Detection and Quantification of Cells using Magnetic Particle Imaging and Magnetic Microspheres

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Medical Biophysics
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Abstract

Magnetic particle imaging (MPI) is an emerging imaging modality that specifically detects superparamagnetic iron oxide nanoparticles (SPIOs). Our lab has shown that cell tracking with magnetic resonance imaging (MRI) has very high sensitivity, but low specificity and quantification of iron labeled cells is difficult. MPI cell tracking could overcome these limitations. A Momentum™ MPI system was installed at Robarts in August 2019 and this is the first project to be completed using MPI. In Chapter 2 a series of in vitro experiments are reported which tested the repeatability and reproducibility of imaging SPIO labeled cell samples. There are no reports of the use of micron-sized iron oxide particles (MPIO) for cell tracking by MPI. Therefore, in Chapter 3, MPIO was evaluated for in vivo detection and quantification of cancer cells in the mouse brain by MPI. In Chapter 4, limitations of these studies and plans for future work are discussed.

Keywords

Magnetic Particle Imaging (MPI), Superparamagnetic Iron Oxide (SPIO), Micron-Sized Iron Oxide Particles (MPIO)
Summary for Lay Audience

The purpose of this thesis is to explore a new imaging tool called magnetic particle imaging (MPI) to study its ability to detect iron-labeled cancer cells that have been dispersed in the brain or cell samples. MPI directly detects iron oxide nanoparticles and can be used to provide a measure of the iron content and number of iron labeled cells in the brain. Since MPI is so new it is not known what type of iron particle will be ideal for cell detection. I explore two different iron particles in this thesis. I labeled breast cancer cells that spread to the brain with large micron-sized iron oxide particles and studied how well these cells could be detected. I compared this particle with the current gold standard for MPI which is called Vivotrax. Experiments were conducted on iron labeled cell samples to determine ways to improve image quality, to see if MPI would damage cells during scanning, and to compare how imaging and analysis were affected when different users conducted scans and analyzed the same data. Iron labeled cancer cells were then injected into mice to see how well MPI would be able to detect cells that were dispersed in a mouse brain. Images from MPI and MRI were compared to determine the benefits and limitations of these complementary imaging tools. Major limitations of the studies and ideas for future work are also discussed.
Co-Authorship Statement

The work contained herein was carried out by the author, under the supervision of Dr. Paula Foster. This included cell culture, cell labeling, Perl’s Prussian Blue and Nuclear Fast Red tissue staining, MPI imaging, operation of the 3T MRI system, mouse anesthesia, data analysis and manuscript preparation.

Julia Gevaert and Olivia Sehl acted as user 2 and user 3 in the Chapter 2 experiment to conduct MPI scans and analysis on in vitro cell samples to test user variability when using MPI. They also acquired RELAX data for MPIO and Vivotrax shown in figure 2.2. Dr. Ashley Makela conducted MPI scans at Michigan State University when waiting for the MPI system to be installed at Robarts Research Institute for experiment 1 in Chapter 3. Dr. Amanda Hamilton and Natasha Knier conducted all intracardiac injections on mice using ultrasound guidance in Chapter 3. Dr. Paula Foster detected cells entrapped in the brain vasculature in MR images for experiments in Chapter 3.
Acknowledgments

I would like to start by thanking my supervisor Dr. Paula Foster. Without you I would have never been able to get through my Masters. The endless nights and weekends you dedicated to helping me understand concepts, editing and helping me prepare is extremely appreciated. I am very lucky to not only have you as a supervisor but also as an incredible role model. I can see why so many students past and present hold you in high regard because your leadership and mentorship are like no other.

To the entire Foster Lab, you all helped play a major role in my success as a master’s student. Natasha, you were always there to reassure me that everything would be okay and helped me believe in myself when imposter syndrome got the best me. I especially appreciate you for always realizing when a quick coffee break was needed. Without you my presentations would never have looked so good. Olivia, you were always the one I could turn to when data was not making sense, or I needed help. You were amazing at helping me understand complex concepts and prepping me for my defense. Julia, you started halfway through my masters and soon became my right hand. Together we helped each other navigate through all our courses and how to deal with MPI, especially when it threw us some unexpected curve balls. Whether were in the lab or out of it I know I can always count on you to be there for me. I am so grateful to have met you all and made such long lasting friendships. I cannot wait to see all the amazing and exciting things you ladies do and contribute to the medical imaging field. I would also like to thank Dr. Amanda Hamilton for teaching me every lab technique that I’ve ever known and for answering all my silly questions along the way. I also want to thank the entire CMIG crew for always being supportive, making me laugh and being amazing people to work and learn with.

Lastly, to my family for always supporting me through this entire process. You have always been by my side and I am so lucky to have you to all as amazing cheerleaders. A special shout out goes to Peter who always made sure I was well taken care of and more importantly fed, to make sure my hangry side never got a chance to come out. I could not imagine going through this amazing journey without each and every one of you.
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<th>Description</th>
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<tr>
<td>MPI</td>
<td>Magnetic Particle Imaging</td>
</tr>
<tr>
<td>SPIO</td>
<td>Superparamagnetic Iron Oxide Nanoparticle</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>$^{19}$F</td>
<td>Fluorine 19</td>
</tr>
<tr>
<td>PFC</td>
<td>Perfluorocarbons</td>
</tr>
<tr>
<td>MPIO</td>
<td>Micron-Sized Iron Oxide Particle</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence Imaging</td>
</tr>
<tr>
<td>Fluc</td>
<td>Firefly Luciferase</td>
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<tr>
<td>Rluc</td>
<td>Renilla Luciferase</td>
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<tr>
<td>CCD</td>
<td>Cooled Charge Coupled Device</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>USPIO</td>
<td>Ultra-Small Superparamagnetic Iron Oxide Nanoparticle</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>PPB</td>
<td>Perls Prussian Blue</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Mass Spectroscopy</td>
</tr>
<tr>
<td>HMSCs</td>
<td>Human Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
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<tr>
<td>EMCs</td>
<td>Embryonic Stem Cells</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
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<tr>
<td>NSG</td>
<td>NOD/SCID/IL1r−/−</td>
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<tr>
<td>FFR</td>
<td>Field Free Region</td>
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<tr>
<td>FOV</td>
<td>Field of View</td>
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<tr>
<td>PSF</td>
<td>Point Spread Function</td>
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<tr>
<td>FWHM</td>
<td>Full Width at Half Max</td>
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<tr>
<td>CLUT</td>
<td>Colour Look Up Table</td>
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<tr>
<td>A.U.</td>
<td>Arbitrary Units</td>
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<tr>
<td>WBRT</td>
<td>Whole Brain Radiotherapy</td>
</tr>
<tr>
<td>MIP</td>
<td>Maximum Intensity Projection</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural Progenitor Cells</td>
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<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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Chapter 1

1 Introduction

Parts of this introduction have been utilized from a paper I am co-author on titled “A Perspective on Cell Tracking with Magnetic Particle Imaging”. (This was published in Tomography in November 2020).

