myelination, [36] or lipofuscin density [22].

Presently, the best method to visualize structure in living brains is with the techniques of in vivo MR imaging. High-field (3 Tesla) and ultra-high field (7 Tesla) in vivo MR are beginning to obtain routinely image resolutions $\sim 1 \text{ mm}^3$ by employing multi-channel array coils and parallel-imaging acquisition strategies [71]. While promising, these results are in slice acquisitions, not volumes, and sub-millimeter sensitivity is necessary to distinguish cortical sublayers. Thus, the gold standard to resolve fine structure of the human brain remains in the field of histology.

Histological methods have many drawbacks when compared with MR imaging. As mentioned, they are inherently two-dimensional and require much labor to construct three-dimensional data sets. Sectioning and mounting of tissue is irreversible and is often fraught with distortion, be it tissue shrinkage or tearing. In most histological preparations, a sample may be stained for one feature and counterstained for one, at most, two, additional features. Subsequent histological investigations cannot be performed on the same sample, as the chemical treatments are mostly irreversible and two stains can be mutually-exclusive, as acidic and basic dyes would be.

Furthermore, it would be most informative to map brain structure or activity within the “organ of the mind” with in vivo MR imaging, but these efforts are hampered by the difficulty in correlating existing brain atlases with in vivo MR imaging data. This lack of resolution and cytoarchitectural detail in current MR imaging is one reason many disorders cannot be diagnosed by MR images alone. A diagnosis of Alzheimer’s Disease, the most common form of dementia, is only confirmed post mortem, after quantitation of amyloid plaques and neurofibrillary tangles [63]. MRI cannot diagnose inborn errors of metabolism [105, 124], such as adrenoleukodystrophy, although the lesion pattern is often suggestive of diagnosis. On the other hand, lesion progression in MR often correlates with clinical disease progression and extent of pathology. Here more refined and detailed MR imaging may help unravel disease mechanisms and help monitor disease progression.

High-resolution ex vivo MR imaging is juxtaposed between the length scales of histology and in vivo imaging and well-positioned to ascertain structural details applicable to in vivo properties. Ex vivo imaging of the human brain holds great potential for measuring
histological properties of any tissue and does not suffer from the setbacks that burden histology. In many ways, MR is a superior alternative to histology. It is non-destructive, and acquisition of three-dimensional, digital data sets permit “reslicing” the sample in any desired orientation. Different image contrasts are available by exploiting spin dynamics to produce image weightings dependent on proton density and/or relaxation times. Changing acquisition sequences and scanning parameters does not alter the tissue sample, and multiple measurements with numerous forms of intrinsic and potential extrinsic contrasts are quite feasible.

Augustinack et al. has successfully visualized the islands of the entorhinal cortex, among the first structures to degenerate in Alzheimer's Disease, using ex vivo imaging at 7 Tesla [1]. However, other studies were unable to differentiate primary brain tumors, metastatic deposits, and non-neoplastic space-occupying lesions based on signal intensity alone [148]. In time, perhaps, radiology will emerge as the new pathology. Post mortem imaging is becoming more wide-spread in clinical settings to supplement and to guide traditional pathological analysis [8, 141, 122, 53, 125]. In Japan, “autopsy imaging” is widely used when permission has not been granted for a regular autopsy, and a post mortem image center is on the horizon [40, 64]. However, more detailed, higher-resolution MR images are required [121] before “necroradiologists” [124] will be in high-demand.

1.1 Magnetic resonance microscopy and the human brain

The field of MR microscopy has existed for more than 15 years [27] and has had a recent resurgence of activity. A number of laboratories are investigating the promise of ex vivo imaging of the brain [69, 10, 41, 112, 134], and each laboratory is using a variety of sequences and imaging parameters. There are no standard imaging protocols, partly because of the large changes in relaxation times caused by formaldehyde fixation.

As protein fixation occurs, relaxation times and proton densities in gray and white matter change concomitantly [145, 21], resulting in image contrasts altered from “normal” in vivo imaging. As such, the sequence parameters used for in vivo imaging must be changed to maximize the contrast-to-noise ratio in ex vivo imaging.
CHAPTER 1. INTRODUCTION

There is no consensus in the field regarding the type of image contrast that should be used to discern fine anatomical details. Benveniste claims diffusion-weighted imaging (DWI) is superior to T2*-weighted imaging for rodent hippocampus [10], but Fatterpekar produces remarkable images of fixed human hippocampus using “intermediate” weighted sequences [41], whose parameters combine proton density and T2* weightings. Pfefferbaum and Schumann claim proton density weightings are superior for gray/white contrast [112, 134], but our own results [1] favor T2*-weighting. Development in this area of research through the optimization of data acquisition to highlight structures of interest will be essential for progress in this field.

While in vivo T1-weighted images are sensitive to laminar differences in archicortex [119, 34] and in neocortex [151, 3, 31], present T1-weighted images of formalin-fixed brains display very low contrast, even between gray and white matter [107]. Although many groups have employed ex vivo imaging to glean neuropathologic information, none have published “traditional” T1-weighted images, in which white matter has a larger signal intensity than gray matter [23, 101, 103, 145, 9, 41, 3, 151, 112, 39].

As imaging technology improves and permits smaller voxel sizes, a potential limitation of this ultra-high resolution imaging is poor signal-to-noise ratios (SNR). Recall that SNR is defined as the ratio of the mean signal in a given region of interest to the standard deviation of the background signal. SNR is proportional to voxel volume, and as the standard in vivo voxel size of $1 \times 1 \times 5 \text{ mm}^3$, a volume equivalent of $5 \text{ mL}$, is diminished to an isotropic voxel size of $100 \text{ mm}$ (a volume equivalent of $10^{-3} \text{ mL}$), SNR is decreased by a factor of $10^3$. This loss can be recovered in several ways, including using ultra-high field strengths, averaging many scans, placing small receive coils directly atop the sample, imaging smaller samples of ex vivo brains, or by using contrast agents.

MR microscopy is also restricted by the relatively small inherent differences in relaxation rates, and hence image contrast, of different tissues. The contrast-to-noise ratio (CNR) is defined as the ratio of the difference of mean signals in two regions of interest to the standard deviation of the background signal. As depicted in Figure 1-1, the parameter choice that maximizes SNR for one tissue class, such as white matter, does not necessarily also accomplish maximal CNR between two tissue classes, such as gray and white matter.
1.2. CONTRAST AGENTS IN EX VIVO MR IMAGING

Figure 1-1: Parameter choice that maximizes SNR of white matter may not also maximize CNR between gray and white matter.

1.2 Contrast agents in ex vivo MR imaging

A viable and under-explored solution to improve diminished SNR in submillimeter-resolution ex vivo MR imaging is extrinsic contrast agents. Over 30% of in vivo MR imaging investigations uses a contrast medium [95], and development of new agents is an active area of research. A number of groups have employed various gadolinium-based agents for ex vivo imaging with success [70, 94, 137, 67]. Johnson et al. [70] have achieved six-fold improvement in SNR by injecting a formalin/gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) (trade name Magnevist, Berlex Laboratories, NJ) mixture into rodents, but comment that the observed contrast enhancement is empirical, with the biological specificity of the agents unknown.

In fact, these gadolinium agents are non-specific in their distribution and enhancement [26, 126]. Gd-DTPA goes into the extracellular compartment, but does not cross intact cell membranes due to its size, charge, and hydrophilicity [153, 81]. As shown in Figure 1.1, our studies of Gd-DTPA suggest a similar relative change in the relaxation parameters of gray and white matter. An ideal contrast agent would selectively reduce the T1 or T2 of a specific tissue class or have a much greater effect on one tissue class than others.
CHAPTER 1. INTRODUCTION

The goal of this thesis is to develop target-specific MR contrast agents for use in ex vivo tissue and to characterize their relaxation enhancement and biological specificity. Unlike in vivo imaging, neither toxicity nor passage through the blood-brain barrier is of concern, and agents can be designed for a specific target. Many histological chemicals are used to label neuronal cell bodies, whole neurons and dendrites, neuroglia, as well as myelin [74]. A long-term goal is to modify many of these histochemical methods to transform the traditional histological stains into MR stains, similar to what Modo et al. has accomplished with a stem cell tracking, gadolinium-based contrast agent [97] and Meade has achieved with in vivo, biochemically-activated agents to monitor gene expression [6]. Just as histologists rely on an arsenal of stains to identify cells under a microscope, we would like to develop target-specific MR contrast agents that would selectively improve the contrast of regions of interest.

Applications of these novel contrast agents include improved MR identification and segmentation of adjacent Brodmann areas. This will permit the creation of more accurate, probabilistic-based MR atlases to aid localization of activation in functional imaging studies. In addition, target-specific MR contrast agents can enhance MR sensitivity to pathologies, such as neuronal loss or demyelination, and be used to advance knowledge of pathophysiology. Contrast-enhanced ex vivo scans could also be used to investigate what structural changes are visible with MR, to monitor treatment efficacy, and to aid design of future generations of in vivo contrast agents.

Figure 1-2: Changes in T1 and T2* as a function of gadolinium-DTPA concentration at 7T.
1.3 Overview of thesis

1.3.1 Specific aims

The goal of this thesis is to employ target-specific MR contrast agents to regionally alter relaxation rates, resulting in increased contrast in ex vivo MR microscopy of the human brain, with the aim of providing richer information about cyto- and/or myelo-architectonics than is currently achievable.

To accomplish this goal, a traditional optical myelin stain, luxol fast blue (LFB) MBSN with a paramagnetic copper core, has been introduced as a white-matter-selective MR contrast agent in ex vivo brain tissue. Thus, it is likely for LFB to enhance MR images in a manner sensitive to myelin content.

Next, the LFB methodology was applied to investigate the microstructure of brain tissue in adrenoleukodystrophy (ALD), a confluent demyelinating disorder involving the accumulation of very-long chain fatty acids. Characteristics of the lesion architecture remain crucial to identifying determinants of disease progression.

1.3.2 Chapter outlines

Chapter 2 reviews the biophysics of contrast agents, relevant to the design and implementation of MR agents for ex vivo samples. A brief background is provided for the physical interactions of water with the field-perturbing agents. A literature review is provided for the candidate binding substrates for myelin-specific luxol fast blue (LFB), and the dye's magnetic properties in solution are presented. Lastly, the space of potential, target-specific contrast agents for ex vivo MR imaging is explored.

Chapter 3 reviews traditional histological processing: fixation, dehydration, sectioning, thin-slice staining, dehydration, and mounting. Next, a novel protocol for en bloc staining is presented, detailing a methodology for staining, differentiating, and preparing blocks of formalin-fixed tissues for subsequent MR imaging. This method enables MR microscopy of contrast-agent-stained blocks of brain tissue.

Chapter 4 describes the first application of LFB as a white-matter selective MR contrast agent. Blocks of formalin-fixed, human visual cortex, free of any documented neurological
disorders, were imaged at 4.7 and 14 Tesla, before and after LFB staining, using the newly-developed en bloc protocol described in Chapter 3.

Longitudinal (R1) and transverse (R2) relaxation rates were determined and images acquired using parameters calculated to yield the largest contrast-to-noise ratio (CNR) for T1 and T2 image weightings. After imaging was completed, samples were sliced in 40-micron sections, mounted, and photographed. The macroscopic and microscopic distributions of LFB were compared to traditional, thin-slice histological preparations.

In Chapter 5, the LFB MR microscopy methods are applied to samples of X-linked adrenoleukodystrophy (ALD) to investigate microstructure and lipid distribution in this demyelinating disorder. X-linked ALD encompasses many of the pathological hallmarks common to many white matter disorders: confluent demyelination, inflammation, gliosis and necrosis, in addition to normal appearing white matter and intact subcortical arcuate fibers. Thus, imaging ALD specimen offers a unique opportunity to improve detection and localization of substrate accumulation to advance the understanding of the pathophysiology, immune response, and treatment efficacy in leukodystrophies.

Longitudinal (R1) and transverse (R2, R2*) relaxation rates were determined and images acquired using parameters calculated to yield the largest contrast-to-noise ratio (CNR) for T1, T2, and T2* image weightings. Remarkable findings were compared with quantitative immunohistological staining and electron micrographs.

Chapter 6 summarizes the findings of this thesis and describes preliminary work on a neuron-specific MR contrast agent, gadolinium-thionine (GdT). This agent combines the large magnetic moment of gadolinium, which possesses 7 unpaired electrons, with the neuron specificity of thionine, a traditional optical Nissl stain.

This thesis provides a foundation for future work in dual histological/MR stains. The results presented in this thesis suggest target-specific contrast agents will enable more detailed MR images, which will permit 1) the construction of better MR atlases to advance understanding of the normal brain, 2) increased specificity to allow MR histopathologic investigations and advance knowledge of pathophysiology, and 3) the design of future target-specific in vivo contrast agents.
Chapter 2

Biophysics of target-specific magnetic resonance contrast agents

"All science is either physics or stamp collecting."

- Ernest Rutherford

The purpose of this chapter is to provide an overview the biophysics of contrast agents, relevant to the design and implementation of MR agents for ex vivo samples. A brief background is provided for the physical interactions of water with the field-perturbing agents. Next, a literature review is provided for the candidate binding substrates for myelin-specific luxol fast blue (LFB), and the dye’s magnetic properties in solution are presented. Lastly, the space of potential, target-specific contrast agents for ex vivo MR imaging is explored.

2.1 Physics of MR contrast agents

Both destructive and constructive effects of magnetic field inhomogeneities are well-documented and are frequently encountered by neuroimagers. Macroscopic variations are present themselves as unwelcomed artifacts, caused by poor shimming, air-tissue interfaces, such as the sinuses or ear canal, or metallic implants. Microscopic variations are more of
a boon for enhancing MR image contrast and are responsible for the effects of exogenous agents, such as gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) or iron oxide, as well as endogenous agents, such as deoxyhemoglobin, which produces the famous blood oxygen level dependent (BOLD) contrast in functional MRI [106, 86].

As water protons diffuse through the sample environment, they interact with fields produced by the orbital and spin angular momentum of the contrast agent. As these momentum quantities are proportional to the magnetic moment of the contrast material, and thus inversely proportional to mass, electrons produce fields 10^3 larger than those produced by protons. Materials, such as transition metals (manganese, iron, and copper), have unpaired electrons and will produce a net magnetization in the presence of an external field.

The magnetization magnitude of the material is measured by a quantity called magnetic susceptibility, denoted by $\chi$, and is defined by the following equation:

$$M = \chi \times B_o,$$  \hspace{2cm} (2.1)

where $B_o$ is the external magnetic field and $M$ is the magnetization produced by the material [55]. This susceptibility will determine whether a compound is diamagnetic ($\chi < 0$), paramagnetic ($\chi > 0$), ferromagnetic ($\chi >> 0$ and diameter>10 nm), or superparamagnetic ($\chi >> 0$, diameter<10 nm) and thus its net effect on the effective field:

$$B_{eff} = B_o + \Delta B = \gamma(\omega_o + \Delta\omega),$$ \hspace{2cm} (2.2)

where $\gamma$ is the gyromagnetic ratio for the imaged proton (the $^1H$ signal from water in most brain imaging studies), $\omega_o$ equals the Larmor frequency (the frequency at which the protons precess around the main field), and $\Delta\omega$ corresponds to the contrast agent-induced frequency shift.

Diamagnetic materials, which include most small, organic compounds, consist of paired electrons only and cannot contribute to the induced magnetic field via spin angular momentum, as electrons (fermions) are required by the Pauli Exclusion Principle to have anti-
2.1. PHYSICS OF MR CONTRAST AGENTS

symmetric wavefunctions [46]. Thus, MR contrast agents are commonly paramagnetic, including all lanthanide-based agents, such as Gd-DTPA, or superparamagnetic, such as monocrystalline iron oxide nanoparticles (MION).

Contrast agents are not imaged directly; rather, their presence is inferred via increases in longitudinal (R1) and transverse (R2, R2*) relaxation rates and the resultant changes in signal intensity. Quantifying these relaxation changes helps estimate agent concentration and distribution. The amount of contrast enhancement is influenced by many factors: size and geometry of environment surrounding agent, agent concentration and relaxivity, field strength, and choice of pulse sequence and parameters. A contrast agent alters the longitudinal and transverse relaxation rates of the solvent nuclei. The observed longitudinal or transverse relaxation rate will be a sum of the diamagnetic and paramagnetic contributions, given by

\[ R_{\text{obs}} = R_d + R_p. \]  

(2.3)

The diamagnetic term is given by the relaxation rate of the solvent in the absence of the contrast agent, 1/T\(_i\), where i=1 for longitudinal and i=2 for transverse relaxation rate. The paramagnetic term represents the effect of the dipole-dipole interactions between the solvent protons and the fluctuating field generated by the agent’s unpaired electron spins. The strength of the interaction is proportional to the \(n^{th}\) power of the agent concentration [95]. The constant of proportionality measures the efficacy of the agent and is called the relaxivity and denoted by \(r_i\). Thus, the equation for the observed relaxation rate becomes

\[ 1/T_{i,\text{obs}} = 1/T_{i,d} + r_i[\text{agent}]^n, \]  

(2.4)

[17]. The proximity of the solvent protons to the contrast agent influences the strength of the dipolar interaction. The mechanism of interaction and contribution to the relaxivity can be classified according to the location of the solvent protons as inner-sphere, second-sphere, or outer-sphere effects. Inner-sphere mechanisms occur when solvent protons bind in the first coordination sphere of the agent and spread its paramagnetic influence to the bulk solvent by exchange. If the solvent molecules are not as strongly bound to the agent, but
are instead weakly attached by hydrogen bonds to the agent or to other solvent molecules within the first coordination sphere, the effects are classified as second-sphere. Lastly, the bulk unbound solvent can also undergo paramagnetic interactions when diffusing past the contrast agent. These are outer-sphere effects \[18, 95\].

