Detection of Iron in Biological Samples Using Magnetic Force Microscopy

THESIS

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By

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Abstract

Iron is crucial for several physiological processes in the human body. Despite its many benefits, if not shielded from surrounding cellular components, iron can cause significant damage. Ferritin, the main iron storage protein, can contain up to 4500 iron atoms in its 8 nm core. Dysregulation of iron in pathological tissues is typically characterized using histological techniques such as Perl’s iron staining or immunohistochemistry (IHC) for ferritin. While these methods successfully detect ferritin, they offer a low spatial resolution and a frequently observed mismatch between ferritin IHC and iron stain. In this study, magnetic force microscopy (MFM) is proposed as a histological technique that allows for the detection of iron at higher spatial resolution (<200 nm) by using the magnetic properties of the ferritin iron core.

MFM is an atomic force microscopy (AFM) based technique available on most commercial AFM equipment. In MFM, a magnetically coated AFM probe is used to scan the sample surface at a user defined lift height to detect the long range magnetic forces. This mode of MFM, also called direct MFM (D-MFM), was used to detect iron in rodent tissue. Perl’s staining allowed for mapping of iron rich or iron poor regions on adjacent tissue sections for MFM imaging. Transmission electron microscopy (TEM) of the tissue confirmed the presence of iron in the sections. D-MFM was able to detect the densely packed ferritin in the lysosomes, but not the ferritin dispersed throughout the cytoplasm. The MFM signal was also found to be dependent on the tissue fixation method.
The requirement to scan samples at multiple lift heights in D-MFM is time consuming and increases the probability of contaminating the sample or the probe. As an alternative to D-MFM, we propose indirect magnetic force microscopy (ID-MFM). ID-MFM uses an ultra-thin membrane to create a barrier between the sample and the probe, thus reducing the risk of contamination and eliminating scans at multiple lift heights. ID-MFM was used to scan in-vitro ferritin samples. However, the magnetic signal of ferritin was not detected due to the effect of the ultra-thin membrane shielding the magnetic signal, a low density of immobilized ferritin, and a significant variation in iron content of the in-vitro ferritin cores. We propose that ID-MFM would be compatible with the strong magnetic signal observed from iron-rich lysosomes in tissue sections.
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Finally, I would like to thank my parents, Mark and Caryn Ollander. There are no words to describe how much their support, advice, and encouragement mean to me.
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Chapter 1: Introduction

1.1 Storage of Iron in the Human Body

Iron (Fe) is an essential metal involved in a variety of physiological functions in the human body. The proper storage of iron in the body is important for two main reasons: 1) to prevent toxic interactions of iron with other components in the blood/cells and 2) to control the release of iron as the body requires it.

Iron in the body is either protein bound or non-protein bound. Non-protein bound iron (free iron) is found as an intracellular labile iron pool (LIP). In addition, free iron can be circulating in the plasma as low molecular weight complexes known as non-transferrin-bound iron (NTBI). High levels of LIP and NTBI are known to be harmful and associated with cellular damage. Excessive amounts of free iron are dangerous to cellular components given their propensity to create free radicals such as the formation of the hydroxyl radical (‘OH) from the superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Large quantities of free iron can cause organ failure, arthropathy, or endocrine dysfunction.

The proteins responsible for storage of iron are ferritin, transferrin, and ferroportin. Ferritin is the major iron-storage protein found in living organisms including animals, plants, and bacteria. It consists of a ~12 nm diameter protein shell with 24 subunits and can store up to 4500 iron atoms in its 8 nm core (Figure 1.1).
Apo ferritin is the protein shell of ferritin depleted of iron. The formation of ferritin occurs by uptake of Fe(II) by the apoferritin shell. Apoferritin provides locations where initial oxidation can occur, thereby accelerating the formation of ferrihydrite.\textsuperscript{4} There are 11 binding sites for metals in the apoferritin molecule.\textsuperscript{4} Once ferrihydrite binds, it will compete with other sites to store iron atoms. This results in the formation of ferrihydrite crystals within the apoferritin shell resulting in the formation of ferritin. Ferritin is dispersed in the cytoplasm or aggregated in lysosomes inside the cell macrophages.

**Figure 1.1: Schematic of Ferritin.** A ferritin molecule consists of a 12 nm protein shell and an 8 nm iron filled core.

Ferritin is known to exhibit variations in size, shape, and content of its core.\textsuperscript{5} This variation can be observed in different tissue types, as well as within the same type of tissue. Additionally, variation in its protein subunits (H vs. L) is found across tissues. The H subunit is found in greater quantities in the heart, while the L subunit is found in greater
quantities in the liver and spleen. L subunits are involved with the formation of the iron core, and H subunits are integral to the oxidation of Fe(II) to Fe(III). Ferritin units that have a greater quantity of H subunits have a lower average iron content with fewer than 1000 iron atoms/molecule. Alternatively, when there is a larger quantity of L subunits, the ferritin core will have a higher average iron content (1500 iron atoms/molecule).

Ferritin releases its iron stores with the help of a chelator. Chelators, such as ascorbic acid, either interact with iron on the outer surface of ferritin or they enter the ferritin molecule to break the bonds within the core. All the chelators known to be involved in the release of ferritin do so by entering the molecule and interacting with the core.