1.1 Motivation and Overview

Magnetic Particle Imaging (MPI) is an emerging imaging modality that directly detects superparamagnetic iron oxide nanoparticles (SPIOs). The Foster lab has been developing cellular tracking techniques using Magnetic Resonance Imaging (MRI) for over a decade. These cellular tracking techniques have used SPIOs and fluorine-19 ($^{19}$F)-based contrast agents to label cells for multiple applications including immune and stem cell tracking for cellular therapies. However, these cell tracking techniques using MRI come with some limitations. SPIO-based MRI cell tracking has very high sensitivity, but low specificity. SPIOs produce negative contrast in MRI, producing signal loss, or signal voids, in MRI. This creates an issue as other anatomical features also appear as regions of signal loss in MR images. This includes the air in the lungs, cortical bone, and late-stage hemorrhage. Therefore, signal void due to iron labeled cells is difficult to differentiate from other regions of low signal. Another challenge for MRI cell tracking is the ability to accurately quantify the number of cells being detected. The signal loss produced by iron-labeled cells is indirectly detected through its relaxation effects on protons, making it impossible to reliably quantify the local tissue concentration of SPIO. $^{19}$F based cell tracking uses perfluorocarbons (PFC) to label cells. The number of $^{19}$F atoms can be directly measured from $^{19}$F MR images and related to cell number. $^{19}$F MRI has high specificity, but low sensitivity; thousands of cells per voxel are needed for detection. MPI has the potential to overcome challenges related to MRI-based cell tracking because it has high specificity (only detects SPIOs) and high sensitivity to nanogram quantities of SPIOs,
which translates to hundreds of cells. Further improvements in sensitivity are expected, especially as tailored SPIOs are developed. Importantly, the MPI signal is linearly related to iron mass allowing for quantitation of the amount of iron from images, and with knowledge of the amount of iron/cell after SPIO labeling, the cell number can be calculated.

In August of 2019, Canada’s first MPI system was installed at Robarts Research Institute and I was the first student in the Foster lab to have a research project focused on the use of MPI for cell tracking. My work focused on using MPI for the detection and quantification of breast cancer cells labeled with micron-sized iron oxide particles (MPIO) in the mouse brain. This nomenclature for MPIO is accepted and commonly used in the cell tracking field.

1.2 Imaging Modalities for In Vivo Cell Tracking

A number of imaging modalities have been used for imaging cells in preclinical models. The most common are bioluminescence imaging (BLI), positron emission tomography (PET) and magnetic resonance imaging (MRI). Magnetic particle imaging (MPI) is just emerging as an in vivo cell tracking modality. BLI is a low-cost modality with high throughput. The most valuable feature of BLI for cell tracking is the ability to differentiate between dead and viable cell populations. For BLI, cells are engineered to express a luciferase transgene, this is often done using Firefly luciferase (FLuc) or Renilla luciferase (RLuc), which reacts with a correlating substrate (D-luciferin for Firefly luciferase or h-coelenterazine for Renilla luciferase). The reaction produces light as a product of enzyme-based oxidation of the substrate and photons are collected by a highly sensitive cooled charge coupled device (CCD) camera that converts photons into electrons. This signal is usually overlaid on top of an x-ray or a computed tomography (CT) image for anatomical reference. However, BLI has its limitations. BLI is limited by low resolution and the inability to image larger animals and humans because of poor tissue penetration of optical signals resulting in limited depth penetration. PET imaging has the advantage of very high sensitivity and unlimited depth penetration. PET provides functional images through
the detection of gamma rays that are emitted from radioisotopes. These isotopes are of varying half-lives and energies and have been widely used for targeted imaging. However, PET images have high background activity and are of relatively low resolution.\textsuperscript{15} PET images are also usually paired with MRI or CT images for anatomical information. The use of isotopes for PET exposes the subjects to ionizing radiation. This can be concerning for imaging cells, especially in the context of cell therapies. In comparison to other cell imaging techniques, MRI has the advantage of high resolution and high tissue contrast. MRI is considered safe and uses no ionizing radiation. This allows for serial imaging. Cellular MRI relies on contrast agents which present some limitations. Proliferation of cells leads to diminished signal and signal can remain visible from cells even after death.\textsuperscript{8} MRI has been developed for cell tracking in the Foster lab and is described in more detail below. A schematic showing the relative sensitivity and resolution for these preclinical imaging systems used for cell tracking is shown in Figure 1.1.

![Figure 1.1: Schematic showing a comparison of resolution and sensitivity of the common imaging modalities used for cell tracking.](image)

### 1.3 MRI Cell Tracking

Cell tracking with MRI uses contrast agents for labeling specific cells, thereby enhancing their detectability.\textsuperscript{16,17} The most commonly used agents for cell tracking with MRI are magnetite (Fe$_3$O$_4$)-based superparamagnetic iron oxide nanoparticles (SPIOs). MRI cell tracking was first demonstrated by Bulte et al.\textsuperscript{18} and Yeh et al.\textsuperscript{19} in the early 1990s
by internalizing iron oxide particles within cells and detecting the resulting negative signal contrast. The presence of SPIOs in cells causes a distortion in the magnetic field and leads to abnormal signal hypo-intensities in iron-sensitive images (T2- and T2*-weighted images are most often used). The resulting signal void occupies a much larger spatial region than the actual size of the individual cells; this is commonly referred to as a blooming effect. This has led to high detection sensitivity, with single cell detection possible under certain conditions.21,22

The SPIOs used for MRI are usually classified by hydrodynamic size: 1) ultra-small superparamagnetic iron oxide nanoparticles (USPIO) (5-50 nm), 2) superparamagnetic iron oxide nanoparticles (SPIO) (50-150 nm), and 3) micron-sized iron oxide particles (MPIO) (0.5 – 2 μm). Worth noting here is that it is the iron core size, not the hydrodynamic size, that is most important in MPI, this is described in detail below. Of the SPIO used for MRI cell tracking, MPIO have the highest iron content per particle, as they have approximately 1pg Fe/particle. This makes one MPIO particle roughly equivalent to 1.5 million standard SPIO or 4 million USPIO. Although there are no FDA approved SPIO for MRI, one agent is currently approved off-label for MRI cell tracking. This is Ferumoxytol, which is a USPIO used to treat iron-deficiency.24 As will be described later, many MPI cell tracking studies have used the same SPIO that have been used for MRI.

There are two strategies for labeling cells with SPIO. Cells can be labeled in vitro during cell culture by incubation with the SPIOs, typically overnight. Uptake of SPIOs by cells occurs through endocytosis.20 Once internalized SPIOs are compartmentalized in endosomes in the cell cytoplasm. This strategy is used when cells are subsequently injected or implanted and then imaged.25 Some cells require other methods for efficient uptake. Transfection agents can be used to enhance uptake of SPIOs into cells. Transfection agents work by coating the outer surface of SPIOs to improve the rate of iron uptake as well as the amount of iron taken up by cells. Our lab commonly uses protamine sulfate in conjunction with heparin (both FDA approved) to improve cell labeling.26 Alternatively, SPIO can be administered intravenously (IV) and phagocytic cells of the reticuloendothelial system (macrophages) take up SPIOs in situ.27 This is typically used
for imaging macrophages and inflammation. For the work in this thesis cells were labeled in vitro with MPIO.

Labeling efficiency can be tested a few different ways. Staining for iron with Perl’s Prussian blue (PPB) can be used to identify iron positive cells. With this method, intracellular iron appears blue and the cells are counterstained with nuclear fast red and appear red. The labeling efficiency can be then be determined by analysis of cells on slides by microscopy and calculating the percentage of blue cells. The mass of iron per cell can be measured using inductively coupled plasma mass spectroscopy (ICP-MS) or spectrophotometric techniques, these report an average value for the amount of iron per cell; some cells will contain more, or less, iron.

It is important to assess the impact of SPIO on cell phenotype and function. Viability can be easily assessed using trypan blue exclusion assay. This assay works off the principal that live cells with intact membranes will not take up the blue dye while dead cells will because of their compromised membrane integrity. Relatively low toxicity has been reported in the literature for SPIO labeling of cells in general. The Foster lab have previously shown that most cells can be efficiently labeled with iron with no negative impact on viability, migration, maturation or differentiation, when using a variety of SPIOs. Roach et al. showed that hepatocytes could endocytose MPIO, achieving iron content as high as ~55 pg/cell, with >75% viability, and extensive morphological and functional assays indicated that labeling was benign to the cells. Some cells are more sensitive to labeling with iron than others. Rohani et al. demonstrated differences in activation and migration of dendritic cells labeled with MPIO. Using flow cytometry they were able to determine the optimal amount of MPIO for labeling to minimize cell apoptosis and maximize detectability by MRI. When testing a new cell and SPIO combination it is important to carefully calibrate the ideal iron loading which produces sufficient intracellular iron for detection, without adversely affecting cells. For MPI cells are labeled the same way and analysis methods are also the same.
1.4 Tracking MPIO-Labeled Cancer Cells in the Mouse Brain by MRI

Our lab has used MPIO extensively to label human breast cancer cells (MDA-231-BR) for tracking by MRI in the mouse brain.\(^{36-38}\) In this animal model MDA-231-BR cells are labeled with MPIO particles by co-incubation for 24 hours – labeling with MPIO results in approx. 20 pg of iron/cell, determined by ICP-MS. Then they are injected into the left ventricle of the mouse heart with ultrasound guidance. Approximately 15-20% of cells have been shown to arrest in the brain vasculature after intra-cardiac administration. This model is used in Chapter 3 were MPI is used to detect MPIO-labeled cells arrested in the mouse brain on the day of the injection.