The so-called “T1 agents” predominantly increase R1 via dipole-dipole interactions between the electron and proton magnetic dipoles. These interactions were first observed by Bloch \[16\] and are well-described by Solomon, Bloembergen \[18\], and more recently by Caravan \[28\]. Short-range interactions are necessary to affect R1; thus, water protons must sample the environment within the coordination sphere of the paramagnetic agent. When diffusion is minimal or agents are compartmentalized, this access to coordination site is restricted, and R1 effects are reduced. In addition, compartmentalized agents produce long-range field inhomogeneities that accelerate the loss of phase coherence and increase R2 and R2*.

Small changes in compound concentration can result in large contrast effects, especially from susceptibility-sensitive sequences \[154, 20\]. Pulse sequence choice can enhance paramagnetic effects, especially complex R2 changes, from selected tissue components. Spin echo (SE) and gradient-recalled echo (GRE) pulse sequences, the workhorses of imaging, have very different sensitivities to magnetic susceptibility.

The SE sequence initially excites the sample magnetization with a 90° radiofrequency (RF) pulse and recruits a 180° pulse a time TE/2 later. After an additional time TE/2 has elapsed, this combination of pulses has the desirable effect of completely refocusing all spins that have strayed from the original Larmor frequency due to static field inhomogeneities. This concept is based on the original pulse sequence ingeniously envisioned by Hahn \[56\], who realized that any two RF pulses in succession would refocus a portion of the original transverse magnetization. A cartoon on the cover of Physics Today, volume 6, number 11, November, 1953, entitled “A Race Track Analogy of the ‘Spin Echo’ Effect” remarkably illustrates this effect in a series of images. The first depicts the 90° pulse as the firing of a starting pistol at the beginning of a race. Then, all runners proceed at their own pace, some more quickly, others more slowly. Next, a time TE/2 later, a second pistol is fired, signalling the racers to reverse direction. With the assumption that all runners still proceed
2.2. BINDING AND MAGNETIC PROPERTIES OF LUXOL FAST BLUE

at their own unique pace, they realign in the final image, back at the starting line after an additional time, TE/2.

Thus, SE sequences reduce signal losses caused by inhomogeneities in the main field as well as frequency-dependent inhomogeneities produced by field perturbations. Although these acquisitions are less disturbed by intravoxel linewidth increases, they can still be affected by non-static phase inhomogeneities resulting from diffusion effects that accumulate during longer echo times.

GRE sequences excite spins with an initial RF pulse that is not necessarily 90°. Typically, GRE sequences have shorter mixing times than SE sequences, as they do not use a π-pulse for refocussing phase losses, but instead compensate for read-out phase dispersion with gradient balancing. As such, GRE sequences are highly sensitive to static and dynamic magnetic field inhomogeneities [7, 86]. Advanced shimming can mitigate inhomogeneities in the main field, but increases in intravoxel linewidth have more of an effect on GRE acquisitions.

2.2 Binding and magnetic properties of luxol fast blue

Histology is somewhat of a dark art or a black box. It is an applied field, with many methods encountered accidentally and passed from one histologist to another. The theory of many stains is not well-understood, but this does not preclude techniques from being put into practice as long as they consistently produce similar results.

2.2.1 Myelin affinity of luxol fast blue

Klüver’s early work concerned porphyrins, naturally occurring structures in the white matter of vertebrates that emit a spectral signature at 625 millimicrons [75]. These porphyrins are not distributed uniformly and are thought to be characteristic of sensory areas, as they are present in cranial nerves II, V, VII, and VIII but absent in III, IV, and VI. There is a species-specific intensity variation in the olfactory tract. Furthermore, this spectral signature is absent in the peripheral, non-glial segment of nerves; thus, porphyrin presence could be correlated with neuroglia or oligodendroglia.
Klüver observed free porphyrins to bind free porphyrins, and tried a number of porphyrin compounds as potential myelin dyes [76]. He found luxol fast blue MBSN, a synthetic dye manufactured by duPont to combine with myelin most effectively. Neither a structure for LFB MBSN nor a hypothesis for its binding specificity were given in Klüver and Barrera's seminal paper; thus, it is probable they were in contact with duPont to learn that LFB is a phthalocyanine, with similar structural appearance to porphyrins.

Porphyrrins and phthalocyanines are members of a class of conjugated cyclic systems of methine groups\(^1\) called annulenes [159]. Most of this family of hydrocarbons is aromatic in character; this has implications for certain chemical properties similar to benzene derivatives, the most pertinent for our studies being the magnetic ring-current effects observable by NMR [159]. Methine groups may be replaced by aza groups\(^2\); annulenes having 18 pi electrons and four nitrogen atoms are called porphyrins and those with 18 pi electrons and eight nitrogen atoms are called phthalocyanines [159].

Conn's tome on biological stains notes that myelin staining is reduced by acetylation, amine blockage, and alkaline hydrolysis [60]. Factors affecting staining of the aza[18]annulene class of dyes, to which LFB is a member, include:

1. electric charge
2. acid-base properties
3. lipophilicity
4. extent of conjugation
5. dye size

The precise binding site of copper phthalocyanines to myelin remains uncertain, but many potential substrates have been suggested, including phospholipids [111], neurokeratin and proteolipid proteins [80], and lipoproteins [132]. In these proposed acid-base interactions, the base of the substrate replaces the base of the dye. In addition, non-polar amino acids

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\(^1\)A methine group is a carbon atom bound by two single bonds and one double bond, where one of the single bonds is to a hydrogen atom.
\(^2\)An aza group is a nitrogen atom bound by one single bond and one double bond.
Figure 2-1: Chemical structure of luxol fast blue (LFB), modeled after The precise binding site of copper phthalocyanines to myelin still remains uncertain, but many potential substrates have been suggested, including phospholipids [111], neurokeratin and proteolipid proteins [80], and lipoproteins [132]. In these proposed acid-base interactions, the base of the substrate replaces the base of the dye. [60].

and fatty acid residues have been suggested as substrates [33]. The chemical component within LFB responsible for the specific binding has also been debated, with copper and acidic groups the major candidates [33]. What follows is a chronological overview of work addressing the specific binding of LFB in myelin.

Pearse [111] described LFB MBSN to be an amine salt of a sulphonated copper phthalocyanine and first revealed its structure, shown in Figure 2-1. He compared staining properties of LFB MBSN to another copper phthalocyanine dye, methasol fast blue, and observed similar results, although at a slower rate. He successfully stained myelin sheaths, as well as erythrocyte capsules, elastic fibers, eosinophilic granules, lipofuscin, and heparin. To probe the specificity of these dyes, he added drops of saturated LFB MBSN, methasol fast blue, or one of “three closely related dyes” in absolute ethanol to commercial samples, in a variety of solvents, of the following: As copper phthalocyanine dyes preferentially stain myelin constituents lecithin (phosphatidylcholine) and sphingomyelin, it was conjectured that the dyes preferentially bound phospholipids that contain the strong base choline. Pearse states, “its specificity, among lipids, for those containing choline has not been established with absolute certainty...[but] any lipid compound giving negative results
CHAPTER 2. BIOPHYSICS OF TARGET-SPECIFIC MR CONTRAST AGENTS

Table 2.1: Preferential binding of luxol fast blue and methasol fast blue. From [111]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Solvent</th>
<th>Precipitate</th>
<th>Binding speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>sphingomyelin</td>
<td>CHCl₃/MeOH</td>
<td>dark blue</td>
<td>30 min</td>
</tr>
<tr>
<td>lecithin (phosphatidylcholine)</td>
<td>etOH</td>
<td>dark blue</td>
<td>10 min</td>
</tr>
<tr>
<td>cephalin (phosphatidyl-ethanolamine)</td>
<td>CHCl₃</td>
<td>none*</td>
<td>n.a.</td>
</tr>
<tr>
<td>cerebroside</td>
<td>C₅H₅N</td>
<td>fine, pale blue</td>
<td>24 hrs</td>
</tr>
<tr>
<td>phosphorylcholine</td>
<td>etOH</td>
<td>fine, pale blue</td>
<td>18 hrs</td>
</tr>
</tbody>
</table>

*A cephalin-phthalocyanine product formed if chloroform used as solvent.

may be presumed choline-free." [111] In addition, basic dyes used as counterstains, such as neutral red, enhanced the appearance of myelin and changed lipid-bound dyes into a blue-black or deep purple. A proposed explanation for this attachment is that the base of the lipoprotein—and not the lipid itself—is what binds to the dye and replaces its base. Thus, LFB MBSN acts as both a stain and a mordant. In conclusion, Pearse’s study suggested the base of lipoprotein replaces the base of copper phthalocyanine dyes.

Some had conjectured that the copper core was responsible for LFB’s binding properties; however, Salthouse disproved this when he classified LFB MBSN as a diarylguanidine salt of a sulphonated copper dye and compared its staining properties with diarylguanidine salts of sulphonated azo dyes, LFB ARN and LFB G, neither of which contain copper [128, 129, 130]. LFB ARN was shown to have similar solubility properties to LFB MBSN; they are both:

1. insoluble in water and hydrocarbons
2. soluble in lower alcohols (methyl, ethyl, and propyl)
3. very soluble in glycols and nitrogenous bases ethanolamine and choline
4. extremely soluble in the phospholipids, lecithin (phosphatidylcholine) and cephalin (phosphatidylethanolamine)

It was discovered that LFB ARN and LFB G form complexes stoichiometrically with phosphatidylcholine and phosphatidyl-serine, and LFB ARN, LFB G, and luxol fast black L to combine with phosphatidyl-ethanolamine. LFB G did not reveal specific binding for
phospholipids, but instead a selectivity dependent on the solvent used. Specifically, Salt- 
house measured binding properties of LFB G combined with phosphatidylcholine, phos-
phatidylserine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin in methanol, 
ethanol, isopropanol, and isobutanol.

To summarize, Salthouse’s work suggests that only phospholipid-containing areas are 
stained with the LFB dyes and that it is not the copper in LFB MBSN that elicits binding, 
but rather the structure of the diarylguanidine salt.

In 1970, Lycette measured the binding capacity of various biological lipids and ma-
terials to LFB ARN [91]. Three concentrations of lipids were used, combined with a 
matched solvent, dried on a glass strip, and then sprayed with a 0.04% concentration of 
LFB ARN, instead of the standard 0.1% w/v. Experiments found LFB ARN to bind 
strongly with phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol to 
form isopropanol-insoluble complexes. Neutral fats, methyl and cholesterol esters of fatty 
acids, cholesterol, saturated hydrocarbons, and, surprisingly, sphingomyelin and cerebro-
sides did not bind the dye to any significant extent.

Comparison of chemical structures of dye-binding and nonbinding substances indicate 
the phosphate group of phospholipids is essential for the reaction, possibly because of 
phosphate-containing metabolic precursors of phospholipids [91]. The experimental findings 
are in contrast to findings of Salthouse and Pearse, perhaps due to the lower concentration 
used, or a pH difference. Another odd result is the low bound fraction for phosphatidyl-
choline; Lycette suggests the quaternary amine of phosphatidylcholine may interfere with 
the binding reaction.

An additional mystery was how lipid solvents influence myelin staining with any LFB 
variant and the implications this has for the dye’s substrate. Two experimental results 
produced four different interpretations: When used on fresh tissue, lipid solvents were 
observed to abolish myelin staining [77, 80], but when used on formalin-fixed tissue, lipid 
solvents produced no change in LFB staining [33, 132].

According to Klüver, this confirmed Pearse’s results that LFB stains phospholipids. 
Koenig hypothesized that not only was myelin staining abolished by extraction methods, 
but the neurokeratin network was also removed. He reasoned that as proteolipid is the
major component of neurokeratin, and as fixation denatures the proteolipid protein, its extraction would be prevented; thus, it is reasonable for neurokeratin to be the binding site for myelin stains. However, a more recent study has localized proteolipid protein to the cytoplasmic side of the membrane bilayer [51].

Clasen notes similarities between neurokeratin and the substrate, but is not convinced the two are identical. Myelin contains non-polar amino acids and fatty acid residues that are not extractable by lipid solvents [74]. Clasen proposed an alternative binding method in which the dye gains access to non-polar amino acids and fatty acid residues [33] by virtue of their water insolubility. Once in site, dye could be held in place by Clathrate bonds (cage-like traps formed by water molecules), with R groups of non-polar amino acids preventing hydrogen bonding between the dye and glycine groups of peptide chains. In summary, Clasen proposed the chemical basis of myelin staining results from the acid dye and not the specific chemical structure of LFB. His studies synthesized a series of acid dyes that all stained myelin.

Lastly, Scholtz doubted the primary role of phospholipids in LFB MBSN staining, as “otherwise there would be a decrease in staining [in fixed tissue]. Their presence may contribute an inhibiting factor and removal may expose new binding sites.” However, he did not realize that not all phospholipids are removed from fixed tissue [74].

Thus, the current knowledge of LFB MBSN suggests 1) copper is not important for binding myelin, 2) acid dyes are important for binding myelin, 3) the exact substrate appears to remain an open question, but choline is most probably involved.

2.2.2 Relaxivity of luxol fast blue in solution

Four concentrations of LFB3 were prepared by modifying a standard histological protocol [74]. Solvent blue 38 powder (Acros Organics, Morris Plains, NJ) was added to a 20:1 mixture of 95% ethanol and 10% acetic acid. The standard protocol suggests preparation of a 0.1% w/v LFB solution. We increased the concentration of LFB until saturation was reached at approximately 1% w/v. The LFB solution was heated at 56°C for 24 hours and then filtered with 90 μm filter paper. Three additional concentrations were created by

3Unless otherwise noted, “LFB” will refer to LFB MBSN in this document.
diluting 1% preparation with ethanol-acetic acid to form 2.13, 1.42, and 0.62 mM concentrations.

These values were confirmed with inductively-coupled plasma atomic emission spectrophotometric (ICP-AES) techniques [68] by quantifying the amount of copper in each sample. Prior to ICP measurement, the 1% LFB solution was diluted by 100x, and a reference sample of 20:1 ethanol-acetic acid was prepared to ascertain matrix effects. Results are shown below in Table 2-1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Copper (µg/g)</th>
<th>Copper (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFB in etOH</td>
<td>180.8 ± 1.5</td>
<td>2.84 ± 0.02</td>
</tr>
<tr>
<td>Aqueous LFB</td>
<td>20.78 ± 2.89</td>
<td>0.33 ± 0.05</td>
</tr>
</tbody>
</table>

It was unexpected that LFB would be substantially soluble in water, as literature cites only a slight solubility. We were able to produce a 0.12% w/v solution of aqueous LFB by heating the mixture in a 56°C water bath before filtration. Later measurements after filtering determined a concentration of 0.33 mM. Three aqueous dilutions of 0.25, 0.17, and 0.08 mM were also created for relaxivity measurements.

Four solutions were each placed in a 3 mm NMR tube, positioned within a water-filled holder, and scanned using a 20-mm birdcage coil in a vertical 89-mm bore 14T magnet (Magnex Scientific, Oxford, England) with a 100 G/cm gradient coil. Scans were repeated in a horizontal 33-cm bore 4.7T magnet (Magnex Scientific, Oxford, England) with a 40 G/cm gradient coil using a 72-mm birdcage coil. An inversion-recovery prepared spin echo sequence was used to deduce longitudinal relaxation times (TR/TE/TI = 5000/7.5/6-5000 ms at 14T and 2000/5.2/9-2000 ms at 4.7T). Regions of interest (ROIs) were drawn in the central area in each tube; means and standard deviations were plotted against TI. Data were fit to solutions of the Bloch equation for inversion-recovery or spin echo sequences using the Levenberg-Marquardt method [118], and T1 determined for each sample. Relaxivities were then estimated by performing linear regression using equation 2-3.

Monte Carlo simulations were performed to estimate measurement uncertainty. Zero mean, unit variance pseudorandom numbers were generated, transformed to have the same
statistics as the measured data, and propagated through the fitting routine. This procedure was repeated 1000 times, and the standard deviations of the resulting relaxivities computed. Longitudinal relaxation rates of LFB in solution increased linearly with LFB concentration. Calculated longitudinal relaxivities for LFB in ethanol solution or aqueous solution are displayed in Table 2-3.

Table 2.3: Longitudinal relaxivities of luxol fast blue (LFB) in ethanol-based or aqueous solutions, at 4.7 and 14 Tesla field strengths.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Field strength (T)</th>
<th>R1 (mM⁻¹/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFB in etOH</td>
<td>4.7</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Aqueous LFB</td>
<td>4.7</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>LFB in etOH</td>
<td>14</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Aqueous LFB</td>
<td>14</td>
<td>0.09 ± 0.04</td>
</tr>
</tbody>
</table>

As expected, longitudinal relaxivity was greater at lower field strength and transverse relaxivity increased with field strength. These longitudinal relaxivities are consistent with those reported by Lauffer for chelated copper compounds at 1.5T, specifically, 0.12 s⁻¹/mM for Cu(II)-DTPA and 0.21 s⁻¹/mM for Cu(II)-EDTA \[87\]. The measured longitudinal relaxivity of aqueous LFB was about one-third lower than ethanol-based LFB at 14T, and one-fourth lower at 4.7T.