Other proteins responsible for iron storage and transport include transferrin and ferroportin. Transferrin is a protein which can bind up to two iron atoms. It is comprised of 679 amino acids divided into two lobes (N-lobe and C-lobe), with two domains per lobe. Each lobe has one iron binding site. The primary function of transferrin is to transport iron throughout the body. Its ability to bind and release iron is influenced by many factors including the pH and temperature. For two iron atoms to bind with transferrin, bicarbonate must be present and the pH needs to be between 7.5 – 10. When iron is released, the two domains of each lobe rotate to change the conformation of the protein from “closed” to “open”.

Ferroportin transports elemental iron between tissues. It is comprised of 571 amino acids with several transmembrane segments. It is associated with tissues that are involved in iron transport such as the macrophages in the red pulp of the spleen. However, several
features of ferroportin are still unknown including how it controls iron transport, the location of the iron binding sites, and its structure.\textsuperscript{11,12}

1.2 Dysregulation of Iron

The dysregulation of iron is associated with several cardiovascular and neurodegenerative diseases. Increased iron deposition is observed in the tissue of patients with hereditary hemochromatosis,\textsuperscript{3} abdominal aortic aneurysm,\textsuperscript{13} multiple sclerosis,\textsuperscript{14} Parkinson’s disease,\textsuperscript{15,16} and Alzheimer’s disease.\textsuperscript{17} Iron is also involved in injury and wound healing. Following a traumatic injury to the spinal cord, the amount of free iron rapidly increases in the first 20 to 30 minutes.\textsuperscript{18,2} Quantifying the spatial location, its ligation state (protein bound vs free iron), and oxidation state of iron in tissues is critical to understanding the pathogenesis of chronic diseases and acute injuries.

1.3 Biochemical Characterization of Tissue Iron

Iron is detected in tissue sections through the utilization of histological stains such as immunohistochemistry (IHC) or through histochemical stains including Perl’s or Turnbull. Ferric iron (Fe\textsuperscript{3+}) in the tissue sections interacts with the potassium ferrocyanide solution in the Perl’s stain producing a blue pigment. Perl’s stain has been used in several studies to detect ferric iron.\textsuperscript{19,20,21} Similarly, ferrous iron (Fe\textsuperscript{2+}) has been stained in tissue sections using a potassium ferricyanide solution in the Turnbull’s stain.\textsuperscript{22,23,24}

Ultrastructural techniques, like transmission electron microscopy (TEM), can improve the detail and resolution of iron detection in tissue. Ferritin cores have been
studied using TEM in several pathological conditions, including Alzheimer’s and Parkinson’s diseases. However, TEM is not routinely used to characterize iron in histological sections due to the stringent sample preparation that is required.

1.4 Magnetic Characterization of Tissue Iron

Ferritin iron can be detected in tissue sections by utilizing its magnetic properties. The magnetic properties of the ferritin iron core have been characterized in both in-vitro and in-vivo studies. These nanoscale iron deposits are understood to be primarily superparamagnetic in nature with an antiferromagnetic sub-lattice arrangement.

Superparamagnetism is the phenomena that occurs when a ferromagnetic material only has a single domain due to its particle size (typically less than 300 Å in diameter). These materials do not display a net magnetization in the absence of an external magnetic field but achieve a high magnetic moment characteristic of ferromagnetic materials in the presence of a field. Superparamagnetic particles have magnetic anisotropy, a preferred orientation of their magnetic moments. There are two orientations for the magnetic moments that are aligned in an antiparallel configuration. At temperatures above the Néel relaxation temperature, the magnetic moments will overcome the energy barrier resulting in a change in their direction. The Néel relaxation time (\(T_N\)) is the average amount of time between changes in the direction of the magnetic moment and is determined by

\[
T_N = T_0 e^{\frac{KV}{k_B T}}
\]

where \(T_0\) is the attempt time and is a characteristic of the material, \(K\) is the magnetic anisotropy, \(V\) is the volume, \(K_B\) is the Boltzmann constant, and \(T\) is the temperature.
Antiferromagnetic materials are magnetically ordered. They have a net magnetic moment of zero. Neighboring magnetic moments within the material are equal but opposite in direction. Above the Neel relaxation temperature, antiferromagnetic materials will be paramagnetic in nature. Antiferromagnetic materials are typically transition metals such as chromium and manganese oxide.

1.4.1 Superconducting Quantum Interference Devices

Superconducting Quantum Interference Devices (SQUID) are magnetometers that use superconductors and Josephson junctions to detect small magnetic fields. SQUID studies of ferritin have shown significant variations in the size of the ferritin core, which directly affect the magnetic properties.\(^{28}\) At 310 K, a magnetic susceptibility of \(3.81 \times 10^{-6} \text{ m}^3/\text{kg Fe}\) was observed.\(^{29}\) SQUID results also suggested that the ferritin core was comprised of two parts: an ordered interior with superparamagnetic moments surrounded by a disordered core surface.\(^{29}\) The interior portion could have a Neel relaxation temperature greater than 37°C. Changes in temperature would result in changes in the sizes of the core layers.