A number of publications have resulted from studies which used this model in the Foster lab. For example, in 2006, Heyn et al. demonstrated that MPIO labeled MDA-231-BR cells could be detected in the mouse brain at the single cell level.\(^{39}\) In a subsequent paper, the ability to monitor non-proliferating, iron-retaining cancer cells was demonstrated.\(^{21}\) Later, Murrell et al. used MRI to image MPIO labeled breast cancer cells in mice treated with whole brain radiotherapy (WBRT). Early, prophylactic WBRT eliminated tumour growth and MRI showed that non-proliferative, MPIO-retaining cancer cells were unresponsive to WBRT and persisted in the brain for up to 100 days.\(^{40}\) At this time there are no published reports of the use of MPI to detect MPIO labeled cells in vivo.

1.5 Limitations of MRI Cell Tracking with SPIOs

As described briefly before, there are several limitations of MRI cell tracking with SPIO to be aware of. The first is low specificity due to other low-signal regions in images, i.e., SPIO-labeled cells in the lung or in a region of hemorrhage cannot be detected as these regions appear black like a signal void would. Ultra-short echo time imaging methods developed for producing positive contrast from iron-labeled cells have similar problems with specificity.\(^{41}\) Second, quantification of iron-induced signal loss is complicated. Our group as well as others have shown that signal loss produced by SPIO-labeled cells is only
linear at low iron concentrations. Typically, the degree of contrast (how black the void is) or the volume of signal loss (how big a void is) is measured from these images. Although some studies have used MR-based relaxometry to show a linear relationship between R2* values and SPIO-labeled cell concentration in samples the in vivo cell quantification by this method is more complicated. Overall, with iron-based MRI cell tracking there are issues with specificity and cell number cannot be accurately determined when quantified. For these reasons we pursued MPI in this thesis as a quantitative in vivo imaging method for cancer cell tracking.

1.6 Cell Tracking with Magnetic Particle Imaging

MPI is a new imaging modality which directly detects SPIOs. MPI cell tracking has the potential to address the limitations presented by SPIO-based cell tracking. First, the MPI signal is only generated from SPIOs. This results in positive “hot-spot” contrast, similar to PET. There is also no signal attenuated from biological tissues meaning there is no background signal that may affect the specificity of MPI. Second, the MPI signal is linearly quantitative with iron concentration, and therefore the amount of iron in a region of interest can be measured from MPI images and, with information on the amount of iron/cell, the number of SPIO labeled cells can be calculated. In addition, MPI uses no ionizing radiation which is important for repeat, longitudinal imaging of cells, especially in the therapeutic cell setting.

1.7 The MPI System

MPI was first presented as a novel imaging modality by Weizenecker and Gleich in 2005. The Momentum™ MPI system we use at Robarts Research Institute (Magnetic Insight Inc, Almeida, California, USA) works by exploiting the nonlinear magnetization of the SPIOs by using weak magnets (mT) and strong gradient fields (T). The MPI system has three main components. The magnets, the transmit and receive coils and the control console. MPI is built around a gradient magnet system. Two opposing
Electromagnets form a selection field, which is a strong gradient magnetic field (~ 6 T/m) that contains a field free region (FFR) near the isocenter where the magnetic field passes through zero (Figure 1.2). The selection field magnetically saturates the magnetization of all SPIOs except for those SPIOs in the FFR, which experience no magnetic field. The FFR is rapidly rastered over the imaging volume by changing the current through electromagnets. When the FFR traverses a location containing SPIOs, the SPIO's magnetization changes nonlinearly, producing a magnetization curve characteristic of the SPIO used. This change induces a voltage in a receiver coil, and the detected voltage can be assigned to the instantaneous FFR location to produce an image, providing spatial localization. The voltages induced are linearly proportional to the number of SPIOs at the FFR location over a wide range of concentrations, enabling quantification of SPIOs.

Figure 1.2: Two opposing electromagnets form a selection field, which is a strong gradient magnetic field (~ 6 T/m). A field free region (FFR) is created near the isocenter where the magnetic field passes through zero.
MPI can produce both 2D and 3D images. 2D projection images are acquired by shifting the FFR along the y-axis by translating the sample bed. The FFR is shifted along the x-axis by changing the electric field strength. These scans are acquired in a grid formation from left to right and top to bottom to create a 2D image. The direction of the projection can also be chosen (coronal- 0 degrees, or sagittal- 90 degrees). Projection images give the sum of the signal along the direction chosen and the signal is resolved in the two orthogonal direction to the projection (x, y plane). The acquisition time for 2D images ranges between 1-5 minutes depending on the scan mode (described in Chapter 2).

3D tomographic images are acquired by projection reconstruction similar or x-ray or CT. The number of equi-spaced projections and angle can be chosen by the MPI user starting as low as 28 projections and as high as 75 projections and angles between 0-360 degrees. Increasing the number of projections increases the scan time as well as increase the signal to noise ratio (SNR). Our system typically uses 35 projections when acquiring a 3D scan. Filtered back projections are used to create a full 3D tomographic image. The signal is resolved along all 3 orthogonal directions (x, y, z plane). The entire gantry containing the 2 electromagnets can be rotated around the sample bed as the sample bed is being translated in and out of the bore for the FFR to translate across. These scans are slower taking anywhere from 15-30 minutes. 3D images are typically used when acquiring in vivo data for accurate localization of signal as well as providing better sensitivity and resolution. The scan modes on the Momentum™ MPI system are described in Chapter 2.

1.8 MPI Resolution and Sensitivity

The resolution of MPI is driven primarily by the gradient strength and the SPIO properties (core size and relaxation). There are no voxels in MPI like in MRI, instead resolution is related to the size of the FFR. If you increase gradient strength in MPI you reduce the size of the FFR, this leads to higher resolution but lower sensitivity since there is less iron, and no signal, contained in a single FFR position. Similarly, a decrease in gradient strength leads to a larger FFR, lower resolution but higher sensitivity (Figure 1.3). The relationships between gradient strength and resolution is illustrated in Figure 1.4 for
three core sizes. Theoretical modeling based upon the Langevin theory of SPIOs predicts that resolution improves with increasing core size. However, Tay et al. found that improved resolution with increasing magnetic core size follows the prediction up to approximately 25 nm when the effects of SPIO rotational times become significant due to relaxation effects (Figure 1.5). For this reason, SPIO with a core size close to 25 nm are currently considered to be ideal for MPI.

Figure 1.3: Schematic showing how the gradient strength can impact the resolution of MPI by changing the size of the FFR. (A) The gradient strength is strong at 6 T/m making the FFR small. (B) The gradient strength is weak at 3 T/m where the FFR is much larger.
Figure 1.4: The relationship between gradient strength and resolution is shown for four different core sizes.