2.3 Overview of additional ex vivo MR contrast agents

Histological dyes that could potentially be transformed into MR contrast agents must possess target specificity and either a paramagnetic core or a functional group that is available for bioconjugation to a paramagnetic core. As noted in the previous section, LFB is an example of the former class of dyes, as it includes a copper ion at the center of its porphyrin ring. The base structure of LFB can be modified to include other paramagnetic cores as well, such as iron(II), manganese(II), or manganese(III). Others have synthesized MnPcS₄, a tetra sodium salt of manganese tetra sulfo phthalocyanine, following a modified method of Webber and Busch \[127\].

The greater the number of unpaired electrons in a metal, the larger its paramagnetic
2.3. OVERVIEW OF ADDITIONAL EX VIVO MR CONTRAST AGENTS

moment. As such, the most widely-used contrast agents for MR imaging are forms of gadolinium, followed by iron oxide and manganese chloride \[95\]. The majority of rare earth elements, those with an atomic number between 57 and 71, are highly paramagnetic. Furthermore, the lanthanide series, a subset of these elements of which gadolinium is a member, has coordination numbers greater than six and sometimes as high as 12. The coordination number is the number of donor atoms that surround a metal ion. The rare earth elements include Lanthanum (La), Cerium (Ce), Praseodymium (Pr), Neodymium (Nd), Promethium (Pm), Samarium (Sm), Europium (Eu), Gadolinium (Gd), Terbium (Tb), Dysprosium (Dy), Holmium (Ho), Erbium (Er), Thulium (Tm), Ytterbium (Yb), and Lutetium (Lu).

Dyes that do not contain a paramagnetic element but do possess reactive groups include cresyl violet, thionine, toluidine blue, neutral red, and chromoxane cyanine R \[74, 60\]. These dyes may be conjugated to another amino group on a linker molecule, which could then react with a chelating moiety. A linker would be appropriate when a dye’s low nucleophilicity hinders direct conjugation with a chelate, or if the chelate could alter the tissue-specificity of the dye \[95\]. Suitable chelates include linear and macrocyclic chelating moieties, for example 1,4,7,10-tetraazacyclododecane-N,N,N,N-tetraacetic acid (DOTA), ethylenediamine tetraacetic acid (EDTA), and diethylene triamine pentaacetate (DTPA); other suitable macrocyclic chelating moieties are described in \[96, 140\].
Chapter 3

Tissue preparation for visualizing cyto- and myeloarchitecture

"The true mystery of the world is the visible, not the invisible."
- Oscar Wilde

This chapter reviews traditional histological processing: fixation, dehydration, sectioning, thin-slice staining, dehydration, and mounting. Next, a novel protocol for en bloc staining is presented, detailing a methodology for staining, differentiating, and preparing blocks of formalin-fixed tissues for subsequent MR imaging. This method enables MR microscopy of contrast-agent-stained blocks of tissue.

3.1 Fixation

The first step in tissue processing is fixation. Tissue is fixed so that it may be preserved from future deterioration, including shrinkage from dehydration, osmotic swelling, or other damage due to bacteria, mold, or autolysis. Another motivation for fixing tissue is providing resistance to subsequent histological treatments, such as embedding in wax, sectioning, staining, and mounting.

Many methods are used to fix tissue, including heating or microwaving, freezing, or
treated with chemicals. These chemicals are either solids in aqueous solution or else organic liquids. For most anatomical investigations, liquid fixatives are used [74]. The number of suitable chemicals is fewer than ten, and of these, either plain formaldehyde, plain osmium tetroxide, or a mixture of other fixatives are routinely used for fixation of neurological tissues. Each of these substances have advantages and disadvantages that must be balanced for the intended application. These considerations fall into three general categories: 1) rate of fixative penetration, 2) subsequent shrinkage or swelling of tissue, and 3) excessive hardening of tissue [74]. A common compromise is to concoct a cocktail of chemicals.

One essential function of a fixative is to stabilize the proteinaceous part of the cell framework within tissue. Coagulant fixatives, such as ethanol, mercuric chloride, and chromium trioxide, produce a coagulum when mixed with albumin [2]. This coagulation distorts organelles and secretory granules [74] and thus does not yield a true representation of living tissue. Non-coagulant fixatives, such as formaldehyde, osmium tetroxide, potassium dichromate, and acetic acid, preserve organelles within an insoluble gel, formerly cellular cytoplasm [74]. However, tissues fixed in this manner may suffer shrinkage and cracks if embedded in paraffin.

According to Kiernan, the “most useful” fixative for most histological and histochemical is a neutral-buffered, aqueous solution of formalin, a commercial fluid containing formaldehyde at about 40% weight per volume [2], with a small amount of methanol, typically 10% volume per volume [74], as a stabilizer. As such, the specific fixative action of formalin will be reviewed; other fixatives are discussed in detail in [2, 74]. Note: in the literature and practice, the terms “formaldehyde” and “formalin” are oft used interchangeably. That convention is also followed in this thesis.

3.1.1 Action of formalin

Formaldehyde is a gas with the chemical formula \( \text{HO(H}_2\text{CO)}_n\text{H} \), where \( n \) ranges between 6 and 100. The monomer is most abundant at standard concentrations. When water is added, methylene hydrate \( \text{(HOCH}_2\text{OH)} \) forms [74]; after lengthy storage times, accumulation of methylene hydrate produces paraformaldehyde, which possesses more than 100 chains of \( \text{H}_2\text{CO} \) molecules.
3.1. FIXATION

Formaldehyde has been used to “tan” leather for over 150 years [14, 44], and its reactions with proteins have been well-documented by studying its interactions with single amino acid peptides [2]. Polyglycine, alanine, polyglutamic acid, and tyrosine all bind little formaldehyde [150]. Polyglutamine has been shown to bind more formaldehyde than any other known structure [59]; the suspected binding site is the amino group, as the deaminized version binds little formaldehyde. Lysine is the most available amino acid with amino groups [2] and is thought to facilitate the following reaction:

$$-(CH_2)_4NH_2 + H_2CO = -(CH_2)_4NH \cdot H_2COH,$$

(3.1)

forming hemiacetals and related adducts [150]. Additional reactions can occur by which the free hydroxymethyl groups of the hemiacetals interact with favorable functional groups of proteins [59]. The final COH group in these newly-formed products is reactive and chemically-stable methylene bridges can be formed between two protein chains:

$$-NH - CH_2 - NH,$$

(3.2)

This bridge formation hardens the proteins and is central to the fixative action of formaldehyde and the formalin mixture.

As formaldehyde is not a coagulant, many hydrophilic groups retain their relation to water [150]. Most lipids are preserved. Specific actions of formaldehyde include supplement of the amino groups of phosphatidyl ethanolamines [74], and could also include oxidizing the double bonds of unsaturated lipids on the fatty-acid chains [2]. However, this latter role could be performed instead by atmospheric oxygen [74]. Lipid preservation can be enhanced and phospholipid solubility reduced by adding calcium ions to the formalin solution [111], mechanism unknown. No strong reaction occurs with carbohydrates [74], although proteins associated with glycogen are fixed such that further dissociation by water is prevented [2].

Formaldehyde penetrates quickly, with a stability constant $K = 3.6$ [2]. However, its rate of cross-linking proteins is much slower, taking 1-2 weeks at room temperature [74]. Other unfavorable consequences of formalin fixation include slight swelling of gelatin-
CHAPTER 3. TISSUE PREPARATION FOR MICROSCOPIC ANALYSIS

albumin \[2\] and a strong hardening \[74\].

Cells directly in contact with the fixative can lose blebs of cytoplasm, although deeper layers are well-preserved \[2\]. Many report that the cytoplasm is "rendered finely granular," mitochondria and lipid droplets emerge unscathed, and nuclei remain "lifelike" \[2\]. Formalin's high marks in structural preservation permit morphological analyses of fixed tissue akin to studying living structures, although at higher resolutions and with greater contrasts.

Long storage periods degrade tissues by producing excessive hardening and loss of stainability by acidic dyes, due to a reduction in available binding sites by formaldehyde residency. Also, a "formalin pigment" may arise from the acid degradation of haemoglobin to haematin \[74\], but is removed by treatment with picric acid or alkalis \[89\].

Formaldehyde is compatible with ethanol, mercuric chloride, and acetic acid \[2\]. It quickly reduces chromium tetroxide and slowly reduces potassium dichromate and osmium tetroxide, commonly used as a post-fixative for electron microscopy \[74\]. Tissue will be very transparent to electrons, so an "electron blocker" of uranium acetate or potassium permanganate is needed to provide EM contrast.

3.1.2 Effect of fixation on relaxation times

There is a consensus within the literature that the gray-white matter contrast in MR images of formalin-fixed brains differs from that of in vivo MR images \[145, 15, 41, 107, 39, 1, 157\]. However, the exact mechanisms underlying this contrast change are not well-known. Few studies have examined MR properties, such as longitudinal and transverse relaxation times, spin density, and diffusion coefficient, as a function of fixation time.

Tovi and Ericsson studied short-term (two days) effects on three brains and long-term (11 weeks) effects on two brains at 0.5 Tesla \[145\]. Their experiments showed initial decreases in both gray and white matter T2 of about 25 and 18%, respectively, but only gray matter T1 decreased (12%). Over the eleven-week period, T1 appeared to decrease continuously in gray and white matter, although only four data points were taken. T1 values had decreased by approximately 48% in gray matter and 40% in white matter. T2 measurements were performed more regularly, although starting values were very different in the two samples: gray matter had a T2 of 90 and 120 ms, white matter, 60 and 90 ms.
3.1. FIXATION

The values at the eleventh week converged for the two samples: \( \sim \)72 ms for gray matter and 55 ms for white matter. These experiments show little change in T2 after 5-6 weeks, but are not conclusive for T1 changes.

Blamire et al. measured relaxation times in three brains weekly, over a period of five weeks [15]. This study confirmed the reduction in gray and white matter T2 values, but showed them to converge at the end of the five-week testing period. In addition, spin density was estimated, and the ratio found to increase from 1.19 at week 1 to 1.54 at week 5.

Most recently, Yong-Hing et al. examined three brains at 1.5 Tesla over a three-week period, at non-uniform intervals [157]. The decrease in T1 was very similar for gray (39%) and white (40%) matter, while T2 decreased more in gray (50%) than white (38%) matter. However, the end-point T2 values were not equal. The rate of change in relaxation times was largest during the first 50 hours, slightly greater for T2 values, and plateaued after 300 hours. Furthermore, a decrease in proton density was estimated of 24% for gray and 31% for white matter. Discrepancies between the findings of [15] and [157] could be explained by a number of factors, including differences in acquisition sequences, post mortem intervals, regions of interest, or fixation protocol.

The study by Yong-Hing et al. was the first to investigate the apparent diffusion coefficient (ADC) as a function of formalin fixation. Surprisingly, values did not change with fixation time and remained \( 50 \times 10^{-7} \text{cm}^2/\text{s} \) for gray and \( 30 \times 10^{-7} \text{cm}^2/\text{s} \) for white matter. In addition, a mathematical model of formalin diffusion was applied to calculate a \( D_{\text{formalin}} \) of approximately 52.6 mm²/h. This predicts an upper bound of 2,500 hours, or 14.8 weeks, for formalin to completely diffuse throughout a standard-sized brain.

T1 weighting appears well-positioned to reveal microstructural information, as the processes of formalin-penetration and the beginning stages of fixation are visible as a bright band where formalin has successfully penetrated through tissue [145, 157]. This phenomenon is not clearly distinguishable upon T2 weighting. Furthermore, it is the ex vivo T1 contrast that reverses from in vivo, beginning during the second week of fixation and completing on day 11 [145]. Gray matter remains hypointense to WM, as in vivo, and there is little change in T2 weighting between day 4 and week 11.

Many attribute T1 contrast reversal to the T1 reduction in gray matter [145], as the
gradual signal increase of gray matter after the second week of fixation could not be explained completely by T2 changes, which are relatively larger for gray than white matter.

Tovi's hypothesis is that decreased water content and increased "static surfaces" that add cross-relaxation and/or hydrodynamic mechanisms result in shortened relaxation times. Although dehydration of tissue is known to reduce relaxation rates in liver and spleen [143], significant hydration differences were not found between unfixed and formalin-fixed tissues [144].

3.2 Histological staining

Once fixation is complete, in order to produce standard, dye-stained histological slides, tissues must undergo an elaborate, labor-intensive processing. Traditional histology is performed on thin tissue slices, typically 20 microns or less in thickness, so that sufficient light may pass through the sample from the microscope to illuminate objects of interest.

Water-based samples are not rigid enough to be sliced, even after fixation. Thus, samples must be hardened by freezing or embedding in wax. If they are to be frozen, a cryoprotectant, such as glycerol, is first added to prevent damage from water expansion, and a freezing microtome or cryostat used to slice the tissue in 20-100 μm sections. If samples are to be wax-embedded, the pretreatment is much more complex. Samples must first be dehydrated in a series of increasing alcohol content, then equilibrated with a solvent miscible with paraffin, such as chloroform, and finally infiltrated with paraffin before being blocked out in solidified wax [74]. A rotary, rocking, or sledge microtome is then used to slice the wax block in sections as thin as 4 μm. An advantage of the paraffin technique over the frozen one is thinner slices, but a disadvantage is the higher temperatures that may damage heat-labile substances such as lipids and enzymes [74].

Frozen slices may be dried onto slides stained directly, but paraffin sections again require additional processing. After tissue sections are completely dried onto slides, xylene is often used to remove the wax. Sections are rehydrated through a series of decreasing alcohol content before staining in an aqueous dye, although an ethanol-based dye like LFB would not require this step. Next, differentiation would be performed, if necessary. All slides
3.3 NOVEL EN BLOC TISSUE-STAINING PROTOCOL

that are to be preserved in a resinous medium, including those sliced while frozen, must finally be dehydrated through a series of alcohols and cleared in xylene before mounting and cover-slipping. These multiple steps can alter the tissue by resultant shrinkage or tearing.

3.3 Novel en bloc tissue-staining protocol

In order to perform MR imaging on large tissue samples that have been stained with an MR-visible dye, an alternative staining protocol must be developed. We first began with a standard protocol for LFB staining [74], and heuristically determined the new durations required for successful implementation of each step, as described below. The protocol was developed by staining small tissue blocks, but, with appropriate scaling, the method would be applicable to larger samples, even entire hemispheres. In addition, the process used to assess modification of steps in standard protocols is applicable to any novel dye visible by light microscopy. Immunohistochemical stains may also work, but there are concerns whether these sensitive vectors would penetrate large samples and retain their function [74].

A flow chart in Figure 2 summarizes the novel LFB en bloc staining protocol. The procedure was as follows:

1. Immerse formalin-fixed tissue in 1% luxol fast blue for 24 hours per 1 mm thickness at 56°C.
2. Blot tissue with filter paper.
3. Rinse in 95% ethanol for 5 min and take to doubly-distilled water. Let stand for 5 min.
4. Blot tissue with filter paper.
5. Transfer tissue to 0.05% aqueous lithium carbonate (LiCO). Remove after 400 minutes per 1 cm thickness.
6. Blot tissue with filter paper.
7. Transfer tissue to perfluorocarbon in low-pressure environment and store overnight.
3.3.1 Facilitating passive diffusion

Lateral diffusion is the only available transport mechanism for LFB through fixed tissue. Our initial observations noted the slow process of passive diffusion of LFB at room temperature. In histological protocols using tissue samples 20-50 microns in thickness, the suggested immersion time is 18-24 hours [74]. It was unclear how this time would scale for larger samples, as the permeability of LFB in fixed tissue has not been reported previously.

Cellular physiologists frequently investigate membrane permeability to various solutions. The so-called “tagged water permeability coefficient” can be estimated by measuring the rate a substance, often tritiated water, crosses plasma or phospholipid membranes [108, 102, 30]. The flux of measured substance, in our case LFB, across a membrane is given by Fick’s law:

$$
\Phi_{LFB} = -D_{LFB}A \frac{d[LFB]}{dx},
$$

where \([LFB]\) is the LFB concentration at any point, \(x\), in the membrane, and \(D\) is the apparent diffusion constant of LFB. Integrating the expression yields:

$$
\Phi_{LFB} = \frac{-D_{LFB}A}{\delta x}([LFB]_1 - [LFB]_0) = -P_d A ([LFB]_1 - [LFB]_0),
$$

where \(P_d\) is the diffusion permeability coefficient of LFB for the membrane.

We sought to determine the equivalent permeability coefficient for LFB at room temperature and at a higher temperature, hoping to accelerate LFB penetration. Blocks of human, formalin-fixed, parietal cortex, approximately \(4 \times 4 \times 5\) mm\(^3\) were immersed in a
1% LFB solution, kept at room temperature or placed in a hot water bath maintained at 56°C, following the temperature suggestion of a study optimizing LFB staining [47]. Blocks from each batch were removed, bisected, and photographed at 6-hour intervals.

As these measurements were performed prior to differentiation, LFB concentrations were assumed to be constant throughout the tissue sample, and $[\text{LFB}_1] - [\text{LFB}_0] \approx [\text{LFB}_1]$. Flux through the tissue was estimated, per unit area, by quantifying “blueness” in the tissue, which is an equivalent property to activity for the use of tritiated water. Profiles were drawn manually, perpendicular to the surface of each block, using Matlab (Mathworks, Natick, MA). A uniform spatial penetration from the outer surface was assumed for each block, and verified upon inspection.

Two representative samples, incubated in LFB for 24 hours and then bisected, are displayed in Fig 3-3. The sample kept at 56°C appears on the left, the sample kept at room temperature on the right, and corresponding intensity profiles graphed below. From a series of such profiles, the estimated of LFB through samples was $0.35 \pm 0.02 \mu m/s$ at room temperature and $0.97 \pm 0.02 \mu m/s$ at 56°C.