In-situ characterization of iron in tissues has also been accomplished using SQUID magnetometry. Kirschvink et al. examined magnetic material in human brain samples acquired postmortem from patients, with Alzheimer’s disease and from healthy controls.\(^{30}\) The magnetic particles in the brain tissue were homogenous and diffusely spread across all the lobes in both healthy and diseased tissues. However, a significant difference in the magnetization between the different cortical regions of the brain tissue was not found.
In a more recent study, SQUID was used to investigated the concentrations of magnetite/maghemite and ferritin in formalin fixed brain tissue extracted from healthy and Alzheimer’s patients.\textsuperscript{31} Specifically, the middle temporal gyrus of the temporal lobe was chosen since it has been suggested that it has a higher iron content than other regions of the brain.\textsuperscript{31} At 100 K, the concentration of magnetite/maghemite was 189.5 ng/g and 277 ng/g for the Alzheimer’s and healthy tissue respectively. Although SQUID studies enable magnetism based detection of tissue iron, they are limited to a spatial resolution of several centimeters and fail to provide a high resolution spatial map.

1.4.2 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) provides a noninvasive option for iron detection. Several improved MRI approaches have been employed to detect iron levels including the use of ultra-high magnetic fields (7T) enabling a spatial resolution of a few millimeters. The increased iron accumulation in the basal ganglia that results in multiple sclerosis has been detected using 7T MRI.\textsuperscript{32} Also, 7T MRI has quantified the iron rich macrophages surrounding multiple sclerosis plaques.\textsuperscript{32}

Additionally, MRI based approaches have employed the signal intensity ratio (SIR) to accurately and noninvasively measure iron levels in liver. The intensity of the signal from the liver was compared to the signal from skeletal muscle.\textsuperscript{33} Skeletal muscle had a lower signal intensity than the liver which made it ideal for determining subtle changes in the liver signal.\textsuperscript{33} Additionally, T2 relaxometry studies have been shown to accurately quantify iron concentrations in the liver. Liver iron concentration (LIC) studies have indicated a correlation between a standard liver biopsy and T2 relaxation measurements.\textsuperscript{34}
A MRI based technique, SWeep Imaging with Fourier Transform (SWIFT) pulse sequence, has been used to quantify iron. It is ideal for tissue samples that have a short T2 relaxation time because of the simultaneous RF excitation and signal acquisition.35 SWIFT MRI causes the production of a hyper-intense signal from regions, such as bone, which have low signal intensity.35 The increase in signal intensity allows for the quantification of iron.

Other emerging techniques to characterize tissue iron deposits based on their magnetic properties include Mössbauer spectroscopy,36 electron paramagnetic resonance (EPR),31 and analytical TEM.37 Despite these advances, the biochemical and magnetic characterization of iron deposits require distinctly difference sample handling and preparation methods and cannot be obtained from the same regions. In this work, magnetic force microscopy is proposed as a novel histological tool which can bridge the biochemical and magnetic approaches to map iron deposits in tissue sections at nanoscale resolution.

1.5 Magnetic Force Microscopy

Direct magnetic force microscopy (D-MFM) is an atomic force microscopy (AFM) based technique. Using a probe with a magnetic coating, the MFM tip scans a sample in two passes (Figure 1.2). The first pass collects the traditional AFM topography data and the second pass is at a user defined lift height to detect the long range magnetic signals from a sample. A lift height is the distance the probe is from the sample surface. D-MFM requires direct contact with the sample surface in the first pass and the collection of images at multiple lift heights.
Given its proven ability to detect the magnetic characteristics of solid state devices and magnetic nanoparticles,\textsuperscript{38} D-MFM has been explored as a technique to detect the magnetic signature of biological materials, specifically ferritin. Previous studies have shown that D-MFM can detect isolated ferritin molecules in-vitro.\textsuperscript{39,40,41} However, very few studies exist on the use of D-MFM in mapping iron deposits in tissues.\textsuperscript{42,43}

Indirect magnetic force microscopy (ID-MFM) is a novel technique that can improve upon the limitations of D-MFM. An ultra-thin membrane is used to create a barrier between the sample and the tip (Figure 1.3). This barrier eliminates the need to repeat scans at multiple lift heights and reduces tip/sample contamination. We hypothesize that the long range magnetic forces between the tip and the sample will be detected through the membrane.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{D-MFM_Schematic.png}
\caption{D-MFM Schematic. The probe makes two passes over the sample. The first pass comes in contact with the sample and the second pass is scanned at a user defined lift height.}
\end{figure}
1.6 Scope of Thesis

The goal of this work was to explore MFM as a histological tool to detect, map, and quantify iron in biological tissue samples via the following objectives:

1. Employ D-MFM to map iron in physiological tissue sections and compare the results with proven iron characterization methods such as Perl’s stain and TEM.