Figure 1.5: A comparison of the experimentally achieved spatial resolution with the predicted spatial resolution from the Langevin model reproduced from reference 33 shows an increasing disparity with increasing core size after 24.4 nm.
The relationship between core size and resolution is a little more complicated. The core size determines the type of MPI relaxation that occurs in MPI when SPIOs are magnetized. MPI SPIO relaxation is much different from MRI proton relaxation. Relaxation impacts MPI images because it prevents the SPIO magnetization from instantaneously rotating. Another way to think about it is that relaxation acts against the SPIOs alignment with the applied field, causing a delay in the magnetization response. There are two types on MPI relaxation, Neel and Brownian. Neel relaxation refers to the reversal of the SPIO magnetic moment whereas Brownian relaxation refers to the physical rotation of the SPIO, in response to the MPI excitation magnetic field. The dominant relaxation mechanism depends on the SPIO core size and also the configuration, spacing and orientation of individual cores that make up the effective core size (Figure 1.6). Importantly, the study by Tay et al. described above looked at SPIOs which were single core particles and most SPIO used in MPI are multi-core clusters (described in more detail below). The transition from Neel to Brownian occurs at about 15 nm. In simple terms, resolution worsens with increasing Brownian relaxation because larger SPIOs experience increased viscous drag. The spatial resolution of MPI using currently available commercial SPIOs is approximately 1 mm and custom made SPIOs, tailored for MPI have demonstrated a resolution of 200 µm. Relaxation effects are one of the primary limits to resolution in MPI, as SPIO synthesis for MPI evolves, improvements in resolution are expected.
Figure 1.6: Schematic illustrating the concepts of a single core iron particle, a multi-core particle and the effective core size ($d_c$) and the hydrodynamic diameter of SPIOs.

The sensitivity of MPI also depends on both nanoparticle and scanner specific factors. Nanoparticle factors include the strength of the SPIO magnetization and the rate of SPIO relaxation at the FFR. Stronger SPIO magnetization improves MPI signal and a faster change in magnetization leads to higher MPI signal (Figure 1.7). Cellular sensitivity also depends on the efficiency of the nanoparticle cell labeling (more iron per cell leads to higher sensitivity).
Figure 1.7: Schematic showing the magnetization SPIOs experience within the MPI system. (A) and (B) are comparing strength of nanoparticle relaxation. The magnetization curve models the relaxation behaviour of the nanoparticles in response to the magnetic field. In (A), a stronger relaxation leads to a taller M-H curve (black), which leads to a higher peak intensity (blue). In (B), we see that the M-H curve is not as tall (black) - that means the magnetization of the particle is not as strong, and we get a shorter peak (in blue). (C) and (D) are comparing nanoparticle relaxation rate. This is looking at the steepness of the magnetization curve. In (C), the M-H curve (black) is almost vertical across the origin. This leads to a higher peak (blue) in the point spread function (higher sensitivity). In (D), the M-H curve is not as steep, meaning the particle is taking longer to rotate, leading to a shorter peak (blue) in the point spread function (lower sensitivity).

Scanner specific factors affecting sensitivity include changing (i) the selection field gradient strength, (ii) the amplitude of the excitation field, and (iii) averaging. When changing these factors there are trade-offs between sensitivity and resolution. As described above a decrease in the selection field gradient strength leads to a larger FFR and this leads to higher sensitivity because there is more iron, and so signal, contained in the FFR. The excitation field is the strength of the rapidly oscillating RF field that rapidly moves the FFR across SPIOs to induce a signal in a receiver coil. A higher excitation field amplitude excites the SPIOs more rapidly, allowing for a faster change in magnetization, thereby inducing a larger voltage in the receiver coil. Therefore, a higher excitation field amplitude gives more signal and higher sensitivity. There is a resolution trade-off where resolution
decreases at higher excitation fields because the nanoparticles, due to their relaxation properties, and are unable to respond to the applied field fast enough and smear out the received signal in the spatial domain. On the Momentum™ MPI system the projection images are acquired as 2D panels which are then stitched together to form the entire image. The overlap fraction is how much these panels overlap each other and are best thought of as a method of averaging. In this case a higher overlap fraction results in more signal averages and more signal for higher sensitivity; the trade-off is scan time. The relationship between signal averaging, via overlap fraction, and sensitivity is still under study.

The in vitro detection limit has been estimated at 200 cells labeled with Vivotrax, however, this was based on the detection of 1000 cells in a 100 μL in vitro cell suspension with SNR ~5. Song et al. reported that as few as 250 cells, labeled with a custom-made MPI tailored SPIO (30 pg Fe/cell), could be detected in vivo if a background subtraction method was used. There is still considerable work to be done to demonstrate and evaluate MPI cellular detection limits more closely.

1.9 Nanoparticles for MPI Cell Tracking

As described above, both MPI sensitivity and resolution are closely related to the type of SPIOs. Many of the SPIOs currently used for MPI are similar to those used for MRI cell tracking. Table 1.1 lists some of the SPIOs that have been tested for MPI. The first SPIO that was shown to be useful for MPI was Resovist, which was previously widely used for liver imaging with MRI. Magnetic Insight Inc., the manufacturer of our Momentum™ MPI system now sells Resovist manufactured as Vivotrax. Vivotrax is currently considered the gold standard for MPI and new SPIO are compared against it, since most published studies of MPI have been conducted with this SPIO. Although widely used, it is not considered optimal for MPI. This is because Vivotrax is a polydisperse mixture of particles containing both single iron cores (~5 nm, 70%) and clusters of multiple cores (~25 nm, 30%), leading to a bimodal size distribution. The single cores are too small to magnetize significantly and so the MPI signal predominately originates from the clustered 25 nm multi-core structures (Figure 1.8).
Table 1.1: Characteristics of the SPIOs used for MRI and MPI cell tracking.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Marketing Status</th>
<th>Applications</th>
<th>Core Diameter/Hydrodynamic diameter (nm)</th>
<th>Coating</th>
<th>FWHM *&lt;i&gt;(Δx)&lt;/i&gt; in mT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resovist® Bayer</td>
<td>Approved for clinical use in EU/ Japan</td>
<td>Liver imaging using MRI</td>
<td>3-5 clustered/62</td>
<td>Carboxy-dextran</td>
<td>9.6</td>
</tr>
<tr>
<td>Feraheme® AMAG Pharma</td>
<td>Approved for use by the FDA</td>
<td>Iron replacement therapy/ MR angiography</td>
<td>6-7/28-32</td>
<td>Polystyrene</td>
<td>39.5</td>
</tr>
<tr>
<td>Vivotrax™</td>
<td>Research use only</td>
<td>Liver imaging using MRI/MPI</td>
<td>4.2/62</td>
<td>Carbohydrate</td>
<td>11.4</td>
</tr>
<tr>
<td>MPIO</td>
<td>Research use only</td>
<td>Liver imaging using MRI/MPI</td>
<td>~5-10/0.5-2 μm</td>
<td>Polystyrene</td>
<td>27.4</td>
</tr>
</tbody>
</table>

Figure 1.8: Schematic for Vivotrax. (A) Vivotrax contains both single core particles (~5 nm) and multicore clustered particles (~25 nm). (B) The size distribution is bimodal; ~70% of the cores are single cores and the other 30% is composed of the multicore particles.

Approaches to improve MPI sensitivity include increasing the fraction of these larger aggregates or by fractionation of Vivotrax. The synthesis of homogeneously distributed single-core SPIOs with optimized core diameters is also being investigated. Unni et al. have shown that single core particles with uniform physical and magnetic size distributions can be synthesized by introducing molecular oxygen in the thermal
decomposition synthesis. These SPIOs have improved MPI sensitivity and resolution compare to commercially available SPIOs.⁵⁹

Ziemian et al. have presented a large-scale water-based synthesis of multicore SPIOs stabilized with dextran which they call MC-SPIOs. They also synthesized single core SPIOs in organic media, coated with a poly(ethylene glycol) gallic acid polymer and phase transferred to water (SC-SPIOs). This group showed that the MC- SPIOs and the SC SPIOs had 2.3 and 5.8 fold higher sensitivity than Vivotrax.⁶⁰

1.10 Characterizing SPIOs for MPI

A common first step for characterizing SPIOs for MPI is by using MPI relaxometry. MPI relaxometry measures the net magnetization of SPIOs, by turning off the selection field and applying a negative magnetic field then a positive field, and back. SPIOs in a sample are driven from a negative magnetic saturation to positive, and vice versa. The output is the derivative of the Langevin function, also called the point spread function (PSF). The signal intensity, or height, of the PSF reflects the sensitivity of the SPIO. The full-width half maximum (FWHM) relates to the spatial resolution of the SPIO.⁶¹ A narrower tracer response shown by the FWHM, indicates superior spatial resolution and a greater signal intensity per mass of iron indicates superior sensitivity.