These values are lower than the results of Cass and Finkelstein [30], who obtained a mean value of 6.2 $\mu m/s$ for the diffusion permeability coefficient of tritiated water through thin lipid membranes at 36°C. They had used magnetic fleas to stir the solution, and found decreasing the mixing to decrease permeability. The LFB solution was not stirred, which could contribute to the difference in permeability. Additional differences that could decrease permeability include formalin fixation, the dye having to pass through gray matter in addition to white matter, non-uniform penetration rate due to surface effects, or temperature differences, although this should increase permeability in our samples.

### 3.3.2 Differentiation

The action of lithium carbonate is to help release unbound dye from tissues [74], namely in the superficial layers of cortex where there is not an abundance of myelinated fibers. Little data has been published on the mechanism behind the differentiation resulting from application of lithium carbonate; one hypothesis, proposed by Clasen, suggests the action may be viewed as a combination of splitting hydrogen bonds and salt bridges. [33] This
Figure 3-2: (A) Two representative samples of human brain tissue, immersed in luxol fast blue (LFB) at either 56°C (left) or 22°C (right) for 24 hours and then bisected. Plotted below for each sample is the reciprocal of the blue intensity along a path perpendicular to the surface. LFB was found to have a penetration rate of 1.4 mm per day at the warmer temperature (B), compared to 0.5 mm per day at the lower temperature (C).

Our goal was to determine a duration that would sufficiently remove unbound LFB from the gray matter without over-differentiating white matter. After sufficient LFB staining, subsequent treatment of samples with 95% ethanol removed additional LFB dye from the tissue surface. A five-minute immersion interval was found to be sufficient, regardless of tissue size, as longer durations did not result in further visually remarkable decreases in dye density from the tissue surface. Rinsing tissues in ddH2O removed slightly more LFB, as

view is corroborated in Conn’s, which states dyes in the same aza[18]annulene class as LFB form pseudobases, which have been described as unstable under alkaline conditions [60].
3.3. NOVEL EN BLOC TISSUE-STAINING PROTOCOL

noted from the resulting blue tint of the liquid. Immersion times exceeding five minutes did not appear to remove any additional dye from the tissue.

Tissues, approximately 8x4x4 mm³ in size, were immersed in an 0.05% w/v aqueous solution of lithium carbonate at room temperature for durations between 4 and 570 minutes. After each interval, samples were removed from solution, blotted, photographed, and returned to solution.

Representative images of samples before and after LICO treatment appear in the top of Figure 3-4. After the initial immersion in LFB, samples appear a uniform blue with white matter having a slightly darker color. Following treatment with LICO, gray matter becomes lighter in appearance as LFB is removed, while white matter retains its darker blue hue. The contrast between white and gray matter is plotted as a function of differentiation time in Figure 3-4. An immersion time of 400 minutes per centimeter thickness of the sample was deemed sufficient for the lithium carbonate solution to fully penetrate the sample without over-differentiating the more superficial lamina. This was assessed by bisecting the tissue and comparing the central contrast to that at the surface.

Although a differentiation window around 400 minutes seemed adequate for the sample size tested, there could exist a maximal sample thickness for which the penetration and differentiation would be uniform. This could be impede extending the LFB-MR method (or any en bloc staining method requiring differentiation) to entire hemispheres, which may be larger than the uniform penetration limit. A potential solution has been suggested [43] of cooling the surface of the sample periodically, such that the diffusion rate would be greater in the warmer center of the sample and cooler at the exterior. This remains to be investigated.

3.3.3 Packaging of samples for imaging

Subsequent to staining and differentiation, samples were immersed in a proton signal-free perfluoropolyether (Fomblin LC-08, Solvay Solexis, Thorofare, NJ) in a custom-built, low-pressure environment to remove artifact-inducing air bubbles within tissue. Fomblin is a commercial, industrial-grade lubricant used for mechanical devices. Fomblin is an inert substance, molecular weight of 800 AMU, that is immiscible in water. The chemical formula
Figure 3-3: Samples of LFB-stained human brain tissue were immersed in a 0.05% aqueous solution of lithium carbonate (LiCO) for intervals ranging between 0 and 570 minutes, blotted, and photographed. Plotted above is the blue intensity difference between white matter (WM) and gray matter (GM) as a function of immersion time. Representative images appear for four time points.
3.3. NOVEL EN BLOC TISSUE-STAINING PROTOCOL

for perfluoropolyether oils, obtained from [147], is as follows:

\[
\text{CF}_3\text{O} - \text{[CF(CF}_3\text{)CF}_2\text{O]}_m - (\text{CF}_2\text{O})_n - \text{CF}_3, \tag{3.5}
\]

where \(m\) and \(n\) are integers such that the ratio \(m/n\) is between 20 and 1,000 [92].

As Fomblin does not contain hydrogen atoms, it does not contribute to the acquired MR signal and provides an excellent embedding material. The first documented use of Fomblin in MR microscopy is by Hurlston et al. [62]. Furthermore, Fomblin reduces surface susceptibility artifacts, matches the quality factor ("Q") of the imaging coil better than isotonic saline solutions, and prevents tissue samples from dehydrating. Tissue blocks were wedged between two polyethylene caps (Wilmad Lab-Glass, Buena, NJ) to prevent motion, immersed in Fomblin, and given a minimum of 24 hours at room temperature to equilibrate.
Chapter 4

Luxol Fast Blue: A white-matter specific MR contrast agent

“The first principle is that you must not fool yourself, and you are the easiest person to fool.”

Richard Feynman

This work details the first application of luxol fast blue (LFB), an optical stain for myelin, as a white matter-selective MR contrast agent for human ex vivo brain tissue. Blocks of formalin-fixed human visual cortex were stained using a newly-developed en bloc protocol, described in Chapter 3, and imaged with an isotropic resolution between 80 and 150 microns at 4.7 and 14 Tesla. Longitudinal (R1) and transverse (R2) relaxation rates in LFB-stained tissue increased proportionally with myelination at both field strengths. The changes in R1 provided larger contrast-to-noise ratios (CNR), per unit time, on T1-weighted images between the deeper, more myelinated cortical layers (IV-VI) and adjacent, superficial layers (I-III) at both field strengths. Specifically, CNR for LFB-treated samples increased by 229 ± 13% at 4.7T and 269 ± 25% at 14T when compared to controls. Also, additional cortical layers (IVca, IVd, and Va) became resolvable in 14T-MR images after LFB staining. Once imaging was completed, samples were sliced in 40-micron sections, mounted, and photographed. Both the macroscopic and microscopic distributions of LFB were found to mimic those of traditional histological preparations. Our results suggest cell-
specific contrast agents will enable more detailed MR images with applications in imaging pathological ex vivo samples and constructing better MR atlases from ex vivo brains.

4.1 Introduction

Here we report work on luxol fast blue MBS (LFB), a traditional histological stain for myelin first used more than 50 years ago [76]. The known chemical specificity of LFB was discussed in detail in Chapter 2. We have introduced a novel role for LFB as a contrast agent for MR imaging. The molecular structure of LFB contains a paramagnetic copper core whose unpaired electrons contribute to local magnetic fields, increasing proton relaxation rates. LFB binds to myelinated axons throughout the central nervous system our hypothesis is that in regions where LFB binds due to chemical affinity, the proton relaxation enhancement will be especially pronounced.

Selectively staining myelin in the human brain enhances the appearance of what neuroanatomist W. J. H. Nauta called the “neocortical warp and woof,” or the superposition of radial and tangential organizations of neocortical axons [104]. The patterns of these fiber connections, as well as the thickness, prominence, and composition of the six layers of neocortex serve as a metric for dividing the brain into cytoarchitecturally uniform areas. The most widely-used parcellations of neocortex are maps delineating regions based on differences in neuronal density, [24, 149] myelination, [36] or lipofuscin density [22].

All maps illustrate that function follows form in the neocortex, and regions of the brain involved in processing primary sensory information possess a more distinct pattern of myelination and cellular packing (specifically of granule cells) than dysgranular cortex or agranular cortex, where layer IV is poorly developed. Layer IV, or the internal granular lamina, is comprised completely of granular neurons and lacks pyramidal neurons. Granular neurons, also called “stellate” neurons for their star-shaped appearance, receive specific thalamic afferents on dendrites whose ramifications are localized near the somata. Layer IV is most distinctive in the primary visual area (Brodmann area 17), where it contains a prominent group of tangential fibers called the line of Gennari, after Francesco Gennari, the Italian medical student who discovered this signature mark of primary visual cortex in 1776 [104].
This axon plexus is a cell-poor band that divides two sublayers rich in granule cells. In the neighboring regions of primary visual area, these striations merge and the line of Gennari vanishes.

The appearance of layer IV, which varies widely throughout the neocortex, is thus a hallmark of the cortical region in which it resides. As such, we have chosen the visual cortex, and specifically the primary visual area, as a testing ground for our myelin-specific contrast agent. LFB holds great potential as an MR contrast agent for use in imaging with a dual role in correlating MR images with histology.

4.2 Materials and Methods

4.2.1 High-resolution MR imaging

Samples of visual cortex were obtained from four human brains in which the following criteria were met: 1) unrestricted permission for autopsy had been given; 2) no clinical suspicion of neurologic disease; 3) no clinical evidence of blood borne pathogens, including HIV, hepatitis, or prion disease; and 4) gross examination of the brain at the time of removal did not indicate the presence of previously unsuspected pathology. Mean age of samples was 55 years (range: 42-65); two males and two females were used. The brain tissue was fixed in formalin for more than eleven weeks, which is the minimum time required for transverse and longitudinal relaxation rates to reach equilibrium [145, 15].

Blocks of tissue, approximately $3 \times 1 \times 1 \text{ cm}^3$, were removed from samples along the calcarine sulcus. Tissue samples were processed in the following three subsequent manners and imaged after each preparation: 1) formalin treatment only, 2) en bloc staining protocol, using an ethanol-acetic acid solvent containing no LFB, and 3) en bloc staining protocol using 1% LFB in ethanol. The steps of the en bloc protocol, introduced in Chapter 3, are as follows:

Procedure:

1. Immerse formalin-fixed tissue in 1% luxol fast blue for 24 hours per 1 mm thickness at 56°C.

2. Blot tissue with filter paper.
3. Rinse in 95% ethanol for 5 min and take to doubly-distilled water. Let stand for 5 min.

4. Blot tissue with filter paper.

5. Transfer tissue to 0.05% aqueous lithium carbonate (LiCO).
   Remove after 400 minutes per 1 cm thickness.

6. Blot tissue with filter paper.

7. Transfer tissue to perfluorocarbon in low-pressure environment and store overnight.

Prior to imaging, ex vivo tissue samples were wedged between two polyethylene caps
(Wilmad Lab-Glass, Buena, NJ) to prevent motion. A proton-signal-free perfluoropolyether
(Fomblin, Solvay Solexis, Thorofare, NJ) was added in a low-pressure environment to remove
artifact-inducing air bubbles within the tissue. Tissue was given a minimum of 24 hours at
room temperature to equilibrate within the perfluoropolyether.

Magnetic resonance imaging was performed on a vertical bore 14T Bruker/Magnex
system, using a 10-mm diameter birdcage coil and on a horizontal bore 4.7T Bruker/Magnex
system with a 72-mm diameter birdcage coil. Inversion-recovery prepared spin echo (IR-SE)
sequences were used for T1 mapping (for both 4.7 and 14T: TR=5000 ms and TI=9, 50, 100, 200, 300, 400, 500, 700, 900, 1200, 1500, 1800, 2500, 3000, 5000 ms) and multi-echo
spin echo (MSME) sequences for T2 mapping (for both 4.7 and 14T: TR=2000 ms and
TE=10-80 ms, in steps of 10 ms). These acquisitions were three-dimensional with an
isotropic resolution of 150 μm.

Regions of interest were drawn manually, using Matlab (Mathworks, Natick, MA) to
encompass each visible layer. Care was taken to avoid edges or artifacts, and ROIs included
outer gray matter (layers I-IVa), inner gray matter (layers IVc-VI), white matter, and
background regions. Classification of ROIs was later validated by aligning histological
sections to MR images. Means and standard deviations of ROIs were plotted against TI or
TE. Data were fit to solutions of the Bloch equation for inversion-recovery or spin echo
sequences using the Levenberg-Marquardt method [118], and T1 and T2 were determined,
respectively, for each ROI in each sample preparation. A paired, two-tailed Student’s t-test
4.2. MATERIALS AND METHODS

assessed the significance of numerical differences in relaxation rates arising from staining tissues with LFB. Differences were considered significant if $P < 0.005$.

Next, calculated relaxation rates were used to determine sequence parameters that maximize contrast per unit time (square-root of TR) for both T1- and T2-weighted imaging sequences. Solutions of the Bloch equations were used for an inversion-prepared spin echo sequence to achieve T1 weighting, selecting inversion times for each preparation equal to the mean of WM, GMi, and GMo T1s. A linear search of contrast space was performed (TR range 500-5000 ms) to select the repetition time for each preparation that maximized the root mean squared (RMS) contrast at each field strength. For T2 weighting, a spin echo sequence was used, selecting an echo time for each preparation equal to the mean of WM, GMi, and GMo T2s. Similarly, a linear search of contrast space was performed (TR range: 200-2000 ms) to determine the repetition time that maximized the RMS contrast for each preparation and field strength. Finally, images were acquired at both field strengths using the determined parameters for maximal contrast (listed in Figs.4-5 and 4-6), and the RMS CNR was computed for each sample preparation.

In addition, three 80-micron isotropic MR images of formalin-, ethanol-, and LFB-prepared samples were acquired at 14T with intermediate weightings. The following sequences were used for each sample: spin-echo (TR/TE = 2000/20.5 ms, NEX = 8), gradient-echo (TR/TE = 200/22.7 ms, $\theta = 30^\circ$, NEX = 8), and inversion-prepared spin echo (TR/TE/TI = 5000/7.2/300 ms, NEX = 8). Acquisition bandwidths and readout gradient directions were identical for these sequences. To investigate laminar characteristics, averages of signal intensity along 12 profiles perpendicular to the cortical surface were calculated, using Matlab, for the same region of each image. A similar profile analysis was conducted on an image from the most recent histological segmentation of human primary visual cortex by Braak [22] to help identify lamina in MR images of LFB-stained samples. This was considered an appropriate comparison because Braak's sample was considerably large for histological analysis (800 microns in thickness) and was subjected to a similar en bloc staining for lipofuscin granules. Furthermore, Braak's samples were stained using an aldehydefuscin stain enhanced by treatment with Astrablau, which is, in fact, a copper phthalocyanine dye [19] with a structure similar to LFB.
4.2.2 Validation of LFB distribution and concentration

After MR imaging was completed, two samples were sectioned into 40-micron slices with a freezing microtome. Sections were mounted on glass slides, dehydrated in progressive ethanol concentrations (70%, 95%, and 100%), cleared in xylene, and coverslipped. No additional myelin stains were added to the sectioned slices; one sample was counterstained with a 0.1% w/v solution of cresyl violet, following a standard protocol [74]. Bright-field microscopy was performed using a Nikon Eclipse 50i microscope. Images were collected using a charge-coupled device camera (Photometrics, Tuscon, AZ) and analyzed with SPOT 4.0 Advanced Version Software (Diagnostic Instruments, Sterling Heights, MI). Additional infrared images having 1-micron in-plane resolution were obtained with the LI-COR Odyssey (Lincoln, NE) to aid registration between MR and histological images using normalized mutual information (Amira, ZIB, Berlin).

In other LFB-stained samples, inductively-coupled plasma atomic emission spectrophotometric (ICP-AES) techniques [68] were employed to determine the concentration of LFB in solution and tissue samples by quantifying the amount of copper in each sample. LFB-stained, LICO-differentiated samples were segmented into regions of white matter or gray matter, based on optical density. Tissue segments of white matter, weighing 50.2 mg, and gray matter, weighing 44.5 mg, were digested in two separate phials containing 2 mL of a 1:1 mixture of 100% nitric acid and 100% sulfuric acid. After 48 hours, all tissue fragments had been fully digested, and each solution was diluted with Triton-X (Sigma-Aldrich, St. Louis, MO) to a final volume of 20 mL. A reference sample of the nitric-sulfuric-Triton solution was also prepared to estimate matrix effects. Measured tissue concentration of LFB was then compared to measured solution concentration to compute partition coefficients for gray matter and white matter, following the method of [73].

4.3 Results

4.3.1 High-resolution MR imaging

Three MR images acquired at 14T of an LFB-stained, lithium carbonate (LICO) differentiated sample of human visual cortex appear in Fig.4-1. Four distinct regions are
apparent in the T2*-weighted image (Fig.4-1 A) and the T1-weighted image (Fig.4-1 B); these areas resemble lamina, stratifying into parallel bands of differing thickness, and follow the convolutions of the lingual gyrus. The innermost area is white matter (WM); the more superficial, thick band is the heavily myelinated layer IVb. Placed between the white matter and layer IVb are layers IVc through VI, collectively referred to in this thesis as inner gray matter (GMi). Although only three cortical regions are discernable in this image, there are as many as 14 subregions in human primary visual cortex [22]. There is no consensus in the literature concerning the specific subdivisions for this area, and the nomenclature varies among authors and depends upon the histological techniques used (see Plate 9 in [22] for a comparison of 11 metrics of subdivision.)