2. Evaluate the effect of fixatives on D-MFM signal.

3. Develop an indirect magnetic force microscopy (ID-MFM) technique to detect ferritin in-vitro.
Chapter 2: Materials and Methods

2.1 Animals

A rodent model of spinal cord injury (SCI) was used for direct MFM (D-MFM) studies. The SCI model has been described previously. 44 Adult male Sprague Dawley rats (n=5) were anesthetized using 80 mg/kg ketamine and 10 mg/kg xylazine. They were transcardially perfused with distilled water and subsequently fixed in a 250 ml primary fixative consisting of 4% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer. Studies were carried out on spleen, liver, and spinal cord extracted from naïve or injured rats. In another set of experiments, the animals were perfused with 4% paraformaldehyde and the extracted tissue was immersion fixed in 4% paraformaldehyde for 2 hours.

2.2 Perl’s Stain

The extracted tissue was segmented into 3-5 mm pieces and embedded in Optimal Cutting Temperature (OCT) compound. They were “snap frozen” in liquid nitrogen and stored at -80°C until they were cryo-sectioned. The tissue was cut to 10 µm thick sections and collected on Superfrost Plus microscope slides (Fisher Scientific). OCT compound was removed by first rinsing the sections in distilled water three times, followed by several rinses in 0.1 M phosphate buffered saline (PBS), and incubated in 25% hydrogen peroxide in methanol for 15 minutes. The tissue was then rinsed several times in PBS, incubated in 0.1% Triton X-100 in PBS for 10 minutes, and then rinsed. The samples were stained using a 2% potassium ferrocyanide solution in 2% hydrochloric acid and incubated for 30
minutes. Samples were washed with water, dehydrated in an ethanol series, cleared with xylene, and cover-slipped with Permaslip (Alban Scientific). An Axioplan 2 (Zeiss) microscope with a AxioCam color camera (Zeiss) was used to image the slides.

2.3 Transmission Electron Microscopy

Extracted tissue was immersion-fixed in 2% glutaraldehyde overnight and post-stained for 1 hour in 1% osmium tetroxide (Electron Microscopy Sciences). A graded ethanol dehydration series (30-100%) was used to dehydrate the tissue. An acetone transition solvent followed by an infiltration series in Spurr’s resin (Electron Microscopy Sciences #14300) was applied to the tissue. Polymerization of samples was completed in a 65°C oven. The resin blocks were then thick sectioned at 750 µm and stained with Methylene Blue-Azure II and Basic Fuchsin stain. Regions of interest in these samples were determined and sections were cut to 40 nm thin sections on an ultramicrotome (Leica Ultracut, Leica-microsystems). The sections were collected on 200 mesh copper grids. A JEM-1400 TEM (JEOL Ltd.) at 80 kV was used to image the samples. The TEM was equipped with a Veleta digital camera (Olympus Soft Imaging Solutions, GmbH). Energy dispersive spectroscopy (EDS) was also used to image these tissue sections. For EDS, a Tecnai F20 field emission 200 kV TEM/scanning-TEM (STEM) and X-Twin lens (FEI) equipped with an EDAX XLT windowless silicon drift detector (SDD) was used.

2.4 Direct Magnetic Force Microscopy

The extracted tissue embedded in OCT, as described above, was cut to 10 µm thick tissue sections and collected on polylysine coated glass coverslips (0.17-0.25 mm thick).
To remove OCT, the tissue sections were washed three times with PBS, two times with ultrapure water, incubated in ultrapure water for one hour, washed two more times with ultrapure water, and air dried overnight.

To evaluate the effect of different sample preparation protocols on MFM signal, a subset of tissue sections was (1) immersion fixed in paraformaldehyde, embedded in OCT, and thick sectioned or (2) immersion fixed in glutaraldehyde, embedded in resin for TEM, and thin sectioned. The tissue sections were then imaged using direct MFM.

The polylysine glass coverslips, with tissue sections, were attached to magnetic stubs and secured to the JV scanner of a Multimode AFM equipped with a Nanoscope IIIa controller (Bruker). The magnetic field at the base of the scanner was approximately 2500 Gauss. The reflected light module of the AFM allows for the visualization of the tissue sections. The Perl’s stained images were used as maps to determine iron rich areas of interest.

High moment MFM probes (ASYMFMHM, Asylum Research) were magnetized prior to imaging using an external magnet (900 Gauss) for 5 minutes. High moment probes and standard moment probes (NSC18, MikroMasch) were tested using a magnetic storage disc sample. As shown in Figure 2.1, the high moment probes have a significantly larger MFM phase signal than the standard moment probes which can help increase the MFM force sensitivity.

The AFM was operated in the tapping mode using a drive frequency of 70 kHz for high moment probes. Height, amplitude, and phase images were recorded for each identified region of interest at lift heights of 0, 30, 40, and 50 nm. Images were recorded
with 512 lines per scan direction using scan speeds of 2 to 3 Hz and scan sizes ranging from 5 to 20 µm.