1.11 Quantification of MPI Signal

Quantification of the mass of iron from MPI images is one of the main advantages of this modality for cell tracking. The first step for quantifying iron is to measure the MPI signal from the image. MPI images should be displayed in full-dynamic range to ensure all of the signal is included when choosing the region of interest (ROI). The ROI is traced manually by referencing a colour look-up table (CLUT) customized for MPI images in Horos using a maximum intensity projection (MIP) for 2D images or slice by slice for 3D
images. The output provides mean signal and ROI volume. The MPI signal for the ROI is calculated by using the following equation:

\[
\text{MPI signal} = \text{Mean signal in ROI (A. U.)} \times \text{ROI volume (mm}^3 \text{ or mm}^2) 
\]

To convert MPI signal to iron content requires a calibration. A calibration line can be constructed by measuring the MPI signal from a series of diluted samples of known iron content. This is explained in more detail in Chapter 2. Other groups have calibrated the MPI signal using a single reference tube with a known iron content for calibration placed in the field of view and imaged along with the sample or animal being imaged. A disadvantage to this method is that the amount of iron in the reference sample needs to be close to the amount of iron expected in the experimental sample. For in vivo experiments this may be unknown.

For a sample prepared with a known number of cells, the average iron content per cell can be measured using ICP-MS. These measurements must be acquired using the same cells used for in your imaging experiment. Subsequently, an estimation of cell number from in vivo MPI images can be calculated:

Number of cells in vivo

\[
\text{Number of cells in vivo} = \frac{\text{iron mass measured by MPI from ROI (\(\mu g\))}}{\text{iron mass per cell measured by MPI or ICP-MS from cell sample (\(\mu g/cell\))}}
\]

1.12 Cell Tracking with MPI

Mentioned below are some of the early MPI cell tracking papers. Most papers have been of the proof-of-principle nature, demonstrating detection of cells in various models, but few have quantified iron content or cell number. So far MPI has been used to image stem cells, pancreatic islets, neural progenitor cells (NPCs), breast cancer cells, T cells, and tumour-associated macrophages (TAMs).
The majority for the cell tracking papers have studied stem cells. The first MPI cell tracking paper published was on human embryonic stem cells (hESC) that were labeled with Resovist by Saritas et al. Resovist, rebranded as Vivotrax is currently the gold standard SPIO for MPI cell tracking. They subcutaneously injected labeled cells into a postmortem mouse at two different injection sites. 1.0 x 10^5 cells were injected on the back of the neck and 2.0 x 10^5 cells on the right hind limb. The mouse was then imaged using MPI where images showed no depth attenuation, and no background signal from mouse tissue. This demonstrated for the first time that MPI had the potential to detect iron labeled cells as high contrast regions in mice. They quantified the MPI signal and found that the ratio between signal intensities was 2 to 1; the injection site containing 2.0 x 10^5 cells showed twice the signal than the 1.0 x 10^5 cell injection.12

Zheng et al. for the first time used MPI to longitudinally monitor and quantify the biodistribution and clearance of stem cells in vivo. Human mesenchymal stem cells (hMSCs) were labeled with Resovist and injected in cell numbers ranging from 5 x 10^6 to 8 x 10^6 into the mouse tail vein. MPI whole body images of the mice were taken over the course of 12 days. In the first hour of imaging this group saw that MPI signal accumulated in the lungs of mice. As they continued to image out until 12 days, they saw that signal in the lungs decreased and relocated to the liver as early as 24 hours post injection. To validate the biodistribution of Resovist in mice, the mice were sacrificed, and organs were excised and imaged using MPI. Ex vivo MPI of the organs confirmed that signal was accumulating in the lungs, liver and to a lesser degree in the spleen. This study was the first to show the biodistribution and clearance of Resovist labeled cells using MPI.54

Bulte et al. have shown that neural and mesenchymal stem cells (MSCs) can be tracked with MPI using three different SPIOs Feridex, Resovist and UW particles. Feridex and Resovist are both commonly used for MRI cell tracking and have also been used in clinical trials. However, these two iron oxide particles are no longer commercially available. This led to the motivation to develop and experiment with UW particles which are a MPI specific tailored SPIO synthesized by this group. They were able to detect as few as 5.0 x10^4 UW labeled stem cells in vivo using MPI when cells were injected into the midline
of the skull. When compared to MRI scans of the same mice the MPI data was in good agreement with the location of the regions of hypo-intensity in the MR images.\textsuperscript{53}

NPCs have been imaged by Zheng et al. 5.0 x 10^5 NPCs were labeled with Resovist and stereotactically implanted into the forebrain of two immunosuppressed rats. To study the migration and clearance of these cells they implanted an equivalent number of labeled NPCs near the lateral ventricle in a third animal. A fourth rat acted as a control, no cells were injected, instead free Resovist was injected into the forebrain. MPI scans of the brain were acquired over time and signal was detected in the brain up until day 87. MPI images showed high contrast and no background signal from animal anatomy. MPI images were quantified using a calibration curve to quantify iron content. Total MPI signal from the cell graft in the first 2 rats had non-significant decay over time. Rat 3 showed the presence of iron posterior to the implant site and significant signal clearance compared to the first 2 rats, indicating the movement and clearance of NPCs were through the ventricular system. The control animal receiving free Resovist showed no MPI signal in all the scans.\textsuperscript{51}

Nejadnik et al. were the first to demonstrate that Ferumoxytol, a USPIO used clinically for anemia and approved off-label for MRI cell tracking, can be used to track cells by MPI. In this study MSCs were labeled with either Vivotrax or Ferumoxytol and implanted into the parietal skull bone of mice and MPI images were compared with MRI. MPI showed that the iron content significantly declined over time, which correlated to the loss of cells at the implant site which was confirmed by histology. MRI showed no significant change in the region of signal loss over the same time.\textsuperscript{62}

Sehl et al. used MPI and MRI to monitor the fate of Ferumoxytol labeled MSCs along with^{19}F MRI after IV PFC administration to simultaneously image the inflammatory response against the transplanted MSCs. This group used calibration lines to quantify iron content from MPI images. A decrease in MPI signal over a 12 day period was observed. This work is exciting as it is the first time these modalities have been compared. It shows promise for therapeutic monitoring as this multimodality imaging approach allows for the visualization of MSC delivery to target, and the ability to quantify inflammation and measure the amount of MSCs at a specific location.\textsuperscript{63}
MPI has also been used to image other therapeutic cell types. For example, Wang et al. demonstrated that MPI can be used for the quantitative detection of Vivotrax labeled baboon pancreatic islets after transplantation into the liver or under the kidney capsule. Mice were imaged on day 1 using MRI to confirm labeled cells were transplanted successfully before being imaged postmortem on day 1 or day 14 using MPI. The MPI signal in the kidney decreased between days 1 and 14. This likely corresponds with a decrease in the islet mass, which is normally observed during the first two weeks due to graft deterioration. In the same mice they also observed an increase in MPI signal in the liver, thought to be due to the accumulation of dead islets in the kidney releasing iron which is cleared by the liver. Iron content in the liver and kidney was calculated using a calibration curve, similar to the methods used in this thesis. The number of labeled islets could not be estimated because the islets for phantom and islets for mice came from different batches and were of different quality. They concluded that MPI would be a suitable method for imaging these transplanted islet grafts as there is no depth attenuation and zero background tissue signal.55

Song et al. synthesized Janus iron oxide particles for MPI cell tracking. These Janus particles showed 3 times the MPI signal intensity of Vivotrax and 7 times of Ferumoxytol. Janus particles were used to label human cervical cancer HeLa cells where 30 000 cells were subcutaneously injected into the abdomen area and 2500 cells were subcutaneously implanted on the back of mice. MPI images were able to detect signal in the abdomen with high contrast. The SNR was 16.1. With the 2 500 labeled cells, MPI signal was decreased but and the SNR was 1.3. When 250 cells were implanted into the back MPI signal was able to be visualized after using background subtraction.

