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# Magnetic mapping of iron in rodent spleen

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**Abstract**

Evaluation of iron distribution and density in biological tissues is important to understand the pathogenesis of a variety of diseases and the fate of exogenously administered iron-based carriers and contrast agents. Iron distribution in tissues is typically characterized via histochemical (Perl's) stains or immunohistochemistry for ferritin, the major iron storage protein. A more accurate mapping of iron can be achieved via ultrastructural transmission electron microscopy (TEM) based techniques, which involve stringent sample preparation conditions. In this study, we elucidate the capability of magnetic force microscopy (MFM) as a label-free technique to map iron at the nanoscale level in rodent spleen tissue. We complemented and compared our MFM results with those obtained using Perl's staining and TEM. Our results show how MFM mapping corresponded to sizes of iron-rich lysosomes at a resolution comparable to that of TEM. In addition MFM is compatible with tissue sections commonly prepared for routine histology.


*Key words:* Ferritin; Iron; Lysosomes; Magnetic force microscopy; Spleen; Transmission electron microscopy

Iron (Fe) is an essential metal involved in a wide spectrum of physiological and pathological processes, e.g. oxygen transport and enzymatic reactions.[1] The oxidation state, mineral composition, and confinement of iron play a crucial role in maintaining iron homeostasis. Ferritin is the major iron storage protein found in mammalian tissues, comprising of a nanoscale iron core with up to 4500 iron atoms inside a ~12 nm protein shell. The organs with the largest stores of ferritin (iron) in the human body are the spleen, liver, and bone marrow. In pathological conditions, elevated levels of localized iron deposits can be found at sites of injury,[2] in cardiovascular diseases,[3] malignancies[4] and neurodegenerative diseases.[5] A precise understanding of the quantity and quality of iron distribution in tissues is important for clinical diagnosis and treatment as well as for applications involving delivery of iron-based carriers or contrast agents.

Histological evaluation of ferritin (iron) in biological tissues is commonly accomplished using ferritin-immunohistochemistry (IHC) and/or histochemical iron staining (e.g. Perl's or Turnball's stain). These techniques rely on light microscopy for detection, wherein the observed iron-rich regions are several microns in size. In certain pathologies, a mismatch between ferritin-IHC and histochemical iron staining has been reported,[6,7] which could arise due to multiple factors such as the presence of iron-deficient apoferritin or deposition of iron beyond the confinement of a ferritin core.[8] Additional factors like antibody type(s) and concentrations, incubation times, and signal enhancement reagents may affect the signal intensity and spatial resolution obtained using IHC or histochemical stains.

High-resolution mapping of nanoscale iron deposits in situ can provide a more accurate insight into tissue iron deposits. This can be achieved using ion or electron microprobe based ultrastructural techniques such as secondary ion mass spectroscopy (SIMS)[9] or analytical transmission electron microscopy (TEM).[10] Like the Perl's stain, these approaches are largely based on the chemical composition of the iron particles. However, a major limitation with these ultrastructural techniques is that they require stringent sample preparation protocols not compatible with the rapid and multi-label histological evaluation

*Abbreviations:* AFM, atomic force microscopy; IHC, immunohistochemistry; MFM, magnetic force microscopy; OCT, optimal cutting temperature; TEM, transmission electron microscopy; EDS, energy dispersive spectroscopy.

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achievable via light microscopy. The ability to map iron deposits at nanoscale level along with IHC on adjacent sections would be especially advantageous for a more accurate histological evaluation.

One of the unique features of ferritin (iron) deposits which remain un-exploited in histological analysis is the magnetic nature of its iron core. Several in-vitro studies using techniques such as SQUID magnetometry,[11] Mossbauer spectroscopy, vibrating sample magnetometry,[12] and nuclear magnetic relaxometry[13] have elucidated the superparamagnetic character of ferritin with an antiferromagnetic sub-lattice arrangement of $Fe^{3+}$ in its core.[14] The superparamagnetic behavior of ferritin above its Néel temperature (>240°K) arises due to an increase in the number of uncompensated spins resulting from thermal disorder and a linear contribution to its magnetization by an externally applied field.[15,16] Magnetic moment of $Fe^{3+}$ atoms residing in the ferritin core has been determined to be 3.8 $\mu_B$ (Bohr magneton),[13] with the entire core possessing a moment of ~200-350 $\mu_B$.[15,17]

Magnetic properties of ferritin have been utilized in magnetometry and relaxometry studies to evaluate the overall iron content ex-vivo in a variety of tissues such as spleen,[18,19] brain,[20] and liver.[21] Non-invasive MRI imaging[22] has also been able to track endogenous ferritin iron deposits in-vivo.[23] However, these studies fail to provide information on spatial distribution of iron deposits, which is essential to understand iron regulation and function at the subcellular level. Magnetic mapping of tissue sections would be especially advantageous to spatially characterize iron-deposits in situ in histological sections or biopsy samples. It could help resolve a mismatch between ferritin-IHC and histochemical stains as well as that between the iron content of serum ferritin and tissue ferritin in certain pathologies.[24,25]