**Figure 2.1: High Moment and Standard MFM Probe.** The high moment probes shown a significantly higher phase signal than the standard moment probes. The high moment probes also resulted in distortion of the magnetic signal.\(^{48}\)
2.5 Indirect Magnetic Force Microscopy (ID-MFM)

Commercially available pure silicon, silicon dioxide, and silicon nitride TEM windows and x-ray diffraction windows were tested for their compatibility with ID-MFM. A comprehensive list of the commercially available non-porous windows used is shown in Table 2.1. Silicon nitride is commonly used as a wear resistant material given its strong mechanical properties.\textsuperscript{45} It is also dense which prevents the diffusion of materials through the film.\textsuperscript{45} Previous studies have shown how silicon nitride can be used as a 7 nm thick protective layer for magnetic materials without adversely affecting the magnetic characteristics.\textsuperscript{45,46} In addition, silicon nitride is transparent to light and electron optics and can enable multimodal imaging and manipulation of samples.\textsuperscript{47} For these reasons, silicon nitride membranes were used for ID-MFM.

Ensuring that the membrane remains intact was critical for the ID-MFM experiments. The TEM membranes are shipped and stored in the “upright” orientation, in which Side A is facing up. To apply a sample to the membrane, membranes were suspended in air using reverse action forceps and flipped to the “upside down” orientation as shown in Figure 2.2. Then, a 1-2 µL drop of sample was deposited on the membrane (Side B) and allowed to air dry. Once samples were dry, the membrane was repositioned in the “upright” orientation and secured to a metallic stub using double sided tape. The membranes were imaged on Side A, as shown in Figure 1.3.

The stubs were secured to the JV scanner of the AFM operated with a Nanoscope IIIa controller (Bruker). The reflected light module of the AFM allows for the visualization of the windows. High moment MFM probes (ASYMFMHM, Asylum Research) were magnetized prior to imaging using an external magnet for 5 minutes. The AFM was
operated in the tapping mode. Height, amplitude, and phase images were recorded for regions of interest. 512 lines per direction were recorded at scan speeds of 2 to 3 Hz. The scan sizes were between 5 and 20 µm. As a control, non-magnetic AFM probes were also used to scan samples. Following the completion of the MFM studies, the membranes were imaged with a JEM-1400 TEM (JEOL Ltd.). The sample used for ID-MFM was purified horse spleen ferritin (Sigma-Aldrich).

<table>
<thead>
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<th>Company</th>
<th>Product Number</th>
<th>Membrane Material</th>
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Table 2.1: Commercially Available TEM Windows. Membranes from multiple manufacturers were tested for their compatibility with ID-MFM. A variety of membrane materials, thicknesses, and sizes were used.
2.6 Image Analysis

Image J (NIH) was used to analyze the light and electron microscopy images. A threshold was set for the Perl’s stained images to select for regions showing a strong iron stain. These regions were hand traced to determine their area using the particle analysis feature of ImageJ. The lysosomes in TEM images were hand traced to determine their
area. The intensity of the lysosomes was also measured by determining the average pixel value (0-255) in those regions.

The D-MFM images were exported from the Nanoscope software as tif files. The regions showing a phase shift (negative or positive) were hand traced using ImageJ to determine their area. The magnitude of the phase shift was measured in the same regions using the section analysis feature of the Nanoscope software. In addition, this software was used to measure the roughness (variation in phase across a region of interest) for areas with a negative phase. A student’s two-tailed paired t-test was used to determine the significance of the phase and area at 30, 40, and 50 nm lift heights. A significant p-value was <0.05 across the lift heights.

The ID-MFM images were also exported from the Nanoscope software as tif files. Any regions with a negative phase shift were analyzed. ImageJ was used to hand trace the area and the section analysis feature on the Nanoscope software was used to determine the phase of the same region.
3.1 Detection of Iron in Rodent Tissue Using Direct MFM

3.1.1 Characterization of Iron Using Histological Techniques

Perl’s staining was done on OCT embedded spleen tissue for naïve rats. These images were used as a map to identify iron rich regions when imaging adjacent sections with MFM. The Perl’s staining and analysis of the naïve spleen are shown in Figure 3.1. Regions with a large iron content appear blue in the images. High magnification images were set to a threshold to determine the frequency of high intensity regions (Figure 3.1 B and C). The majority of the dark stained regions were 20 to 40 µm² in area (Figure 3.1 D).
Unstained naïve spleen tissue was also imaged with TEM. Electron dense ferritin iron cores could easily be identified in the TEM images. Lysosomes within a macrophage consisted of aggregates of ferritin (Figure 3.2 A). The surrounding cytoplasm consisted of a monodisperse population of ferritin (Figure 3.2 B). The density of iron content varied between lysosomes (Figure 3.2 C). To quantify the iron content in the lysosomes, arbitrary pixel units were used to measure their pixel intensity. Approximately 75% of the measured lysosomes had a pixel intensity value less than 35 which corresponds to a high iron density.

**Figure 3.1: Perl's Staining of Naïve Spleen Tissue.** Iron rich and iron poor regions are detected. The area of the majority of iron rich regions was between 20 and 40 µm². 48
Also, about 70% of the lysosomes measured were less than 0.2 \( \mu m^2 \) in area. Lysosomes that had a relatively small area were typically more iron dense.

To confirm the presence and relative density of iron in the unstained spleen tissue, Energy Dispersive X-ray Spectroscopy (EDX) was used. The characteristic x-rays peaks of iron (\( K_\alpha = 6.4 \) keV and \( K_\beta = 7.05 \) keV) were detected in both the cytoplasm and the lysosome (Figure 3.3). The lysosomes had higher iron peaks than the cytoplasm, which was consistent with the higher density of particles observed in TEM images and analysis.