Figure 4-1: MR images with T2-weighting (A), T1-weighting (B), and intermediate weighting at 14 Tesla of an LFB-stained, LiCO-differentiated sample of human visual cortex. Three regions of interest used for analysis are clearly visible in all images: white matter (WM), inner gray matter (GMi), comprised of layers IVc-VI, and outer gray matter (GMo), comprised of layers I-IVa. The Line of Gennari, layer IVb that is heavily myelinated in visual cortex, is also prominently displayed. In image C, the u-fibers are also resolvable and appear darker than the underlying white matter. Images have 150 micron isotropic resolution and were acquired using (A) a gradient echo sequence (TR/TE = 80/15.2 ms, $\theta = 30^\circ$); (B) an inversion-prepared spin echo sequence (TR/TE/TI = 5000/5.96/320 ms); and (C) an inversion-prepared spin echo sequence (TR/TE/TI = 5000/5.96/120 ms).
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The outermost region encompasses layers I through IVa and is referred to in this chapter as the outer gray matter (GMo). An additional dark band is discernable in the intermediate-weighted image (Fig.4-1 C), separating the WM and GMi. This is a layer of u-fibers, especially prominent in visual cortex.

Longitudinal and transverse relaxation rates were calculated in these three regions of interests (ROIs) for MR acquisitions of samples prepared successively with formalin, ethanol, and LFB at 4.7 and 14T. As seen in Fig.4-2, LFB was more effective in increasing the longitudinal relaxation rates in all ROIs than either formalin or ethanol preparation at both field strengths. At 4.7T, the percentage increase in R1 for the LFB samples compared to the ethanol preparation was 199% for WM, 129% for GMi, and 78% for GMo. At 14T, the percentage increases in R1 were more modest than at the lower field strength and equaled 30% for WM, 25% for GMi, and 5% for GMo. All changes were statistically significant \( p < 0.005 \) and suggest LFB effected an increase in R1 proportional to myelination.

LFB was also more effective in increasing transverse relaxation rates in all ROIs than either formalin or ethanol preparation at both field strengths. However, the increase in R2 effected by LFB was a much smaller percentage change than that observed in R1. At 4.7T, the percentage increase in R2 for the LFB samples compared to the ethanol preparation was 8.4% for WM, 1.3% for GMi, and 0.5% for GMo. At 14T, R2 increased by 59% for WM, 42% for GMi, and 27% for GMo. Changes seen at higher field are larger in part because of the increased field strength but could also result from rehydration effects, which lower R2 values in the ethanol-prepared, LICO-differentiated sample compared to the sample treated with formalin only. Although, significant hydration differences were not found between unfixed and formalin-fixed tissues [144]

The root mean squared (RMS) contrast between adjacent regions, per unit time, for formalin-, ethanol-, and LFB-treated samples at 4.7 and 14T are displayed in Fig.4-3 a. These values were obtained via simulations of the Bloch equations for T1- or T2-weighted sequences, as explained in the previous section. The LFB preparation has the largest contrast per unit time for T1- and T2-weighted imaging at 4.7T, although the propagated error from the fitted parameters reduces the significance of the gain in contrast. At 14T, signal differences are larger, and the LFB preparation produces a highly significant increase
Figure 4-2: Comparison of relaxation rates in white matter (WM), inner gray matter (GMi), and outer gray matter (GMo) for three differing preparations of fixed human tissue samples: 1) formalin alone, 2) 95% ethanol immersion and 0.05% lithium carbonate differentiation, or 3) LFB immersion and 0.05% lithium carbonate differentiation. (A) At 14T, R1 increased significantly ($p < 0.05$) for all ROIs (WM, GMi, and GMo) for all comparisons (formalin-etOH, formalin-LFB, and etOH-LFB). (B) At 14T, R2 increased significantly for all ROIs and all comparisons except GMo between formalin and etOH preparations. (C) At 4.7T, R1 increased significantly for all ROIs and all comparisons except GMo between formalin and etOH preparations. (D) At 4.7T, R2 increased significantly for all ROIs and all comparisons except GMi and GMo between etOH and LFB preparations.
in contrast for T1-weighted imaging. However, the LFB did not enhance T2 contrast compared to the formalin preparation.

Experimental values of contrast per unit time for each preparation, displayed in Fig.4-3 b, confirm the simulation predictions for contrast: LFB produces a significant increase in T1 laminar contrast per unit time at both field strengths but does not significantly increase T2 contrast above that of controls. The improvement in laminar contrast can be seen in the T1-weighted images of visual cortex prepared with ethanol or LFB, displayed in Fig.4-4. These LFB-MR images are the first images of ex vivo tissue in which white matter appears brighter than gray matter, as typical for in vivo T1-weighting. This traditional weighting is achieved more so in the LFB preparations (Fig.4-4, B and D) than in the ethanol controls (Fig.4-4, A and C). The brightening of WM aids identification of the WM-GMi boundary at both field strengths. Furthermore, GMi is more distinct from GMo in the LFB preparations, due in part to the enhanced detection of heavily-myelinated layer IVb, which appears brighter than adjacent lamina at both field strengths. In addition, GMi has a larger signal intensity than GMo at 14T, presumably because of its increased myelination. Thus, LFB enhances intralaminar contrast on T1-weighted images by increasing the signal intensity in proportion to myelination.

Differences in laminar contrast can also be seen between T2-weighted images of visual cortex prepared with formalin or LFB (Fig.4-5). WM and GMi regions appear slightly darker in the LFB-treated samples (Fig.4-5, B and D) than in the formalin controls (Fig.4-5, A and C). At 4.7T, LFB darkens the appearance of GMi, which enhances the GMi-GMo transition but blurs the boundary between WM and GMi. At 14T, there are marked differences in the appearance of GMi in the control and LFB data sets. In the control image, GMi is fairly homogenous, with a slightly larger signal intensity near the fundus of the gyrus. However, in the LFB sample, the uniformity of GMi is interrupted by fine lines having a perpendicular orientation to the laminae. These fine lines are consistent in location and orientation with the radial fascicles of the cerebral cortex. These myelinated axons are known to extend from the white matter to layer III in slender bundles [104], and have been documented to be present in higher density and greater thickness in mammalian visual cortex [146, 66]. The radial lines in the LFB-stained MR images appear to extend slightly
Figure 4-3: Comparison of theoretical (a) and experimental (b) contrast per unit time in T1- and T2-weighted images at 4.7 and 14T using optimal parameters (listed in Figs.4-4 and 4-5). An asterisk denotes a significant ($p < 0.05$) change in contrast per unit time when compared to samples imaged at the same field strength and image weighting.
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Figure 4-4: T1-weighted MR images of tissues stained with ethanol and differentiated with lithium carbonate (A,C) exhibit less contrast between adjacent laminar regions, white matter (WM), inner gray matter (GMI), and outer gray matter (GMO) than samples stained with LFB and differentiated with lithium carbonate (B,D). Images with 150 micron isotropic resolution were acquired using an inversion-prepared spin echo sequence, choosing an inversion time at each field strength equal to the mean T1 of all regions of interest for each sample preparation. Samples A and B were imaged at 4.7T, samples C and D at 14T.

past the line of Gennari. The accumulation of contrast agent along these radial fibers could have contributed to compartmental susceptibility effects, resulting in the stippled appearance of the more myelinated, inner layers in the MR images of LFB-prepared samples.

In the high-resolution images, additional laminar structure not resolvable in control data becomes apparent in the LFB-treated sample (Fig.4-6). Two structures were considered resolvable according to the Rayleigh criterion if two peaks are separated by a minimum having a height no more than 75% of the smaller peak height  [90]. Images of the ethanol-treated control sample reveal some laminar structure (Figs.4-6 A-C). A laminar profile perpendicular to the cortical surface of the spin echo acquisition is plotted in Fig.4-6 D. A dip in signal intensity (73% of peak height) corresponds to layer IVb. This is the only feature resolvable according to the Rayleigh criterion. A broad local maxima appears for subjacent layers and no additional subdivisions can be made. In the profile through the
4.3. RESULTS

Figure 4-5: T2-weighted MR images of control tissues treated with formalin only (A,C) compared to tissues stained with LFB and differentiated with lithium carbonate (B,D). Samples A and B were imaged at 4.7T, samples C and D at 14T. White matter (WM) and inner gray matter (GMI) regions appear darker in the LFB-treated samples, which, at 4.7T, enhances the transition between GMI and outer gray matter (GMO) but blurs the WM-GMI boundary. In addition, at 14T, the GMI in the LFB sample has a stippled appearance, presumably due to compartmental susceptibility effects caused by accumulation of contrast agent along radial fibers. Images are 150 micron isotropic resolution and were acquired using a spin echo sequence, choosing an echo time at each field strength equal to the mean T2 of all regions of interest for each sample preparation.

A gradient echo image (Fig.4-6 E), a narrow dip appears in the location of layer IVb as well as a small peak that could correspond to a more superficial layer. A profile through the more T1-weighted image (Fig.4-6 F) reveals a series of shallow peaks and troughs that could correspond to additional lamina, all superficial to layer IVb.

Profiles through all acquisitions of the LFB-treated sample reveal peaks and troughs corresponding to four distinct lamina (Fig.4-6 J-L, arrows). These additional layers are inferior to the prominent line of Gennari, and alternate in signal intensity compared to the myelinated layer IVb. Specifically, in the T2-weighted spin echo and gradient echo acquisitions (Figs.4-6 G and H), layer IVb appears as a dark stripe and two fine, brighter lines are discernable, separated by a thin, darker layer. In the laminar profiles, the layers appear as a series of peaks and troughs, and are most resolvable in the profile derived from the gradient-echo image (dip-to-peak height ratios of 73%, 80%, and 62%, respectively). In the more T1-weighted image (Fig.4-6 I), there is a lower contrast-to-noise ratio overall,
Figure 4-6: Three separate, 80-micron isotropic resolution MR acquisitions at 14T of ethanol control samples (A-C) and LFB-stained preparations (G-I). Images A and G are spin-echo acquisitions (TR/TE = 2000/20.5 ms, NEX = 8), images B and H, gradient-echo acquisitions (TR/TE = 200/22.7 ms, θ = 30°, NEX = 8), and images C and I, inversion-prepared spin echo acquisitions (TR/TE/TL = 5000/7.2/300 ms, NEX = 8). (D-F, J-L) Averages of signal intensity along 12 profiles perpendicular to the cortical surface were calculated in the same region, illustrated by the rectangle and are plotted to the right of each image. Additional lamina that become resolvable in the LFB preparations are highlighted with arrows (J-L).
4.3. RESULTS

but the averaged profile reveals local maxima and minima at the same locations as in the T2-weighted images, but with a reversal in polarity. Specifically, layer IVb now appears as a local maxima, the next layer as a dip, the next as a peak, and the last as a dip (dip-to-peak height ratios of 87%, 82%, and 77%, respectively). These additional layers follow the course of the line of Gennari, ending abruptly at the V1-V2 transition, as can best be seen in Fig.4-6 H.

A similar profile analysis was performed on an Astrablau-stained image of striate cortex (Fig.4-7 A, modified from [22]). In this inverted image, signal intensity is proportional to lipofuscin density. The peaks and dips appearing in a profile through (Fig.4-7 B) could be identified from the labels on the original image. Progressing from superficial to deep layers, the first, wide dip is unlabeled, but has the proper thickness and location to be layer IVb. The next peak is consistent with layer IVcb; the next trough, layer IVd, and the final peak, Va. None of the additional listed layers (Vb-VIb) could be distinguished.

There is a remarkable resemblance between the profile through the histological preparation and the profile through the gradient echo image of the LFB-prepared sample (Fig.4-7 C). Expressing the abscissa of each graph as a percentage of cortical depth helped identify the additional lamina in the high-resolution MR images of LFB-prepared samples. It is reassuring that Astrablau, the copper phthalocyanine dye found to enhance lipofuscin staining [111, 22], would have such a similar staining profile to LFB.

4.3.2 Validation of LFB distribution and concentration

A high-resolution image acquired at 14T was matched to an infrared image of 40-micron slice preparation and then to a 2x-magnification light microscope image (Figs.4-8 A-C). The alignment is not exact due to tissue damage incurred during slice preparation, common in traditional histology. According to a trained neuropathologist, neither the en bloc staining technique nor the perfluorocarbon embedding appear to have altered the microstructural appearance of the fixed tissue. Furthermore, the macroscopic distribution of LFB dye is similar to standard histologic preparations: the darkest staining areas of visual cortex are the white matter and the heavily myelinated line of Gennari (Fig.4-8 C). The optical density of the tissue appears proportional to myelin content, and the deeper, more myelinated cortical
layers appear darker than the more superficial layers. Upon higher magnification (10×, Fig.4-8 D), the dense meshwork of subcortical white matter contains many blue fibers. At an even greater zooming strength, (40×, Figs.4-8 E-F) individual radial fibers can be distinguished projecting perpendicular to the cortical lamina; closer to layer IVb, horizontal fibers can be distinguished that belong to this tangential net.

In Fig.4-9, a 40-micron histological image counterstained with thionine is matched to a 150-micron MR image acquired at 14T. This histological preparation permitted segregation of eight laminar divisions: layers I, II-III, IVa, IVb, IVc, V, VI, and white matter. These lamina confirm our previous choices of regions of interest first introduced in Fig.4-1.

Table 4.1: ICP-AES concentration Measurements

<table>
<thead>
<tr>
<th>Sample</th>
<th>Copper (μg/g)</th>
<th>Copper (mM)</th>
</tr>
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<tbody>
<tr>
<td>LFB in GM</td>
<td>12.63 ± 0.23</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>LFB in WM</td>
<td>26.32 ± 0.28</td>
<td>0.93 ± 0.01</td>
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</table>

The ICP-AES measurements for gray and white matter copper content are displayed above, in Table 4-1. These are minimal values for the tissue samples, as storage in distilled, deionized water before tissue digestion did cause some contrast agent seepage, as noted by the bluing of the solution. Using these values, the partition coefficient was calculated to be 15.8% for gray matter and 32.7% for white matter.

4.4 Discussion

This work has introduced luxol fast blue as a novel magnetic resonance contrast agent with binding affinity for myelinated constituents of the brain. This specificity for lipid constituents increased the longitudinal and transverse relaxation rates of tissue dependant on myelination, leading to enhanced contrast between lamina possessing varying degrees of myelination. LFB treatment, employed using the newly-developed en bloc staining technique, revealed sublayers IVca, IVd, and Va that were not discernable in control preparations.

While the solution longitudinal relaxation rates of LFB are a fraction of gadolinium
4.4. DISCUSSION

analogues, LFB's efficacy lies in its myelin specificity. The small magnetic moment of copper, due to its spin of 3/2 versus gadolinium's spin of 7/2, limits the relaxivity of LFB. This problem could be ameliorated by replacing the copper in LFB with a metal having a larger magnetic moment, such as manganese. Saini et al. have synthesized MnPcS4 [127], a tetra sodium salt of manganese tetra sulfo phthalocyanine, following a modified method of Webber and Busch [152]. At 1.5T, they measured a longitudinal relaxivity for MnPcS4 twice that of Gd-DTPA. In a similar fashion, LFB-chelated manganese could be synthesized to produce a more effective, white matter specific MR contrast agent. However, caution should be exercised and the effect on T2* contrast considered in designs to replace copper. The subtle susceptibility enhancement was surprising, and replacing the weak copper by a metal having greater susceptibility could overwhelm this gain in contrast.

Target-specific paramagnetic contrast agents hold much promise for aiding cytoarchitectural identification in high-resolution, high-field MR imaging. These agents will advance ex vivo imaging as a technique to create more detailed volumetric MR atlases of normal and pathologic brains. LFB may be especially useful in investigating structural changes in demyelinating disorders, such as multiple sclerosis (MS), because of its chemical affinity for myelinated tissues. These agents will facilitate the use of non-destructive, three-dimensional ex vivo imaging methods as a supplement—or an alternative—to traditional histology methods used for pathological analysis of the brain.
Figure 4-7: (A) An inverted light microscope image (Fig.5, Plate 2 from [22]) of an 800 micron-thick slab of Astrablau-stained human visual cortex. (B) Average signal intensity along 120 profiles perpendicular to the cortical surface were calculated in the rectangular region shown in (A). Arrows have been added to label the peaks and troughs corresponding to the documented lamina (layers IVb, IVcβ, IVd, and Va) in (A). (C) Fig.4-6 K is repeated here for comparison: Average of signal intensity along 12 profiles perpendicular to the cortical surface calculated for the rectangular area shown in Fig.4-6 H.
4.4. DISCUSSION

Figure 4-8: Distribution of LFB among myelinated fibers for en bloc mimics that of traditional histologic preparation. Image A is an 80-micron isotropic resolution gradient echo acquisition (TR/TE = 60/7.7 ms \( \theta = 30^\circ \)) at 14T. Image B is a 21 micron in-plane resolution infrared image obtained to aid alignment of MR and histological images. The red circles in images A and B indicate the approximate location of the 40-micron thick, 2x magnification slice preparation in image C, to which no additional stain has been added. The macroscopic LFB distribution is proportional to myelination and is concentrated in white matter and the line of Gennari, causing these areas to have a darker intensity in light microscopy. Illustrated in image C are three additional regions of interest, which appear at higher magnifications in images D-F. Image D is a 10x magnification of subcortical white matter; image E, a 40x magnification of inner gray matter (GMi) reveals radial fibers (arrows) that retain the LFB stain; and image F, a 40x magnification closer to layer IVb shows tangential fibers (arrows) that retain the LFB stain.
Figure 4-9: 40-micron thick slice preparation of LFB-stained tissue (using en bloc technique) with a cresyl violet counterstain (traditional method). Slice is matched to a 100-micron isotropic resolution MR acquisition at 14T (gradient-echo acquisition: TR/TE = 200/22.7 ms, $\theta = 30^\circ$, NEX = 8). Histological preparation has been segmented into layers based on neuronal density.
Chapter 5

LFB-MRM in X-linked Adrenoleukodystrophy

"A doctor can bury his mistakes but an architect can only advise his clients to plant vines..." — Frank Lloyd Wright

We present a novel method to visualize substrate accumulation in neurolipidoses with magnetic resonance (MR) imaging. High-resolution MR images of adrenoleukodystrophy brain tissue, stained with luxol fast blue (LFB), revealed an additional zone, unseen in images of the same tissue blocks, before staining. T2*-weighted images produced the largest LFB-contrast-enhancement, which exhibited contrast-to-noise increases as high as four-fold. Electron micrographs within this newly-visible zone identified macrophages laden with lipids and myelin debris. Ultrastructural examination revealed LFB particulates attached to intact and degenerating myelin engulfed by macrophages: evidence our technique can assist identification of active disease and inflammation. Furthermore, compartmentalization of LFB within macrophages is consistent with susceptibility-induced proton relaxation enhancement and the resultant regional changes in relaxation rates.
5.1 Introduction

Neurolipidoses are lipid storage disorders in which enzymatic defects result in accumulation of particular substrates within the central nervous system: sulfatides in metachromatic leukodystrophy (MLD) [84], very-long chain fatty acids (VLCFA) in adrenoleukodystrophy (ALD) [99], and glucocerebroside in Gaucher’s Disease [109], for example. Therapeutic interventions, such as gene therapy, enzyme replacement therapy, and lipid-lowering drugs, are under active investigation [100]. However, evaluating the efficacy of these treatments is hampered by the lack of specific biomarkers for substrate accumulation. While conventional imaging techniques can follow disease progression, they are not specific to the substrate.