In this study, we investigate the capability of magnetic force microscopy (MFM) to map ferritin (iron) deposits in biological tissue in situ. MFM is a technique utilizing the principles of atomic force microscopy (AFM). It operates by scanning the surface of a sample with a magnetic probe at user defined lift heights to detect the magnetic signature of the scanned sample. While MFM has been extensively used to characterize magnetic domains in solid state devices[26] and magnetic nanoparticles in-vitro,[27–29] limited studies exist on applications of MFM for examining tissues or cell samples in-situ.[30–33] Previous studies by us[34] and others[32,35,36] have shown how MFM can detect purified ferritin at the single particle level in-vitro. In this study, we examined the rat spleen tissue for ferritin (iron) deposits in-situ using Perl's stain, TEM, and MFM and compared the spatial resolution obtained using these techniques. We demonstrate how MFM can successfully map iron-rich regions in spleen tissue at sub-micron resolution and is compatible with sample preparation typically used for routine histology.

## Methods

### *Tissue collection*

All animal work was performed in agreement with The Ohio State University Institutional Animal Care and Use Committee. Anesthetization of adult male Sprague Dawley rats was accomplished via administration of 80 mg/kg ketamine/xylazine mixture. Rats (n = 3) were perfused transcardially with distilled water followed by fixation in 250 ml of the primary fixative solution consisting of 4% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (consisting of 0.2 M trihydrate sodium cacodylate at pH 7.2, with 1% $MgCl_2$ and 0.5% $CaCl_2$). Spleens were then dissected from the animals, cut into 0.5-1 mm segments and immersion-fixed overnight at 4 °C in primary fixative (as defined above) for TEM processing. Subsequently, tissue was placed in 0.2 M cacodylate buffer for storage before further processing for TEM. For embedding spleen tissue in Optimal Cutting Temperature (OCT) compound, perfusion-fixed tissue (as above) was dissected into 3-5 mm segments and immersed in OCT filled molds, then snap frozen in liquid nitrogen and stored at −80 °C until use.

### *Transmission electron microscopy*

Glutaraldehyde-fixed spleen tissue was processed for TEM analysis, beginning with a 1-h post-stain in 1% osmium tetroxide (Electron Microscopy Sciences (EMS) Hatfield, PA). Samples underwent a graded ethanol dehydration step (30-100%) followed by an exchange into acetone transition solvent and subsequent infiltration series with a Spurr's resin mixture (EMS #14300). Samples were polymerized overnight in a 65 °C oven, after which resin blocks were thick sectioned at 750 μm and stained with Methylene Blue-Azure II and Basic Fuchsin stain[37] in order to identify regions of interest (ROIs) within spleen samples. Blocks were trimmed down to ROIs, and 40 nm thin sections were cut on an ultramicrotome (Leica Ultracut UCT, Leica-Microsystems) and collected on 200 mesh copper grids. Sections were examined without further staining on a JEM-1400 TEM (JEOL Ltd. Tokyo, Japan) operating at 80 kV and digital micrographs were captured using a Veleta digital camera (Olympus Soft Imaging Solutions GmbH, Műnster, Germany). For energy dispersive spectroscopy (EDS), the very same grids were imaged in dark field using a Tecnai F20 field emission 200 kV TEM/scanning-TEM (STEM) and X-TWIN lens (FEI) equipped with a HAADF (High-Angle Annular Dark Field) and an EDAX XLT windowless silicon drift detector (SDD).

### *Perl's iron staining*

OCT embedded tissue was cut into 10 μm thick sections using a HM 505E (Microm) cryostat and mounted onto Superfrost Plus Microscope slides (Fisher Scientific). Sections were rinsed in distilled water 3 times to remove OCT compound, followed by several rinses in 0.1 M phosphate buffer saline (PBS) and 15 min in 25% hydrogen peroxide in methanol. After several more rinses in PBS, tissue was permeabilized for 10 min in a 0.1% Triton X-100 solution in PBS and rinsed thereafter. For staining of ferric iron, a 2% potassium ferrocyanide solution in 2% hydrochloric acid solution was incubated on tissue for 30 min. All samples were rinsed with $H_2O$, dehydrated in an ethanol series, cleared with xylene and cover-slipped with Permaslip (Alban Scientific). Slides were imaged using an Axioplan 2 (Zeiss) microscope equipped with an AxioCam color (Zeiss) camera.