**Figure 3.2: TEM of Naïve Spleen Tissue.** Low magnification TEM images of unstained naïve spleen tissue showed the lysosomes within in the macrophage (A). There was a difference in ferritin density between the cytoplasm and the lysosomes (B) and variation in iron density across lysosomes (C). A majority of the lysosomes were less than 0.2 \( \mu m^2 \) in area and were iron dense (D).48
Figure 3.3: EDX of Naïve Spleen. Data was recorded in a lysosome (A-I) and the cytoplasm (A-II) of the tissue. The characteristic x-ray peaks for iron were observed in both the cytoplasm (B) and the lysosome (C).
3.1.2 Magnetic Mapping of Iron Using MFM

(a) MFM signals corresponded to lysosomal ferritin

The Perl’s stained tissue was used to identify iron rich and iron poor regions on adjacent sections prepared for imaging with MFM. Iron poor Perl’s stained regions did not show an MFM phase signal at a 30 nm lift height (Figure 3.4 A). Regions with a strong Perl’s stain corresponded to an MFM phase signal at a 30 nm lift height (Figure 3.4 B, C, D). A range in the intensity and size of the regions exhibiting MFM phase shift was observed. The majority of regions that showed an MFM phase signal were less than 0.2 µm² in area, which was consistent with the sizes of the lysosomes observed in TEM. The signal from individual ferritin molecules dispersed in the cytoplasm could not be detected in MFM images.

(b) MFM signals corresponded to an attractive force between the probe and the sample

To confirm the long range nature of the magnetic signal, regions were also scanned at lift heights of 0, 30, 40, and 50 nm (Figure 3.5). The phase and area were analyzed for regions with a positive or negative phase signal. The area of the negative and positive phase shifts decreased with increasing lift heights and some of the regions failed to show a phase shift at a lift height of 40 or 50 nm. The decrease in area was significant between all lift heights measured for both the negative and positive phase (Figure 3.6 A). However, the decrease in the magnitude of the phase signal was only significantly different for the negative phase (Figure 3.6 B).

The negative phase shift represented an attractive force between the sample and the MFM probe. In these experiments, the only magnetic field that the samples experienced...
was from the probe. Thus, the probe induced a magnetic moment in the iron particles present in the lysosomes resulting in a negative phase signal. Since ferritin is considered to be superparamagnetic, the direction of magnetization would have been parallel to the direction of the magnetic field of the probe.

The observed positive phase shift could have originated from non-magnetic interactions between the sample and the tip including electrostatic effects, capacitive coupling, and surface topography. Thus, only the negative phase shift was considered to be the true magnetic signal.

(c) Factors Contributing to MFM signal

The average negative phase shift of the naïve spleen tissue was approximately $100^\circ$ which was significantly larger than what was reported in previous work on isolated ferritin. The large phase shift can be attributed to the high iron core density of ferritin in lysosomes. It is interesting to note that a variation in the intensity of the negative phase shifts was observed in different regions of interest, even at a fixed lift height. This variation in MFM signal intensity could arise due to differences in the density of iron present in the lysosomes and/or presence of lysosomes at various depths in the tissue section. Therefore, to analyze if the negative phase shift arising from lysosomes is affected by either of these two factors, the MFM images were analyzed for their roughness (variation in phase across the region of interest) and magnitude of phase as a function of lift height. This is because earlier studies have shown how the roughness of the MFM signal can depend on particle aggregation.
To ascertain if the roughness of the MFM signal depended on its magnitude, phase images were analyzed for their roughness at a fixed lift height of 30 nm. A 0.04, 0.01, and 0.0025 µm² area was selected in fifteen different regions. The roughness at 30 nm (R$_{30}$) for the particles was plotted as a function of the phase for each selected area (Figure 3.7). The roughness for all particles decreased as the selected area was decreased from 0.04 to 0.0025 µm². This suggested that the iron distribution in smaller regions was less heterogenous. For all areas analyzed, the roughness of the MFM signal increased with an increase of the MFM phase signal. This observation is consistent with a higher density of iron particles, which would not only result in a higher phase but also increased roughness of the MFM signal.

The change in the roughness, area, and phase were evaluated at multiple lift heights. The roughness, area, and phase were analyzed for the same five particles at lift heights of 30, 50, and 80 nm (Figure 3.8). The phase and area showed a decreasing trend as the lift height was increased (Figure 3.8 A and C). No trend in the roughness was observed at the different lift heights (Figure 3.8 B). The statistical significance was not determined given the small number of particles included in the analysis (n=5).