As described in the previous chapter, we have developed an ex vivo magnetic resonance imaging technique using luxol fast blue (LFB) as a contrast agent. The lipid-binding properties of LFB, a histological dye widely-used to demonstrate myelin, paired with its paramagnetic copper core, make this dye uniquely suited to measure myelin debris and brain lipid accumulation with MR imaging. We have the opportunity to assess the lipid distribution in X-linked ALD, a disorder that encompasses many of the pathological hallmarks common to many white matter disorders: substrate accumulation, confluent demyelination, and gliosis and necrosis, in addition to normal appearing white matter and intact subcortical arcuate fibers. Thus, imaging ALD specimen offers a unique opportunity to improve detection and localization of substrate accumulation near the leading edge of the neurodegenerative process and to advance the understanding of the pathophysiology, immune response, and treatment efficacy in neurolipidoses. Additionally, imaging ALD presents an opportunity to assess the biochemical nature of LFB binding in the setting of pathology.

X-linked ALD is a heritable disorder that impairs function of the adrenal cortex and nervous system myelin [99]. The genetic defect resides in Xq28, which codes for peroxisomal membrane protein ABCD1. Almost 500 different mutations have been documented [38], but no genotype-phenotype correlations have been established. The estimated incidence of X-ALD is 1:17,000, with no preference for ethnicity [13]. About 35% of patients have the childhood cerebral form of X-ALD, in which they develop normally until age 4-8 and then undergo a progressive neurological decline involving dementia, gait changes, and sensory
deficits, leading, ultimately, to a vegetative state and death within three years [99, 38]. The pathological changes involve severe inflammatory demyelination that begins in the splenium and genu of the corpus callosum and spreads, systematically and usually symmetrically, to the periventricular white matter [131, 116].

The demyelinating process in X-linked ALD has been well-documented and consists of three sequential histopathological zones: a lesion core, a demyelinated zone, and an actively-demyelinating zone [131, 115, 98]. These regions, first described by Schaumburg, are documented below in Table 5-1. The core is necrotic and lacks axons or myelin. Astrocytic gliosis may form where myelinated fibers had once existed, and occasional perivascular macrophages are seen. Adjacent to the core is a zone of inflammation that contains the largest concentration of perivascular plasma cells, lymphocytes, and macrophages. At the leading edge, a more active area of demyelination reveals LFB-positive macrophages. These lesions develop in a well-ordered, symmetric fashion, as the leading edge of demyelination begins in the midline of the corpus callosum and advances outward, sparing subarcuate fibers and cortex [131, 117].

Table 5.1: Schaumburg's Zones of Demyelination in X-ALD

<table>
<thead>
<tr>
<th>Region</th>
<th>Cellular constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion core</td>
<td>Few macrophages, gliosis, necrosis</td>
</tr>
<tr>
<td>Total myelin loss</td>
<td>Macrophages, lymphocytes</td>
</tr>
<tr>
<td>Active demyelination</td>
<td>LFB-positive macrophages</td>
</tr>
<tr>
<td>Normal-appearing white matter</td>
<td>Densely-packed myelinated axons</td>
</tr>
<tr>
<td>Cortex</td>
<td>Normal myelinated fiber density</td>
</tr>
</tbody>
</table>

These inflammatory changes occur in the setting of reduced degradation of very long chain fatty acids (VLCFA) in tissues and body fluids [99]. It is not clear whether VLCFA serve as a trigger to the inflammatory process [25] or as a consequence. Long chain fatty acids have backbones of 14-18 carbon atoms, designated “C14-18,” and comprise the majority of dietary fatty acids. VLCFA have 22 or more carbons [99]; normal, saturated VLCFA occur in highest concentration in myelin lipids and red blood cell sphingomyelin (C26:0 accounts for 1-5% of total fatty acids in brain cerebrosides and sulfatides) [65, 25]. The accumulated VLCFA are saturated and unbranched (mainly C24:0 and C26:0), with
the excess most striking in the cholesterol ester and ganglioside fractions of affected brain white matter and adrenal cortex [65]. Interestingly this does not appear to hold true for the histologically-intact regions of the X-ALD brain. Theda found the phosphatidylcholine fraction to contain a 39 fold excess of C26 in comparison with control, while the other fractions were normal or increased less than twofold [142]. This is suggestive of a role in the pathogenesis of ALD.

5.2 Materials and Methods

5.2.1 Ex vivo sample preparation

Four samples of occipital cortex were obtained from autopsy material from three childhood cerebral ALD cases. One was from a 6-year-old boy who had suffered behavioral changes, gait difficulties, and spastic quadriplegia. The second was from a 9-year-old boy who had presented with blindness, deafness, spastic quadriplegia, and seizures. And the third patient had been a 13-year-old boy, who, 6 months prior to death, had been diagnosed with X-ALD following a rapidly progressive neurologic decline.

All samples, obtained from the Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD) were biochemically confirmed and most demonstrated the inflammatory demyelinating lesions typical of ALD [99]. Mean patient age is 9.6 years; mean post-mortem interval is 10 hours. Brain tissue was fixed in formalin for at least eleven weeks, the minimum time required for transverse and longitudinal relaxation rates to reach equilibrium [145, 15]. Blocks, approximately $6 \times 1.5 \times 1 \text{ cm}^3$, were bisected perpendicular to the cortical surface, along the longest axis. One half was used for MR imaging and electron microscopy, the other for immunohistochemistry.

Samples used in imaging were prepared in two subsequent manners: formalin treatment only and en bloc staining protocol using 1% LFB. The en bloc protocol, introduced in Chapter 3 was modified to perform LFB immersion at room temperature instead of 56°C to minimize lipid phase transitions [133, 123]. In addition, the LFB was noted to leave the ALD tissue much more rapidly during differentiation than from normal tissue; as such, each lithium carbonate differentiation was estimated individually from the apparent surface
5.2. MATERIALS AND METHODS

Gray-white matter contrast. The protocol steps are as follows:

Procedure:

1. Immerse formalin-fixed tissue in 1% luxol fast blue for 48 hours at 20°C.
2. Blot tissue with filter paper.
3. Rinse in 95% ethanol for 5 min and take to doubly-distilled water. Let stand for 5 min.
4. Blot tissue with filter paper.
5. Transfer tissue to 0.05% aqueous lithium carbonate (LiCO). Remove after ~30 minutes per 1 cm thickness.
6. Blot tissue with filter paper.
7. Transfer tissue to perfluorocarbon in low-pressure environment and store overnight.

Prior to imaging, tissue blocks were wedged between two polyethylene caps (Wilmad Lab-Glass, Buena, NJ) to prevent motion. A proton-signal-free perfluoropolyether (Fomblin, Solvay Solexis, Thorofare, NJ) was added in a low-pressure environment to remove artifact-inducing air bubbles within the tissue. Tissue was given a minimum of 24 hours at room temperature to equilibrate within the perfluoropolyether.

5.2.2 High-resolution MR imaging

Magnetic resonance imaging was performed on a vertical bore 14T Bruker/Magnex system, using a 20-mm diameter birdcage coil. Inversion-recovery prepared spin echo (IR-SE) sequences were used for T1 mapping (TR=5000 ms and TI=6, 50, 100, 300, 500, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 4000, 5000 ms), multi-echo spin echo (MSME) sequences for T2 mapping (TR=2000 ms and TE=6.83, 13.65, 20.48, 27.35, 34.13, 40.96, 47.78, and 54.61 ms), and gradient-recalled echo (GRE) sequences for T2* mapping (θ=45°), TR=200 ms and TE=4, 7.73, 11.47, 15.20, 18.94, and 22.67 ms). These acquisitions were performed in five slices having an in-plane resolution of 115x115 μm² and a through-plane resolution of 500 μm.
Regions of interest (ROIs) were drawn manually, using Matlab (Mathworks, Natick, MA) based on MR signal intensity, were later assessed by aligning histological sections from mirror-face blocks to the MR images. As one objective of this study is to investigate the biochemical nature of LFB binding in the setting of ALD, we refrained from performing histology in the same imaged sample. Electron microscopy will reveal tissue substructure at the cellular level, and high-pressure liquid chromatography will allow quantitation of lipid subfractions, including phosphatidylcholine, a leading candidate for LFB’s binding partner that is highly elevated (~39-fold) in very-long chain fatty acids in ALD [142]. Together, this data can illuminate the interactions between LFB and the myelin membrane. Furthermore, ALD is well-known to posses confluent demyelination and symmetrical lesions [131, 115, 99]; as such, it is likely for a section from the adjacent slab to provide a reasonable estimate of zonal demyelination, even if it may originate as far as 200 µm away.

Means and standard deviations of ROIs were plotted against TI or TE and fit to solutions of the Bloch equation for IR-SE, SE, and GRE sequences. The Levenberg-Marquardt method [118] was used to determine $T_1$, $T_2$, and $T_2^*$ for all ROIs in each sample preparation. A paired, two-tailed Student’s t-test assessed the significance of numerical differences in relaxation rates arising from staining tissues with LFB. Differences were considered significant if $P < 0.005$.

Next, calculated relaxation rates were used to determine sequence parameters that maximize contrast per unit time (square-root of TR) for $T_1$-, $T_2$-, and $T_2^*$-weighted imaging sequences. Solutions of the Bloch equations were used for an inversion-prepared spin echo sequence to achieve $T_1$ weighting, selecting inversion times for each preparation equal to the mean $T_1$ for all regions. A linear search of contrast space was performed (TR range 500-5000 ms) to select the repetition time for each preparation that maximized the root mean squared (RMS) contrast for the selected TI. For $T_2$ or $T_2^*$ weighting, a spin or gradient echo sequence was used, respectively, selecting an echo time for each preparation equal to the mean $T_2$ or $T_2^*$ for all regions. Similarly, a linear search of contrast space was performed (TR range for SE: 200-2000 ms; TR range for GRE: 60-200 ms, $\theta$ range: $15-90^\circ$) to determine the repetition time and flip angle that maximized the RMS contrast for each preparation and field strength. Finally, images with an isotropic resolution of 115 mi-
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crons were acquired using the determined parameters for maximal contrast, and the RMS contrast-to-noise ratio (CNR) and percentage change computed for each image weighting and sample preparation.

5.2.3 Histology

Mirror-face blocks were paraffin-embedded and sectioned into five-micron slices. A traditional, thin-slice haematoxilyn and eosin (H&E) staining was performed, counterstained with 0.1% LFB, and differentiated in LiCO₃. A control sample from the occipital cortex of a 12-year-old boy with no history of neurological disorder was also stained in this fashion. Immunohistochemical techniques were employed on the ALD specimen to demonstrate myelin distribution by staining with antibodies to myelin basic protein (MBP); microglia (resting and activated) with ionized calcium-binding adapter molecule 1 (IBA-1); and astrocytes with glial fibrillary acidic protein (GFAP). Double immunostaining was performed for MBP and IBA-1 and demonstrated the relationship between myelin and mononuclear phagocytic cells. Cell or protein density was quantified by sampling 3-4 regions from images acquired with a Leica DMR microscope (Leica Wetzlar, Heidelberg, Germany) and an Optronics MicroFire digital camera system (Goleta, CA), processed in Photoshop (Adobe, San Jose, CA) and ImageJ (NIH, Bethesda, MD).

5.2.4 Electron microscopy

After MR imaging was completed, four regions, each about 4 × 2.5 × 2 mm³, were dissected from one ALD LFB-stained block. These regions, corresponding to lesion, perilesional area, normal appearing white matter (NAWM), and cortex, were prepared for electron microscopy following standard protocols [74]. In addition, control samples of gray and white matter were prepared. Specifically, samples were post-fixed with osmium tetroxide, stained en bloc with uranyl acetate, dehydrated in graded ethanol solutions, infiltrated with propylene oxide/Epon mixtures, flat-embedded in pure Epon, and polymerized overnight at 60°. One micron sections were cut, stained with toluidine blue, and examined by light microscopy. Representative areas were chosen for electron microscopic study, and the Epon blocks trimmed accordingly. Thin sections (~ 1/40 micron) were cut with an LKB 8801
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ultramicrotome and diamond knife, stained with Sato's lead, and examined in a Phillips 301 transmission electron microscope. Additional samples were processed without addition of any metals (osmium tetroxide, Sato's lead, or uranyl acetate) or with only the addition of osmium tetroxide.

5.3 Results and Discussion

5.3.1 High-resolution MR imaging and histology

All four samples had regions of confluent myelin loss. In two samples treatment with LFB revealed an additional zone on high-field MR. Immunohistochemical analysis revealed a corresponding tightly packed zone of perivascular macrophages. The other two samples had no additional zone on LFB-MR or immunohistochemical analysis. We conclude that LFB-MR is able to detect the actively demyelinating edge in cerebral ALD samples.

MR detection of the actively demyelinating edge

In two samples we found confluent loss of myelin, apparent in MR imaging, light microscopy, and immunohistochemistry. The area of myelin loss was surrounded by a region of perivascular macrophages. This region, referred to as the “peri-lesional area,” corresponds to Schaumburg's zone of active demyelination (see Table 5.1) and became visible in MR images after staining with LFB (Fig. 5-2 E, 5-4 D-F, and 5-5 B). Thin-slice LFB preparations corroborate the zonal appearance seen in MR; furthermore, measurements of IBA-1 positive cell density within the zones identified in LFB-MR demonstrate an increase of microglia and macrophages in the peri-lesional area (Fig. 5-1). Electron microscopy, described in a later section, confirms the presence of macrophages laden with lipid droplets, LFB-stained myelin and myelin debris, and linear inclusions (Fig. 5-14). A more detailed analysis of image contrast follows.

Before LFB staining

For both samples, MR images of unstained samples displayed adequate contrast between gray and white matter (Figs. 5-2 A-B, 5-4 A-C). Tissues showed prominent, confluent de-
myelination and subcortical sparing of subarcuate fibers. Parameters chosen to maximize T1 contrast for IR-SE images produced homogeneous, hypointense white matter and hyperintense gray matter (Fig. 5-2 A). Signal intensities are reversed from typical in vivo T1-weighted MR images but are common for ex vivo imaging. Parameters chosen to maximize T2 contrast in SE acquisitions produced slightly hyperintense white matter, which is unusual for T2-weighting in normal ex vivo samples and is suggestive of pathology. Cortical laminar structure is more apparent in the T1-weighted image than in the T2-weighted image; however, striations in the upper part of subcortical white matter just beneath gyral crowns are only apparent on T2 weighting.
Figure 5-2: MR images of an actively demyelinating ALD sample, before (A-B) and after (D-E) LFB staining. (A) and (D) are IR-SE acquisitions (TR/TE/TI=2000/7.57/ ms), (B) and (E) are MSME acquisitions (TR/TE=1000/ms). An MBP stain (C) from the mirror-image sample reveals spared arcuate fibers and a myelinated cortical lamina, possible layer IVb. An IBA-1 stain (F, approximately matched to the rectangular area shown in E), reveals large macrophages and activated microglia.
5.3. RESULTS AND DISCUSSION

After LFB staining

MR imaging the same ALD sample after LFB staining reveals additional features. Post-staining, outer cortical lamina now appear brighter than underlying gray matter in both optimized T1- and T2-weighted images (Fig. 5-2 D-E, respectively). In addition, cortical lamina present more intensely as alternating bands of bright and dark signal intensity (arrows). These findings confirm the normal appearance of cortex in ALD and are consistent with our previous work in normal ex vivo tissue.

Two hyperintense lamina are visible in the middle gyrus on T2-weighting. A single stripe is discernable in the bottommost gyrus and may represent layer IVb, the Line of Gennari, although its thickness and intensity are less than in normal occipital cortex. An MBP stain, from a similar section in the mirror-image block, also captures this lamina in the bottommost gyrus (Fig. 5-2 C, arrow). The intensity of the MBP stain is correlated with the amount of intact myelin: the subcortical arcuate fibers, spared in ALD [115, 99], appear darkest; the cortex is lightly stained; and the white matter is very pale, indicating a substantial degeneration of myelin.