Arami et al. synthesized nanoparticles that targeted cancer cells. Mice were injected subcutaneously with C6 rat glioma cells into the right flank. Tumours had 3-4 weeks to form before mice were injected with the new nanoparticle via the tail vein. They compared the targeting results of the nanoparticle by attaching external magnets to the tumours of mice. Control mice had no magnet attached to their tumor. 1-2 hours after injection mice were imaged using IVIS fluorescent imaging system to evaluate the nanoparticles uptake in tumours and in the liver and spleen. Mice were imaged with MPI ex vivo. Reference
tubes were used for co-registration purposes. MPI images with the lactoferrin conjugated nanoparticles were internalized and retained in mouse tumours, and was enhanced when there was a magnet attached to mice. MPI results of the excised tumors showed that the tumor uptake was best with the combination of magnetic and lactoferrin-assisted targeting showing the greatest uptake.64

MPI has also been used to detect cells labeled in situ by IV administration of SPIOs. Makela et al. saw that TAMs labeled with either Ferumoxytol or Vivotrax could be visualized by MRI. However, strong MPI signal in the liver due to uptake of SPIOs by resident liver macrophages limited the visibility of iron in tumors ex vivo. MPI did detect SPIO-positive TAMs in lung metastases which could not be detected by MRI since the lungs also appeared with low signal intensity in MR images. This group used calibration lines to quantify the amount of iron in MPI images.65

Yu et al. also detected breast cancer cells labeled in situ by IV administration using an SPIO tailored for MPI called LodeSpin. Rats were injected subcutaneously with 7 million MDA-MB-231-luc cells allowing for BLI to confirm formation of tumours before being injected IV with LodeSpin. Rats were split into Groups A, B, and C. Group A rats had tumours in the left lower mammary fat pad and were injected with LodeSpin. Group B had tumors at the right lower flank and injected with LodeSpin. Group C was the control rat that had no tumour and was just injected with LodeSpin. MPI scans were acquired at multiple time points up until day 6. Images showed that SPIOs accumulated in tumors due to their abnormally leaky vasculature. MPI images were quantified using a calibration curve to calculate iron content.66

1.13 Limitations for Cell Tracking with MPI

As described above resolution is currently one of the major limitations of MPI. Ongoing development of MPI tailored SPIOs should improve resolution. A second limitation is the requirement to acquire images with another imaging modality to obtain anatomical information. This can be done by co-registering the MPI images with images
obtained from CT or MRI. A Momentum™ MPI/CT system is now available. Limitations and challenges specific to this project are described in Chapter 4.

1.14 Purpose of Thesis

The purpose of this thesis was to use MPI for tracking MPIO labeled breast cancer cells that are dispersed in the mouse brain. MPI cell tracking using MPIO has not yet been studied. Our previous work using MRI to track MPIO-labeled cancer cells in the mouse brain has motivated these experiments. Although MPIO particles are very different from the SPIOs that have been used so far for MPI cell tracking our preliminary evaluation of MPIO showed favorable MPI characteristics (Chapter 2). MPIO particles also have very high iron content per particle compared to other commonly used SPIOs which could enhance cell detection sensitivity. We expect that MPI will provide complementary information to MRI for cancer cell detection, most significantly MPI will provide quantitative information on iron content and cell number. Our hypothesis is that MPI can be used for quantitative MPI of MPIO labeled cells arrested in the mouse brain.

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Chapter 2

2 Introduction

This section contains a description of experiments conducted to test the precision of the MPI system and the magnetic properties of MPIO and Vivotrax. This was done to characterize these iron oxide particles before deciding if their use would be beneficial to cell tracking applications of breast cancer cells that have metastasized to the brain. Iron oxide particle characterization gave us information about expected MPI signal sensitivity and resolution from relaxometry testing. We were also able to create calibration lines for each iron oxide particle to use for iron content quantification from our in vitro MPI scans. This section also looks into the repeatability and reproducibility of the MPI system to test for uncertainty. This was explored by comparing results from three different users who imaged the same samples over three days to test reproducibility. Testing also included scanning the same sample multiple times and comparing iron measurements to also determine repeatability. We also explore how changing the number of averages used while scanning can help to detect low cell numbers in vitro as well as look into how MPI scans can affect cell viability. The methods and results from each experiment have been combined together in the following sections.

2.1 Instrumentation

2.1.1 The Momentum™ MPI System

Magnetic Insights Momentum™ MPI system was installed at Robarts Research Institute August of 2019 (Figure 2.1). This installation marked Canada’s first MPI scanner. The maximum gradient magnetic field strength is 6.1 T/m. There are 2 transmit channels (X, Z), and 2 receive channels (X, Z) in which samples can be scanned with. The MPI system comes equipped with a digital camera above the sample bed to provide photographs against which the MPI images can be overlayed for rough anatomical reference. The sample bed comes with cut outs where reference tube markers can be placed to help with
co-registration and for insertion of tubing for gas anesthesia and vacuum lines. The maximum FOV is 12 cm x 6 cm x 6 cm.

Figure 2.1: Photo of the Momentum™ MPI system from Magnetic Insight Inc. at Robarts Research Institute. (B) Photo of the sample bed of the MPI system with a sample placed at the 2 cm mark.

2.1.2 Scan Modes

There are six scan modes that can be selected from when conducting MPI scans (Table 2.1). The settings for acquisition parameters used to achieve each of these scan modes are hidden to the user and not accessible. This is meant to make the system user-friendly and turn-key. The scan mode chosen will largely depend on the application you are using the MPI for. They include: (1) Default: this mode has a moderate scan time, and provides images with compromise resolution and sensitivity, used for many different applications. Preview: this mode is used for localizer or scout scans. It is the fastest acquisition scan but comes with the cost of both low sensitivity and resolution. It is typically used first for all imaging sessions. High Sensitivity: this mode is used when expecting low signal (close to detection limit). Higher sensitivity is obtained at the cost of low image resolution. High Resolution: this mode is used when higher resolution is needed. This is obtained at the cost of acquisition time and low sensitivity. Isotropic: this mode is
used when isotropic resolution is desired. The acquisition time is the slowest of all the scan modes as the scan is done with dual channel transmit/receive along X and Z.

<table>
<thead>
<tr>
<th>Scan Mode</th>
<th>Sensitivity</th>
<th>Resolution</th>
<th>Acquisition Time</th>
<th>Gradient Strength (T/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Default</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>5.7</td>
</tr>
<tr>
<td>Preview</td>
<td>Low</td>
<td>Low</td>
<td>Fastest</td>
<td>3</td>
</tr>
<tr>
<td>Fast</td>
<td>Moderate</td>
<td>Low-Moderate</td>
<td>Fast</td>
<td>5</td>
</tr>
<tr>
<td>High Sensitivity</td>
<td>Highest</td>
<td>Lowest</td>
<td>Moderate</td>
<td>3</td>
</tr>
<tr>
<td>High Resolution</td>
<td>High</td>
<td>Highest</td>
<td>Slow</td>
<td>6.1</td>
</tr>
<tr>
<td>Isotropic</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Slowest</td>
<td>5.7</td>
</tr>
</tbody>
</table>

**Table 2.1:** Table summarizing the different MPI scan modes and the kind of performance that can be expected with each mode.