Additionally, the range (z$_{max}$) of MFM (the maximum lift height in which a phase signal is observed) was determined. Multiple regions were imaged on the naïve spleen tissue starting from z=30 nm and thereafter increasing the lift height in increments of 20 nm. Figure 3.9 shows representative MFM images with their section analysis profiles at z=30 nm and at z$_{max}$. The phase at z=30 nm (φ$_{30}$) was plotted as a function of lift height. Only particles with an area less than 0.2 µm² (representative of individual lysosomes) at z=30 nm were considered. A scatter in φ$_{30}$ can be explained by variations in the density of
lysosomes or the depth to which they are embedded in the tissue section. The maximum range of MFM signals determined from this analysis was 120 nm. This suggests that the maximum depth in a tissue section from which MFM signals could be obtained is ~120 nm. MFM can thus be compared to a confocal or a TIRF microscope which enables signals from only a thin z-section of the sample.

(d) MFM signal across various tissue types

To examine MFM signal across physiological tissues, the naïve liver was also imaged with MFM. The phase and area of the magnetic signal were compared at different lift heights between the spleen and liver (Figure 3.10). No significant difference between the spleen and liver were observed at each lift height. This suggests that the iron content of these physiological tissues is relatively similar.
Figure 3.4: MFM of Iron Rich or Iron Poor Regions of Spleen. The topography and phase \((z = 0\) or \(30\) nm) were recorded in multiple regions of spleen tissue sections. Iron poor regions (A) and iron rich regions (B, C, D) were identified using the Perl’s stained map.\(^{48}\)
Figure 3.5: MFM of Naïve Spleen at Multiple Lift Heights. Phase images were recorded at lift heights of 0, 30, 40, and 50 nm to observe the long range magnetic interactions. The phase and area were measured for the regions with negative phase (blue) and positive phase (red).
Figure 3.6: MFM Positive and Negative Phase of Naïve Spleen. Average values of (A) area and (B) phase of n=12 MFM positive regions (red) and n=19 MFM negative regions (blue) were analyzed. Area of the MFM positive and negative regions showed a statistically significant decrease with increasing lift heights (*p<0.05; **p<0.01). There was a statistically significant difference in the magnitude of phase with the increasing lift heights for the negative regions, but not for the positive regions.\textsuperscript{48}
Figure 3.7: Roughness and Change in Phase of Naïve Spleen MFM. 0.04, 0.01, and 0.0025 μm² areas were selected in regions showing a negative phase signal in the MFM images of spleen tissue. The roughness (at 30 nm) for fifteen particles as a function of the phase was plotted. The roughness decreased as the selected area decreased.
Figure 3.8: Phase, Roughness, and Area of MFM Signal at Multiple Lift Heights. The graphs of the magnitude of the phase (A), roughness (B), and area (C) are shown for the same five particles at multiple lift heights.
Figure 3.9: Maximum Lift Height. Regions of interest on the naïve spleen were imaged beginning at a lift height of 30 nm and increasing the lift height in increments of 20 nm until the phase signal was no longer detected. Images are shown for two regions at a lift height of 30 nm and their respective maximums.
Figure 3.10: MFM of Naïve Spleen and Naïve Liver. The phase and area were compared across the naïve spleen and naïve liver. No distinctive difference between these tissues was identified.
3.2 Effect of Tissue Fixation and Processing on MFM Signal

Three common histological protocols used for fixation and processing were tested to compare their effect on the magnetic signature of injured spinal cord tissue. Tissue sections were prepared using one of the following methods:

1) Perfusion fixed with 4% paraformaldehyde and 2% glutaraldehyde followed by no immersion fixation (n=12)

2) Perfusion fixed with 4% paraformaldehyde followed by a 2 hour of immersion fixation in 4% paraformaldehyde (n=8)

3) Perfusion fixed with 4% paraformaldehyde and 2% glutaraldehyde followed by immersion fixation overnight in 2% glutaraldehyde (n=9)

The MFM images of the injured spinal cord tissue using the above protocols are shown in Figure 3.11 at a lift height of 20 or 30 nm as indicated. At least eight regions with MFM signal were analyzed for each fixation method. The MFM phase and area from these samples are summarized in Table 3.1 and also shown in Figure 3.12.

A significant difference in phase was found between all the sample preparation methods (Figure 3.12). However, the area did not show a significant difference between any of the methods. The highest MFM signal was observed for samples with the least fixation (no immersion fixation). These observations are consistent with earlier reports that state that the magnetic properties of biological samples are dependent on the method of fixation.49
Figure 3.11: MFM of Spinal Cord for Different Fixation Methods. AFM topography and phase images (z= 0 or 30 nm) are shown for the three different fixation methods.

Table 3.1: Effect of Fixation on MFM Phase and Area. The values for the average phase and area are reported. *p-value between samples 1-2, 2-3 and 3-1 are indicated in the respective rows.
3.3 Indirect MFM of Ferritin In-vitro

Initial efforts in developing the ID-MFM technique focused on in-vitro horse spleen ferritin. Ferritin was immobilized on side B of the 20 nm thick silicon nitride TEM windows and then imaged with ID-MFM on side A. Several different concentrations of
ferritin (ranging from 1 to 85000 µg/ml) were tested to see if the magnetic signature could be detected with ID-MFM. As expected, the height images in ID-MFM showed no indication of ferritin topography. However, regardless of the ferritin concentration, no distinct MFM signal was observed in ID-MFM (Figure 3.13). 5 nm and 10 nm thick silicon nitride windows were also tested, but no MFM signal was detected (data not shown).