Unlike in normal tissue, LFB staining of the ALD samples enhances subtle structural differences in white matter. Specifically, after LFB staining, two regions are now resolvable in T1-weighted MR that had been indistinguishable in images of the unstained sample. MR signal intensities in white matter had a unimodal distribution before LFB staining but became bimodal after staining. The newly-appearing region also contains several hypointense circles (Fig. 5-2 E, asterisk) and overlaps with IBA-1 positive macrophages and activated microglia (Fig. 5-2F, arrow) in the mirror-image block corresponding to the white rectangular region in Fig. 5-2E.

These findings are consistent with clumped accumulations of contrast agent. It is unlikely for these features to be caused by air bubbles, as this slice is within a central portion of the tissue. One explanation for this added contrast is an accumulation of LFB particulates within macrophages, as seen in [131, 85]. These molecules are known to clump together [48], and could thus produce large susceptibility shifts not refocused by the SE sequence, as explained in Chapter 2. In fact, the GRE images of this sample (Fig. 5-3 A-B) suffered a large susceptibility-induced signal loss specifically in this inner region after LFB staining.
Figure 5-3: Regional accumulation of LFB in ALD demyelinating white matter produces large susceptibility effects on gradient-echo imaging sequence: (A) sample treated with formalin only; (B) identical sample after LFB-staining.

In a second sample, after LFB staining, MR images optimized for contrast (Fig. 5-4 D-F) reveal striking differences from images of the pre-stained sample. The lesion is no longer homogeneous, but has a high core signal intensity on T2 or T2* weighting (low with T1 weighting) and a darker (brighter) region extending below. The lesion reaches well past boundaries seen in the unstained sample and becomes bespeckled near its edges, which are no longer smooth but jagged. Furthermore, subcortical white matter is not only comprised of lesion and non-lesion, but a third, transitory zone has emerged between normal appearing white matter (NAWM) and lesion. This middle zone is discernable in all three image weightings as a dark, non-uniform band encircling the lesion. The band has a honeycomb appearance, especially in T1- and T2-weighted images, and is most pronounced on T2*-weighting, perhaps a result of stronger susceptibility effects that expand and join adjacent pores. GRE MR images acquired before and after LFB staining (Fig. 5-5 A-B) have been aligned to an MBP stain and an H&E stain (Figs. 5-5 C-D) from a similar section in the mirror-image block.

Immunohistochemistry stains were performed to help identify the biophysical constituents
Figure 5-4: LFB-MR microscopy reveals an additional zone in ALD. MR images before staining (A-C) present three major iso-contours of signal intensity; whereas MR images after staining (D-F) present four distinct regions of signal intensity. Scale bar in (B)=1 millimeter and is applicable to all images. IR-SE parameters: TR/TE=2000/7.27 ms; TI: (A)=731 ms, (D)=632 ms. GRE parameters: TR/θ=60 ms/45°; TE: (B)=15.4 ms, (E)=10.8. MSME parameters: TR=1000 ms; TE: (C)=15.6 ms, (F)=15.4 ms.
Figure 5-5: MR images of unstained ALD samples resemble MBP stains, which correspond to myelin density; whereas LFB-MR images superimpose additional information about macrophage and microglia density atop myelin distribution. Images (A) and (B) are the same GRE acquisitions detailed in Fig. 5-3 (B) and (E), respectively, but have been resliced to better match MBP (C) and H&E (D) stains prepared from the mirror-image sample. The MBP stain was double-stained for IBA-1, and (E) corresponds to the rectangular area in (C). A higher magnification of the rectangle in (E) appears in (F).

Contributing to zonal differences in MR signal, T2*-weighted MR images of an unstained (Fig. 5-5 A) and LFB-stained (Fig. 5-5 B) ALD sample were aligned to a 2x magnification MBP stain (Fig. 5-5 C) from a similar section in the mirror-image block. The MBP stain mimics the contrast seen in the unstained, formalin-fixed image. This finding is consistent with Koenig’s proposal that a heavily T2-weighted image is akin to a map of myelin distribution [82]. The reddish-orange color imparted to myelin by this stain confirms the designation of a NAWM ROI, which overlaps completely with the colored myelin, and the lesion ROI, which is devoid of MBP pigment. However, little structural information is suggestive of the contrast seen in the peri-lesional area.

Upon higher magnification (data not shown), a view encompassing demyelination, NAWM, and cortical regions reveal subtle differences within the MBP stain on 4x magnification. The dense meshwork of myelinated fibers gives the NAWM a uniform appearance, except where vasculature has pierced through. There is also a slight darkening of MBP pigment at the transition between cortex and white matter at the location of the arcuate fibers, which are
Figure 5-4: LFB-MR microscopy reveals an additional zone in ALD. MR images before staining (A-C) present three major iso-contours of signal intensity; whereas MR images after staining (D-F) present four distinct regions of signal intensity. Scale bar in (B)= 1 millimeter and is applicable to all images. IR-SE parameters: TR/TE=2000/7.27 ms; TI: (A)=731 ms, (D)=632 ms. GRE parameters: TR/θ=60 ms/45°; TE: (B)=15.4 ms, (E)=10.8. MSME parameters: TR=1000 ms; TE: (C)=15.6 ms, (F)=15.4 ms.
in terms of age, post-mortem interval, and fixation. Although ALD lesions are confluent and systematic in progression, the differences seen in these samples underscore the localized process and regional progression of demyelination.

**Normal control**

We show for comparison the normal LFB distribution in thin-slice preparations as well as IBA-1 density in a normal control sample (Fig. 5-7). Note the deep blue of normal white matter, and the uniform IBA-1 density. MR imaging was not performed on this sample.

**Changes in relaxation rates**

Relaxation rate changes for all samples were analyzed in lesion, peri-lesion (where applicable), NAWM, and cortical ROIs, as shown in Fig. 5-8 A. Changes in longitudinal relaxation rates (R1) were proportional to myelination, with the largest change of $34.5 \pm 1.5\%$ occurring in NAWM, the next largest, $13.4 \pm 0.3\%$, in the peri-lesional area, and the small-
Figure 5-7: a) An LFB preparation of a normal control sample. b) IBA-1 density for normal control sample; for each region: central white matter (cen WM), subcortical white matter (sc WM), and cortex, counts were averaged across four areas, in a 40x-magnification immunohistochemical preparation.

...est in the lesion, $7.4 \pm 1.9\%$ and cortex, $5.8 \pm 3.4\%$. The magnitude of these values most closely paralleled MBP counts (Fig. 5-8 F). Homogeneous transverse relaxation rates ($R_2$) increased a similar amount for lesion and NAWM, $17.8 \pm 1.2\%$ and $17.2 \pm 0.5\%$, respectively, $7.4 \pm 0.2\%$ for cortex, and $4.7 \pm 0.4\%$ for peri-lesion. By far, the largest rate increase was seen for the inhomogeneous transverse relaxation rate ($R_2^*$) of the peri-lesional area and equaled $53.5 \pm 0.8\%$; $R_2^*$ increased $25.4 \pm 1.2\%$ in NAWM, $12.1 \pm 1.3\%$ for lesion, and $7.7 \pm 1.4\%$ for cortex. These values most closely paralleled IBA-1 values (Fig. 5-8 G). Thus, NAWM would be best visualized by LFB imaging with T1 weighting, while the peri-lesion area would also be better emphasized using T2* weighting.

The selective regional enhancement in relaxation rates effected by LFB staining was predicted to increase the CNR over that achieved in unstained samples. Measurements confirmed this hypothesis, and CNR increased in LFB-stained samples for all three image weightings in all samples, displayed in Fig. 5-9. The largest relative change in CNR occurs for T2*-weighting in all four samples: 57.6%, 150%, 198%, and 413% for samples 1-4,
Figure 5-8: Regional relative changes in relaxation rates mimic immunohistochemical measurements. (A) GRE image of LFB-stained ALD sample, displaying numbered ROIs: 1. lesion, 2. peri-lesion, 3. NAWM, and 4. cortex. Values are displayed for measurements made within these four ROIs for (B) MBP, (C) IBA-1, (D) ΔR2, (E) ΔR1, (F) ΔR2*.
respectively. The next largest change, seen in T2-weighted CNR, was 15.2%, 9.3%, 109%, and 54.8%, for samples 1-4, respectively. Changes in T1-weighted CNR were 32.6%, 45%, 8.3%, and 56.3%. These changes suggest LFB affects ALD samples differently depending on their microscopic structure. Samples 1 and 2, in which demyelination appeared more inhomogeneous and less zonal, experienced increases in R2*, while samples 3 and 4, with zonal demyelinating characteristics, experienced even greater changes in R2* in addition to a moderate increase in R2. Furthermore, these gains in CNR are a minimum estimate of the contrast enhancement achievable with LFB, as the parameters used were chosen to maximize overall contrast and not contrast of a specific region.

5.3.2 Electron microscopy

It was uncertain whether these formalin-fixed, en bloc LFB-stained tissue segments would yield informative data, as glutaraldehyde is the standard fixitive used for electron
microscopy (EM), and LFB's properties for EM have not been documented (although copper, as a metal, should be electron-dense).

The patency of some membranes was increased, and more artifactual holes were produced in the sample, but, overall, the cellular structure and geographic relations were preserved and recognizable by pathologists.

On the 1-μm slices, large crystalline structures were apparent throughout the peri-lesion sample (Fig. 5-10 a-b), and a pathologist noted they were larger than the typical linear inclusions seen in ALD. These crystal structures were present throughout the peri-lesion sample, occasionally seen in NAWM or lesion, near the peri-lesion border, and absent from cortex and from normal white and gray matter samples, identically prepared.

Comparison of the light micrograph (Fig. 5-10 a) and the electron microscopic image (Fig. 5-10 b) emphasizes the differences in scale and the dramatic increase in detail available with the electron-based contrast mechanism. The image in Fig. 5-10 c, a close-up of (b), just above the leftmost, shorter crystal, reveals degenerating myelin, some of which appears dotted. An even higher magnification from (c) appears in (d) and better illustrates these particulates, presumed to be LFB. To test the nature of these particulates, in Fig. 5-11, we compare a typical EM preparation of a myelinated, sural nerve, without prior treatment of LFB, and a myelin sheath that has been pre-stained with LFB. The high magnification images well illustrate the alternating, electron-dense electron-light structure of the myelin sheath, with a known periodicity of 12-15 nm [5, 82]. The major dense lines are formed by the apposition of the cytoplasmic surfaces of myelinating processes and represent the extracellular domain; whereas the clear canals of the intraperiod spaces are comprised of hydrophobic constituents and represent the intracellular domain [120].

The dark blebs in the image of the LFB-stained sample are much larger than any of the smaller spots in the unstained sample. Furthermore, the LFB particulates appear to colocalize with the major dense lines rather than the intraperiod spaces. This finding supports Pearse's theory of LFB binding to phospholipids within the myelin membrane [111] rather than Clasen's alternative hypothesis that LFB binds to non-polar amino acids within the hydrophobic domain [33].

As further evidence of LFB's specificity for the myelin membrane, inspection of an axon
5.3. RESULTS AND DISCUSSION

(a) Light microscopy, 10x, scale bar=250 μm.  
(b) Electron microscopy, 3,400x, scale bar=2 μm.  
(c) Electron microscopy, 34,000x, scale bar=500 nm.  
(d) Electron microscopy, 91,000x, scale bar=100 nm.

Figure 5-10: Large crystalline structures seen in actively demyelinating zone of one ALD sample on light microscopy (a) and electron microscopy (b-d).

whose sheath is loosely-wound (Fig. 5-12), reveals several spots confined to the majority of sheaths, and none marking the innermost axon, the axoplasm, or the cytoplasmic background. The outermost sheaths are unstained, and it should be investigated whether this incomplete staining is such from the beginning, after differentiation, or, if the EM preparation has dislodged some LFB from its attachment points.

Eliminating additional metals from the EM preparation protocol permitted further investigation of the electron-dense particulates. Myelin sheaths were still dotted with dark particulates without the lead and uranium treatments (Fig. 5-13). However, when the post-fixing with osmium tetroxide was also removed from the processing stream, very little was visible within the sample. These results suggest the copper within LFB is responsible for
the particulates and that osmium tetroxide is required for EM visualization. Osmium is a strong oxidant that cross-links lipids by reacting with unsaturated carbon-carbon bonds [74] and could serve as a mordant that improves the fastness of LFB to ethanol and enhances the particulate appearance on electron micrographs.

Next we searched the stage for the classic players involved in the inflammatory zone: macrophages and lymphocytes. Many ballooned macrophages were present, containing rough endoplasmic reticulum, mitochondria, lysosomes, and a large nucleus. In ALD, lipids, myelin debris, and linear, cytoplasmic inclusions are consistently found within macrophages [114, 131]. One such macrophage appears in Fig. 5-14, similar to the one seen in Fig. 10 of Schaumburg’s paper [131]. Myelinated, demyelinating, and bare axons fill the space, along with myelin debris, appearing as dark smudges, and slender spicules, which are thin membranes separated by an electron-lucent space [114].

Additional spicules and lipid droplets were located in other macrophages. Some spicules appeared to have a dark substance centrally, whereas in standard EM preparations without LFB, they are typically clear (Fig. 5-15 a). A comparable image of ALD spicules from a preparation that did not pre-stain with LFB is displayed in Fig. 5-15 b, courtesy of Dr. Dickinsin, MGH. These spicules do contain a translucent core; whereas those in (a) appear...
5.4 Conclusion

LFB staining highlights the microscopic structure of ALD samples and is able to identify active demyelination within subcortical white matter in ex vivo MRI. This sensitivity to myeloarchitecture is not seen with other magnetic resonance techniques and heretofore has only been demonstrable by histological methods. We demonstrate with LFB-MR mi-
croscopy a zone of macrophages and myelin debris at the leading edge of active demyelination. This area was especially prominent in T2*-weighted sequences whose contrast is dominated by susceptibility-based mechanisms. We conjecture this enhanced contrast results from two factors: compartmentalization of contrast agent and accumulation of VLCFA.

As described in Chapter 2, susceptibility contrast depends upon the size and shape of compartments containing the contrast agent, the diffusion rate of water through tissue, the susceptibility difference between the tissue and agent, and the specific pulse sequence parameters used.

Electron microscopy localized electron-dense particulates bound to the external lipid bilayers of myelin. These were presumed to be accumulations of LFB dye molecules, whose family of chemicals are prone to aggregation by van der Waals' attractive forces between their phthalocyanine rings [48]. In ALD and other neurolipidoses, intact myelin and myelin breakdown products accumulate within macrophages. Unique to ALD are spicules, or
5.4. CONCLUSION

Figure 5-14: Ultrastructure of a ballooned macrophage within ALD sample. Cytoplasm contains myelin debris and suggestive spicular structures (arrows), a hallmark of ALD [114, 131]. 4,500x, scale bar = 2 μm.

(a) EM with LFB staining. 34,000x, scale bar will = 2 μm.
(b) EM without LFB staining. 34,000x

Figure 5-15: Electron microscopy of spicules (lipid inclusions) in ALD, with (a) and without (b) LFB staining prior to EM preparation. (Figure (b) courtesy of Dr. Dickinsin, MGH)
cytoplasmic inclusions, which are thin, linear structures consisting of an electron-lucent core bounded by an electron-dense membrane [114]. Our electromicrographs were suggestive that LFB particulates may also bind to the spicular membranes, but higher resolution data is required to be conclusive.

To estimate how water spins sample their LFB-containing environment, we calculated the time required to diffuse a distance $R$: $R^2/D$ [12]. We measured an average particulate size of $10.2 \pm 0.1$ nm. When combined with estimates of water diffusion in formalin-fixed tissue, approximately $40 \times 10^{-7} cm^2/s$ [52, 157], this yields a $1/\tau_d$ of $D/R^2 = 3.8 \pm 0.07$ MHz. The field inhomogeneity produced by the paramagnetic particles of LFB was estimated as:

$$\delta \omega \approx \gamma \Delta B_{loc}(r) \approx (\pi H_o)\Delta \chi,$$

where $\gamma$ is the gyromagnetic ratio, $\Delta B_{loc}(r)$ is the volume average field produced around spheres of LFB with radius $r$, and $\Delta \chi$ is the susceptibility difference between pure water and water doped with agent [50]. The magnetic susceptibility of anhydrous copper(II) sulfate ($CuSO_4$) has been measured as $1330 \times 10^{-6} (cgs)$ [88]. When adjusted for the difference in molecular mass between ($CuSO_4$) and LFB, this value becomes $1330 \times 10^{-6} \times 778/159.6 = 6483 \times 10^{-6} (cgs)$. Thus, $\delta \omega = \gamma \Delta H \approx 12.2$ MHz at 14T, and the susceptibility interactions are defined by the intermediate gradient regime, as $1/\tau_D \approx \delta \omega$.

This regime lies between the motionally-narrowed regime, in which diffusion is fast compared to spatial variations in the perturbing field, and the linear gradient range, in which spins sample a small or homogenous region of perturbing fields. Closed-form solutions derived for $\Delta R_2$ in these regimes [49, 93] are not applicable; instead, numerical models are used to balance contributions from both static field variance and spin diffusion. Interestingly, this is also the regime applicable to much of the susceptibility contrast seen in vivo with the endogenous contrast agent, deoxyhemoglobin [154], which is also a porphyrin, like LFB.