## 2.2 Methods and Results

### 2.2.1 Iron Oxide Particles

MPI is a tracer driven imaging technique and yet there are currently no commercially available MPI dedicated tracers. The most commonly used iron particle for MPI is ferucarbotran, originally a MRI contrast agent known as Resovist, and now made available as Vivotrax by Magnetic Insight Inc. In this thesis we analyzed the MPI characteristics of Vivotrax, since it is currently the benchmark against which other iron particles for MPI are compared, and MPIO since this iron particle is the focus of the thesis.

Vivotrax is a polydisperse mixture of particles containing both single iron cores (~5 nm) and clusters of multiple cores (effective core size ~25 nm), leading to a bimodal size distribution (illustrated in Figure 1.8). Only the larger clustered particles are thought to
contribute to the MPI signal. MPIO are micron-sized iron oxide particles called Bangs Beads (also referred to as magnetic microspheres) from Bangs Laboratories Inc, Fishers, IN, USA. The MPIO used in this thesis have a mean hydrodynamic size of 0.9 μm and a broad size distribution (0.5 to 2 μm). These MPIO have many small iron cores (5 - 10 nm) embedded in a polystyrene matrix; the effective core size is larger but has not been determined experimentally. Appendix A contains electron microscopy images, gathered from manufacturers or published manuscripts, for Vivotrax, MPIO and other commercially available particles used for MPI.

2.2.2 Relaxometry

The Momentum™ MPI system at Robarts comes equipped with what is called a RELAX module. This module allows users to characterize SPIOs of interest. As described in 1.11 the RELAX module allows for measurements of the FWHM (spatial resolution) and signal per gram of iron (sensitivity) for individual SPIO.

Relaxometry was performed for MPIO and Vivotrax. Samples of each were made by diluting the iron particle solution in PBS so that the samples contained the same amount of iron (30 mg), and each sample was scanned separately. The RELAX data takes approximately one minute to acquire per sample. This data is shown in Figure 2.2. The amplitude of the MPI signal for MPIO was higher than Vivotrax; the relative sensitivity was 1.5 for MPIO versus 1.0 for Vivotrax. The FWHM was larger for MPIO; the resolution of MPIO was 2.7 times that of Vivotrax; 4.49 mm versus 1.69 mm for a 6.1 T/m gradient. These preliminary results showed that MPIO could be a suitable SPIO for further MPI experiments, with high sensitivity, albeit relatively low resolution.
2.2.3 Measuring the MPI Signal and Calibration for SPIOs

To convert the total MPI signal to iron content requires a calibration. The relationship between MPI signal and iron content can be determined by measuring the MPI signal from a series of diluted samples of known iron content. The same type of SPIO and the same scan mode which will be used for the imaging experiment must be used for the acquisition of images for the calibration. The MPI signal is measured from the image of each sample and a calibration line is constructed by plotting the MPI signal for each sample versus the known values for iron mass in each sample. Subsequently, the equation of this line can be used to determine the iron content associated with MPI signal measured from an ROI.

In this thesis calibration lines were made for MPIO and Vivotrax with different scan modes depending on the experiment. Samples of either Vivotrax or MPIO were diluted in PBS into aliquots of 100%, 75%, 50%, 37.5%, 25%, 10%, 7.5%, 5%, 3.75%, 2.5% of iron. 1μL of each dilution was then pipetted into capillary tubing and 5 samples at

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Iron content (mg/mL)</th>
<th>Sensitivity/iron content</th>
<th>Relative Sensitivity</th>
<th>FWHM (mT)</th>
<th>FWHM (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vivotrax</td>
<td>5.5</td>
<td>38.34</td>
<td>1.000</td>
<td>10.342</td>
<td>1.695</td>
</tr>
<tr>
<td>MPIO</td>
<td>2.8</td>
<td>57.71</td>
<td>1.565</td>
<td>27.404</td>
<td>4.492</td>
</tr>
</tbody>
</table>

Figure 2.2: (A) Point spread functions (PSF) for MPIO and Vivotrax samples of 30 mg iron. MPIO has a higher peak signal indicating higher sensitivity. (B) MPIO has a larger full width at half maximum (FWHM) indicating poorer resolution compared to Vivotrax. (C) MPI image of MPIO sample containing 30 mg iron and (D) Vivotrax with corresponding PSF to the right of the images.
a time were spaced 2 cm from each other on the MPI sample bed to be imaged. The FOV was 12 x 6 x 6 cm. Representative images and calibration lines for MPIO and Vivotrax are shown in Figure 2.3. Linear correlations were conducted between MPI signal and iron content to determine Pearson’s correlation coefficient. The equation for each line (y = mx + b; where m is the slope and b is the y-intercept) (shown below the plots) is used to determine iron content (x) from the MPI signal (y).

![Figure 2.3](image)

**Figure 2.3:** 2D MPI images of a series of diluted samples of Vivotrax (A) and MPIO (B) used to create the calibration lines shown in (C) and (D). The relationship between iron content and MPI signal is linear for both nanoparticles. Equations derived from the calibrations (shown above the plots) are used for calculating iron content in MPI images.
2.2.4 Repeatability and Reproducibility Measurement

To measure the precision of our new MPI system we acquired data for MPIO and Vivotrax to assess repeatability and reproducibility. Repeatability measures the variation in measurements taken by a single instrument and/or person under the same conditions. Samples containing 5 μL of MPIO or Vivotrax mixed with 50 μL of PBS were prepared. The suppliers of Vivotrax (Magnetic Insight Inc.) and MPIO (Bangs Laboratories) indicate that the iron concentrations is 5.5 mg of iron/ml for Vivotrax and 2.8 μg of iron/ml for MPIO, this amounts to an expected value of 27.5 μg of iron for 5 μL of Vivotrax and 14 μg of iron for 5 μL of MPIO.

In the first experiment the samples were placed on the MPI sample bed at the 2 cm mark and scanned six times without moving. In the second experiment the same samples were again scanned six times at the 2 cm mark but between scans the sample was removed and then repositioned. In the third experiment the same samples were scanned six times without moving it after being placed at the 4 cm mark. All data was acquired in default mode. The images for these samples are shown in Figure 2.4 and for Vivotrax in Figure 2.5.
Figure 2.4: (A) Six MPI scans of a MPIO sample scanned at the 2 cm mark on the MPI sample bed, shown in a full FOV. (B) Six MPI scans of a MPIO sample which was scanned and then removed from the scanner and repositioned at the 2 cm mark for each subsequent scan. (C) Six MPI scans of a MPIO sample which was scanned at the 4 cm mark on the MPI sample bed.
Figure 2.5: (A) Six MPI scans of a Vivotrax sample scanned at the 2 cm mark on the MPI sample bed, shown in a full FOV. (B) Six MPI scans of a Vivotrax sample which was scanned and then removed from the scanner and repositioned at the 2 cm mark for each subsequent scan. (C) Six MPI scans of a Vivotrax sample which was scanned at the 4 cm mark on the MPI sample bed.
For each image the total MPI signal was measured and the iron content calculated using calibration lines described in 2.2.2. The individual values for iron content and the average value and standard deviation of these measurements are shown in Table 2.2 for MPIO samples and Table 2.3 for Vivotrax for each experiment. The value for standard deviation reports on the variation in measurements and thus repeatability for these experiments. The smaller the number the lower the variability. Our results show that there was some variation in the measurements for all experiments. There was more variability for MPIO samples compared to Vivotrax.