Various possible causes for the failure of the ID-MFM experiments were examined. First, the presence of ferritin on silicon nitride TEM windows was verified using TEM (Figure 3.14). Next, the density of ferritin (number of particles per unit area) at two concentrations (10 µg/ml and 1000 µg/ml) on the silicon nitride windows was compared (Figure 3.15). The two concentrations of ferritin on the TEM windows yielded a difference that was not statistically significant, which suggests that the density of immobilized ferritin reaches a maximum and cannot be altered by increasing the liquid ferritin concentration. This observation was consistent with the inability to detect the ID-MFM signal for various ferritin concentrations.

The detection of ferritin in spleen with D-MFM was successful, while the detection of in-vitro ferritin using ID-MFM was unsuccessful. To explain this discrepancy, the density of immobilized ferritin on silicon nitride membranes was compared to ferritin particles found in the spleen lysosome and cytoplasm in-vivo. The density of ferritin was largest in the spleen lysosomes and the smallest in the spleen cytoplasm. A statistically significant difference between in-vitro and in-vivo densities was found (p < 0.005).

Further, the TEM images of in-vitro ferritin revealed variation in the density of iron cores between individual ferritin molecules. On the other hand, the iron particles present in the spleen lysosome and cytoplasm exhibit a nearly uniform iron content. The iron
particles were set to a threshold for their intensity in TEM images and the percentage of light versus dark (electron dense) particles was compared (Figure 3.16). No statistically significant difference was found between the ferritin in the spleen cytoplasm and lysosome. However, significant (p < 0.05) variation between the density of the iron in ferritin was observed across in-vitro and in-vivo samples. Ferritin immobilized on the membranes had a higher percentage of lighter particles, indicating a lower iron content. Both the variation in the iron content across in-vitro ferritin molecules and the limitations in the density of ferritin on the window surface would diminish the MFM signal arising from individual ferritin and an ensemble of ferritin. These factors along with a possible shielding effect of the silicon nitride membrane made it difficult to detect ferritin with ID-MFM.

Based on these observations, future studies of ID-MFM should focus on lysosomal ferritin in tissue samples. The D-MFM phase shift of lysosomal ferritin was more than two times higher than that of in-vitro ferritin samples at z=30 nm when using high-moment probes. Thus, the ID-MFM signal from lysosomes could be detected even through a membrane thickness of 5 to 20 nm. However, immobilization of tissue sections on side B of the SiN windows for ID-MFM would require optimization of sample preparation protocols.
Figure 3.13: ID-MFM of Ferritin. Various concentrations of ferritin images with ID-MFM on a silicon nitride TEM window. A distinguishable phase signal was not detected at any concentration of ferritin.
Figure 3.14: TEM of Ferritin. Ferritin on a silicon nitride TEM window. The ferritin concentration was 10 µg/ml (A,C) or 1000 µg/ml (B,D).
Figure 3.15: Number of Ferritin Molecules in Spleen Tissue Vs. on TEM Window. The number of ferritin molecules in the lysosome and cytoplasm of a spleen section was compared to two different concentrations of in-vitro ferritin on a TEM window. The difference in the number of ferritin molecules for the two concentrations of in-vitro ferritin was not statistically significant. However, the difference in the number of particles was statistically different in all other cases (p<0.005).

Figure 3.16: Variation in Ferritin Iron Density. The variation in iron density was compared between the lysosome, cytoplasm, and two concentrations of ferritin (10 µg/ml and 1000 µg/ml). No significant difference between the ferritin in the cytoplasm and the lysosome was observed. However, a significant difference (p <0.05) was found for all other groups.
Chapter 4: Conclusions

This work investigated the strengths and limitations of MFM as a technique to map iron content in biological tissue sections using their magnetic signature. D-MFM could successfully map the densely packed lysosomal ferritin in physiological tissues and was consistent with results obtained using currently available techniques, such as Perl’s staining and TEM. The spatial resolution of D-MFM matches that of TEM. Thus, D-MFM can serve as a bridge between the biochemical and magnetic characterization of iron to localize iron deposits in tissues.

D-MFM can only detect the magnetic signal from densely packed ferritin in the lysosomes and not the monodisperse ferritin in the cytoplasm. Thus, complementing D-MFM results with total-iron assay can help ascertain the various fractions of iron present in tissues. The magnitude of phase shift and the area of the region emanating the MFM signal could be used to quantitatively evaluate the lysosomal iron content in ~120 nm thick layer of tissue. These results could then be used to calculate the lysosomal iron in total tissue volume.

This study also provides insight into the effect of three different tissue fixation methods on the D-MFM signal from lysosomes. The highest D-MFM signal was achieved by preparing tissue without immersion fixation. It is likely that the signal from LIP and cytosolic ferritin is also affected by tissue fixation protocols. These factors should be
considered while evaluating iron content in tissues by biochemical or magnetic characterization approaches.

ID-MFM was tested as a novel approach to MFM. Various concentrations of in-vitro ferritin were examined. ID-MFM sample preparation enables multimodal microscopy using TEM and AFM. Although in-vitro ferritin samples could not be detected using ID-MFM, this approach is a possible option for examining histological samples.
References


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