Our measurements, which conclude a much larger change in $R2^*$ than $R2$, are consistent with observed behavior in this regime for small perturbers and relatively slow diffusion [20]. Furthermore, knowledge that a copper-core LFB produces intermediate-regime susceptibil-
ity has implications for selecting a paramagnetic metal substitution. While we originally expected a copper agent to produce significant effects only on longitudinal relaxation, the additional susceptibility contrast enhancement proved useful for visualizing LFB bound to myelin and myelin debris compartmentalized within macrophages. Although the copper agent is neither the most effective R1 nor R2 agent, it does provide two distinct forms of contrast. Replacing the copper with a stronger paramagnetic agent, such as manganese or iron, would substantially increase R1 effects, but may also increase $\delta \omega$ excessively, resulting in too much phase decoherence, especially at high field strengths where transverse relaxation rates are already quite large. Perhaps a good compromise would be a weaker lanthanide, such as dysprosium or terbium.

The second contributing factor to LFB-enhanced contrast is the presence of VLCFA and the resulting changes in membrane structure and LFB binding. A possible trigger of the demyelinating cascade is thought to involve the membrane accumulation of VLCFA [79], which diffuses freely into phospholipid bilayers and desorbs at a rate inversely proportional to carbon chain length [58]. Binding of the VLCFA has been shown to bring more order to the phospholipids on the myelin membrane, increasing their packing density and potentially altering binding properties [57]. As shown in Fig. 5-16, the VLCFA, drawn in red, may wedge between phospholipids in a straight or kinked fashion and become trapped within the bilayer.

Once positioned in the membrane, these VLCFA affect the dynamics of interactions by altering membrane properties and by themselves interacting with the external milieu.

Studies by Whitcomb on cultured human adrenocortical cells found addition of VLCFA increases the microviscosity of adrenocortical cell membranes [155]. Similar studies have not yet been conducted in neural membranes [99]; based on membrane work by Koenig, it is a logical conclusion that VLCFA-induced increases in membrane viscosity would change the motion of water within the bound monolayer, altering the interaction time scale that determines magnetic resonance relaxation rates [82]. Water-protein collisions do not have a large effect on the solvent tumbling rate [82]; however, the interaction of water with a more rigid surface, such as the VLCFA-adsorbed membrane, could slow rotational motion within the first few monolayers, as seen in studies of water-clay interfaces [156].
Figure 5-16: Schematic diagram representing possible binding modes of C26:0 molecules in a phospholipid bilayer. The acyl chains of the phospholipid (PC) have 16 (sn-1) and 18 (sn-2) carbons. The molecules could fit linearly and penetrate about halfway through the opposing phospholipid monolayer or could bend in the middle of the bilayer [58].
The dominant interaction is with the phosphatidylcholine and sphingomyelin subfractions of the glycerolipids [111, 129], found in the extracellular half of the myelin membrane [5]. The increase in VLCFA is non-uniform; phospholipids undergo the most pronounced changes, especially the phosphatidylcholine subfraction, in which hexacosanoic acid (C26) is elevated up to 39-fold [142]. Model membrane experiments have found the molecular environment of the head group of hexacosanoic acid in phosphatidylcholine vesicles to be identical to shorter chain acids (14-18 carbon chain length) [58]. This suggests VLCFA could provide additional binding sites for LFB. We are currently measuring the lipid fractions within the LFB-stained ALD tissue using a new isolation technique of tandem liquid chromatography (LC/MSMS) [61] and will correlate this with the LFB concentration and observed regional relaxation enhancement.

By probing the biophysical interactions of LFB across many length scales, from the macroscopic scale of MR imaging to the ultrastructural scales of electron microscopy and chemical bonding, our study demonstrates the significance of LFB in targeting macrophages and VLCFA accumulation. It is now possible to monitor substrate accumulation ex vivo as well as identify the site of active demyelination. These findings should be applied in future development of high resolution, target-selective in vivo investigations of cerebral demyelination.
Chapter 6

Summary of results and future work

“Never attempt to teach a pig to sing; It wastes your time and annoys the pig.”

- Robert Heinlein

A n understanding of how to best visualize changes within the nervous system using magnetic resonance (MR) imaging or microscopy requires fundamental knowledge of the normal and abnormal structures as well as the sensitivity of the tool. This thesis has focused on MR contrast enhancement to better visualize subcortical white matter and cortical myelinated fibers in normal ex vivo brains as well as in demyelinating disorders, specifically X-linked adrenoleukodystrophy (X-ALD).

6.1 Summary

The novelty of this work lies in the development of target-specific MR contrast agents. We have drawn from the arsenal of chemicals used by histopathologists, modifying protocols or combining paramagnetic metals with pre-existing light-microscopic stains to create a new class of contrast agents for ex vivo MR microscopy with a specific distribution.

In this thesis, we have
CHAPTER 6. SUMMARY OF RESULTS AND FUTURE WORK

1. Conceptualized the use of histological stains, with or without the attachment of additional paramagnetic or superparamagnetic metals, as magnetic resonance contrast agents.

2. Measured the relaxivities in solution for luxol fast blue (LFB) MBSN, a traditional white matter stain. They were calculated to be $0.15 \pm 0.01$ for $r_1$ at 4.7T, and $0.12 \pm 0.01$ for $r_1$ at 14T.

3. Developed, implemented, and validated a method for en bloc staining of fixed tissue blocks. This method retains the specificity of thin-slice preparations while enabling subsequent contrast-enhanced MR imaging. The development of this en bloc protocol was discussed specifically for LFB but is applicable for modifying any pre-existing histologic protocol for larger tissues.

4. Established LFB as a white-matter-specific MR contrast agent. Relaxation rates increased proportionally to myelination:

   (a) At 4.7T, the percentage increase in $R_1$ was 199% for WM, 129% for GMi, and 78% for GMo, and at 14T, equaled 30% for WM, 25% for GMi, and 5% for GMo.

   (b) At 4.7T, the percentage increase in $R_2$ was 8.4% for WM, 1.3% for GMi, and 0.5% for GMo, and at 14T, equaled 59% for WM, 42% for GMi, and 27% for GMo.

   Furthermore, contrast-to-noise values increased by $229 \pm 13\%$ at 4.7T and $269 \pm 25\%$ at 14T when compared to controls.

5. Acquired the first “true” T1-weighted ex vivo images, with hyperintense white matter and hypointense gray matter, using the LFB-MRM technique.

6. Visualized layers IVa, IVd, and Va in the human primary visual cortex using LFB-MRM. These lamina were not visible in comparable MR images of unstained samples.

7. Identified with electron microscopy dark particulates in intact and degenerating myelin sheaths of LFB-stained tissue. These particulates were not present on the axon, axo-
plasm, cytoplasm, or other cellular or background structures. Furthermore, particulates were absent in samples not stained with LFB.

Our experiments co-localized the LFB particulates with the major dense lines of the myelin membrane and not with the intraperiod lines. This binding location supports Pearse's theory of LFB binding to phospholipids within the myelin membrane [111] rather than Clasen's alternative hypothesis that LFB binds to non-polar amino acids within the hydrophobic domain [33].

8. Discriminated sites of active demyelination in X-ALD samples with LFB-MRM. This area was not distinguishable before staining and has an appearance consistent with Schaumburg’s actively-demyelinating zone: it is located between the NAWM and the region of total myelin loss, contains macrophages filled with lipid droplets, LFB-positive myelin debris, and spicules that appear enhanced on ultrastructural imaging after LFB staining.

This work provides the first circumstantial evidence of abnormal lipid accumulation within the external phospholipid bilayers in human X-ALD brain tissue. The known VLCFA accumulation in X-ALD, the binding properties of LFB, and the model membrane work of Ho and Hamilton [57, 58], suggests that LFB-MR is capable of visualizing VLCFAs within myelin membranes.

Applications of this work include advancement of current ex vivo investigations of fixed human or rodent tissue. "True" T1-weighted images or enhanced susceptibility-weighted images may be used to construct three-dimensional MR Brodmann maps that can be projected into a common space and registered for comparison with any structural or functional imaging scan. In addition, contrast-enhanced ex vivo MR can be used to illuminate the pathophysiology, progression, and treatment efficacy of disorders of the nervous system and beyond. Our method allows specific detection and quantification of substrates, items that are currently limited in histology. This carries great importance for storage disorders and their animal models that are under present investigation.

This work provides an impetus to synthesize additional target-specific MR agents that sensitize MR microscopy to small population changes of neuronal constituents. In this
manner, ex vivo MR microscopy can identify targets to monitor in vivo and assist with the design and testing of future in vivo MR contrast agents.

6.2 Future work

6.2.1 LFB

As suggested in previous chapters, future work with LFB-MR methods include the following:

1. Investigate metal substitutions for copper, being cognizant of tradeoffs between dipolar and susceptibility enhancements;

2. Determine sample size limit for current en bloc staining method and modify to enable staining of hemispheres;

3. Complete lipid analysis of ALD sample, correlating with LFB concentration and relaxation rate increases;

4. Investigate LFB binding in model membranes containing fatty acids of various chain lengths;

5. Perform additional, higher magnification EM to view 1) spicules in ALD tissue, with and without LFB staining, and 2) formalin-fixed ALD brain without LFB treatment;

6. Perfuse-fix lipid-injected mice with LFB to visualize lipid distribution and microstructural changes.

6.2.2 Gadolinium-Thionine

Exciting work is in progress on gadolinium-thionine (GdT), a neuron-specific MR contrast agent that would complement LFB’s white matter specificity. This agent is the marriage of thionine, a popular Nissl stain, and gadolinium-tetraazacyclododecanetetraacetic acid (Gd-DOTA). GdT is patent-pending, and unlike any existing MR contrast agent, it is designed to have a regional distribution in proportion to neuronal density. Applications of
6.2. FUTURE WORK

Thionine (C.I. 52000; M.W. 264)

(a) Thionine, a Nissl stain [74]. (b) Gadolinium-thionine

Figure 6-1: Chemical structures for thionine (a) and gadolinium-thionine (b).

this agent in ex vivo neuroimaging are numerous: characterization of the cortical lamina most affected by normal aging and neurodegenerative disorders, such as Alzheimer’s Disease and schizophrenia, as well as investigation of the progression, pathophysiology, and treatment efficacy of these diseases.

Binding specificity

Thionine\(^1\) is a thiazine dye used for Nissl stains, which highlight neuronal cell bodies and beginnings of dendrites. The dye binds to the endoplasmic reticulum of cells, namely the acid groups in ribonucleic acid [74]. The structure of thionine is shown in Fig. 6-1 a. Thionines specificity is pH-dependent; standard histological protocols use 0.1% w/v solutions at a pH of 4.

Synthesis of GdT

Many synthesis pathways have been attempted to create an MR-visible thionine. Two are described below: the first with extremely low yield (1%) and questionable gadolinium attachment; the second with a larger yield (10-25%) and a more certain gadolinium linkage.

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\(^1\)A footnote about spelling (personal communication from John Kiernan): modern German writers on dye chemistry [159] use the convention of -ine for bases (amines), -ein for compounds in which unsaturation is significant, and -in for uncharged or anionic dyes. Haematoxylin and haematein are uncharged (but can still form anions). Eosin and phloxin are also anionic dyes, so it is wrong to put an -e on the end of either. Thionine is a basic (cationic) dye, so it should never be spelled as “thionin”!
GdT, version 1

The synthesis pathway for Gd-thionine appears in Fig. 6-2. To make thionine MR-visible, the dye was first attached to a chelate and then gadolinium chloride was added. Specifically, 4,7,10-Tris-tert-butoxy-carbonylmethyl-1,4,7,10-tetraaza -cyclooctadec-1-yl)-acetic acid (DOTA) was treated with excess dicyclohexylcarbodiamide (DCC) and N25 hydroxysuccinimide (NHS) in dimethylformamide (DMF) in the presence of diisopropylethyl amine (DIEA) followed by the addition of thionine. The reaction mixture was stirred at room temperature for one day, filtered, and DMF was removed to complete dryness. The residue was treated with gadolinium chloride hexahydrate after Boc group deprotection in TFA for one day to afford the product. Mass spectroscopy was performed (MALDI-TOF MS) (M+H)+; the chemical formula, C\textsubscript{28}H\textsubscript{33}GdN\textsubscript{7}O\textsubscript{7}S, predicts a molecular weight of 769.92 and 769.17 was measured.

![Figure 6-2: Synthesis for first version of Gd-thionine.](image)

The initial synthesis of Gd-thionine produced a very low yield (1%) and required additional techniques to estimate the dyes concentration. Inductively-coupled plasma (ICP) methods measured Gd content and estimated a concentration of 0.032 mM.

Optical methods were also used to deduce thionine content. First, the molar absorptivity for thionine was measured to be 49,860 cm\textsuperscript{-1}, in agreement with published values [54]. Similarities in optical properties of the dyes suggest an identical molar absorptivity for Gd-thionine. Using this value, the initial concentration of Gd-thionine was calculated as 0.48 mM via Beers Law. This disagreement in concentration estimates suggested a higher
amount of free thionine in the dye.

Four optically-determined concentrations of Gd-thionine (0.5, 17.4, 24.3, and 182.7 mM at 4.7T; 60, 120, 240, and 480 mM at 14T) were used to determine a longitudinal relaxivity ($r_1$) of 0.27 s$^{-1}$/mM at 4.7T and 0.3 s$^{-1}$/mM at 14T. Published values for Gd-DOTA $r_1$ are around 3 s$^{-1}$/mM at 4.7T. Using the ICP-determined concentrations would produce relaxivities 15 times larger.

Slices of rat hippocampus 400 micron in thickness were immersed in 0.48 mM or 0.02 mM aqueous solutions of Gd-thionine or 0.1% w/v thionine (0.005 mM) for 48 hours and differentiated in ddH2O for 6 hours. The appearance of the GdT-stained tissues in Figure 6-3 b-c appear very similar to tissue stained with thionine in Fig. 6-3 a. The darkest region in all preparations is the dentate gyrus, which contains the densest region of neurons. Also, the innermost region of white matter remains unstained in the lower GdT concentration. Despite this good distribution in tissue and apparent specificity, the uncertainty in gadolinium attachment and extremely low yield suggested trying a new synthesis pathway.

![Image](image-url)

Figure 6-3: The dentate gyrus in a 400-micron slice of hippocampus is heavily stained with both thionine and Gd-thionine (version 1). (a) 0.005 mM thionine, (b) 0.02 mM Gd-thionine, and (c) 0.48 mM Gd-thionine (showing a close-up of the dentate gyrus).

**GdT, version 2**

Thionine was first dissolved in DMF and dichloromethane (DCM). Triethylamine was added to increase the pH. Next, alpha-amino-gamma-propinoic acid was dissolved in solvent
and water-soluble DCC was added. Deprotection was performed with a 50% solution of TFA (diluted with DCM). DOTA was treated with excess DCC and N25 NHS in DCM and dimethylformamide (DMF) to couple with an amino group. Next, Boc group deprotection of the tertibutyl group was performed in 50% TFA in DCM. Gadolinium chloride in glycine buffer was then added to the reaction mixture and was stirred at room. All intermediate products were purified by column chromatography, and the final step was purified by high performance liquid chromatography (HPLC). Mass spectroscopy was performed (MALDI-TOF MS) (M+H)+; the chemical formula, $C_{32}H_{40}GdN_8O_8S$, predicts a molecular weight of 854.19 and 854.02 was measured. The chemical structure of the final product appears in Fig. 6-1 b.

**Relaxivity measurements**

Five concentrations of gadolinium-thionine (GdT) were used to measure the agents longitudinal and transverse relaxivities at a field strength of 14 Tesla. Inversion-recovery prepared spin-echo, multi-echo spin echo, and gradient recalled echo sequences were used to determine an $r_1$ of $0.87 \pm 0.05$ s$^{-1}$/mM, an $r_2$ of $1.02 \pm 0.03$ s$^{-1}$/mM, and an $r_2^*$ of $0.98 \pm 0.12$ s$^{-1}$/mM in solution; data is shown in Figs. 6-4 and 6-5. These values are closer to the observed relaxivities in solution and imply the correct proportion of gadolinium is present, unlike in the first version of GdT. Additional ICP measurements should be performed to validate the gadolinium concentration.

**Optical properties**

Lambda max ($\lambda_{max}$)was measured with a spectrophotometer for each newly-synthesized dye and compared to thionine. $\lambda_{max}$ was found to be 598 nm for thionine, in agreement with published literature [54]. Our first version of Gd-thionine (GdT1) had an identical $\lambda_{max}$, but the second version (GdT2) decreased to 250 nm. Next, emission spectra were compared using a spectrofluorometer, exciting at $\lambda_{max}$. Results, shown in Fig. 6-6 are nearly identical for thionine and GdT1, but significantly different from GdT2. This result is also suggestive of the successful binding of gadolinium and thionine.
6.2.3 Future work

Now that the properties of this novel neuronal contrast agent have been elucidated, the appropriate concentration for staining tissues must be determined. Once that information is known, tissue blocks may be stained following the standard en bloc staining protocol described in Chapter 2, using deionized, distilled water as a differentiator. Next, changes in tissue relaxation rate before and after staining should be measured.

Finally, MR images should be correlated to histology and the neuronal distribution quantified in normal brains as well as in pathological states, such as Alzheimer's Disease or other neurodegenerative disorders.
Figure 6-4: Five concentrations of gadolinium-thionine (GdT). Multi-echo spin echo sequence, used for r2 calculation at 14T. (TR/TE=1000/47.8 ms).

Figure 6-5: Relaxivity measurements of gadolinium-thionine (GdT) at 14 Tesla.

(a) r1 measurement.  (b) r2 measurement.  (c) r2* measurement.
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Figure 6-6: Comparison in emission spectra, exciting at $\lambda_{\text{max}}$ for a) original thionine dye ($\lambda_{\text{max}}=598$ nm), b) Gd-thionine, version 1 ($\lambda_{\text{max}}=598$ nm), and c) Gd-thionine, version 2 ($\lambda_{\text{max}}=250$ nm).
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