<table>
<thead>
<tr>
<th>MPIO</th>
<th>Experiment 1: Iron Content (µg)</th>
<th>Experiment 2: Iron Content (µg)</th>
<th>Experiment 3: Iron Content (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan 1</td>
<td>18.19</td>
<td>Scan 1</td>
<td>19.10</td>
</tr>
<tr>
<td>Scan 2</td>
<td>18.31</td>
<td>Scan 2</td>
<td>19.49</td>
</tr>
<tr>
<td>Scan 3</td>
<td>17.20</td>
<td>Scan 3</td>
<td>20.82</td>
</tr>
<tr>
<td>Scan 4</td>
<td>16.62</td>
<td>Scan 4</td>
<td>20.69</td>
</tr>
<tr>
<td>Scan 5</td>
<td>17.16</td>
<td>Scan 5</td>
<td>15.49</td>
</tr>
<tr>
<td>Scan 6</td>
<td>17.12</td>
<td>Scan 6</td>
<td>21.41</td>
</tr>
<tr>
<td>Average</td>
<td>17.43</td>
<td>Average</td>
<td>19.5</td>
</tr>
<tr>
<td>Std Dev</td>
<td>0.67</td>
<td>Std Dev</td>
<td>2.15</td>
</tr>
</tbody>
</table>

Table 2.2: Table summarizing the iron content measured from each MPIO sample that was scanned in Figure 2.4, the mean iron content of the 6 samples, and the standard deviation.

<table>
<thead>
<tr>
<th>Vivotrax</th>
<th>Experiment 1: Iron Content (µg)</th>
<th>Experiment 2: Iron Content (µg)</th>
<th>Experiment 3: Iron Content (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan 1</td>
<td>29.72</td>
<td>Scan 1</td>
<td>29.14</td>
</tr>
<tr>
<td>Scan 2</td>
<td>28.99</td>
<td>Scan 2</td>
<td>29.19</td>
</tr>
<tr>
<td>Scan 3</td>
<td>28.77</td>
<td>Scan 3</td>
<td>29.09</td>
</tr>
<tr>
<td>Scan 4</td>
<td>29.13</td>
<td>Scan 4</td>
<td>28.81</td>
</tr>
<tr>
<td>Scan 5</td>
<td>28.62</td>
<td>Scan 5</td>
<td>28.64</td>
</tr>
<tr>
<td>Scan 6</td>
<td>29.04</td>
<td>Scan 6</td>
<td>28.81</td>
</tr>
<tr>
<td>Average</td>
<td>29.01</td>
<td>Average</td>
<td>28.95</td>
</tr>
<tr>
<td>Std Dev</td>
<td>0.38</td>
<td>Std Dev</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 2.3: Table summarizing the iron content measured from each Vivotrax sample that was scanned in Figure 2.5, the average iron content of the 6 samples, and the standard deviation.
Reproducibility is most often defined as the degree of agreement between the results of experiments conducted by different individuals, or different location or instruments and measures whether an experiment can be reproduced. For measuring uncertainty in an instrument reproducibility can also be assessed by changing the conditions of the measurement for the same sample. In this case a measure of reproducibility can be made by performing a repeatability test, changing a variable and repeating the repeatability test (as done above), calculating the average values for each test and calculating the standard deviation of the test averages. This was calculated for experiment 1 versus 2 and experiment 1 versus 3 for MPIO and Vivotrax. The standard deviations were 1.05, and 0.42 for MPIO and 0.11, and 0.43 for Vivotrax.

2.3 In Vitro MPI of SPIO-Labeled Cells

2.3.1 Cell Labeling with SPIOs

Our lab has been developing cell labeling techniques for many years and has experimented with a range of cell lines and SPIOs. In this thesis, MPIO and Vivotrax were the two SPIOs used for cell labeling. For cell culture, cells were maintained at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin antibiotic. Cells were passaged every 2-3 days to prevent overgrowing and to discard dead cells and debris. For cell labeling with MPIO, adherent cells were incubated with 25 μg Fe/mL of MPIO for 24 hours. Cells were then washed three times with Hanks balanced salt solution (HBSS) and then trypsinized with 0.25% Trypsin-EDTA. The cells were then collected and washed another three times with HBSS to remove unincorporated MPIO before being used.

For cell labeling with Vivotrax we have seen that simple coincubation does not produce good labeling efficiency, therefore, labeling was enhanced by the use of transfection agents. A labeling mixture is prepared using 2 falcon tubes of 2.5 mL of serum free DMEM. In tube A, 60 μL of stock protamine sulfate was added and vortexed to mix.
In tube B, 90 μL of Vivotrax was added and vortexed to mix. 20 μL of stock heparin was then added to tube B and then vortexed to mix. Old media was removed from cells and rinse once with HBSS. Tubes A and B were combined and vortexed. This labeling mix was added to the flask and incubated at 37°C, 5% CO2 for 2-4 hours. 5 mL of complete DMEM was added to the flask after the 2-4 hours and incubated at 37°C, 5% CO2 overnight. This protocol has been published by Arbab et al. and implemented for various cell types and SPIO in the Foster lab. On day 2 cells were washed 3 times with HBSS and then trypsinized with 0.25% Trypsin-EDTA. The cells were then collected and washed another three times with HBSS to remove unincorporated Vivotrax before being used. For both labeling methods, cell viability after labeling was assessed using the trypan blue exclusion assay and labeling efficiency was assessed by staining for iron with Perl’s Prussian blue (PPB) and subsequently counting the number of positively stained cells in 3 random 40x magnification fields under the microscope. Representative PPB images of MPIO and Vivotrax labeled cells are shown in Figure 2.6.

![Figure 2.6](image)

**Figure 2.6:** (A) Representative Perl’s Prussian Blue (PPB) staining of MDA-231-BR cells labeled with (A) MPIO and B) Vivotrax shown at 40x magnification.

### 2.3.2 Reproducibility for MPI of Cell Samples

Reproducibility was also assessed by scanning MPIO-labeled cell samples on three separate days by 3 different MPI users. This looked at both variations in the measurements of the same samples on different days, and at variations in the quantification of images by
different users. Cell samples were created by co-incubating MDA-231-BR cells with 25 μg Fe/mL of MPIO particles. Three samples were made each containing $2.5 \times 10^5$ MPIO labeled MDA-231-BR cells. Each user scanned the three samples three times, three days in a row. Images were acquired in 2D high sensitivity mode with a FOV of 12 x 6 x 6 cm, each sample was placed at the – 2cm center line.

Each user individually quantified their data using the same quantification protocol and calibration line. Figure 2.7 shows all of the MPI scans along with the values for iron content measured from MPI. Table 2.4 shows the data for all measurements. The average values for iron content for each user are compared in Figure 2.8. There was no significant difference in these measurements day to day or from sample to sample for Users 1 and 2. The measurements from User 3 were significantly higher than Users 1 and 2. The higher values for User 3 may be attributed to the user drawing a larger ROI area when measuring the mean MPI signal, which is used in the following equation to determine total MPI signal and subsequently iron content.

$$\text{MPI signal} = \text{Mean signal in ROI (A. U.)} \times \text{ROI volume (mm}^3 \text{ or mm}^2)$$

When it comes to choosing the ROI the manual outlining of an ROI is user dependent and the signal interpreted as true signal may vary between users. A way to help mitigate this user variability is by having consistent protocols and training when it comes to teaching users best quantification practices.
Figure 2.7: MPI images of three samples of $2.5 \times 10^5$ MPIO labeled cells imaged on three different days and the iron content quantified by User 1 (A), User 2 (B), and User 3 (C). For each user there was no significant difference between the measurements made for each sample on each day or for the measurements made between days.
Table 2.4: Table summarizing the iron content calculated for each sample that was scanned in Figure 2.7, the average iron content of the three samples scanned on each day and the standard deviation calculated by each user.

**Figure 2.8:** The average iron content measured for the three cell samples by each user on each day of the experiment. **The values measured by User 3 were significantly higher than those measured for Users 1 and 2, on each day.**
2.3.3 SPIO Labeled Cell Detection Limits

To assess differences in the detection limits between MPIO and Vivotrax labeled cells we compared samples containing the same number of cells using the same MPI imaging parameters. Eighteen cell samples were prepared for each of the MPIO and Vivotrax labeled MDA-231-BR cells; three samples of six different cell numbers: 2.0 x 10^5, 1.0 x 10^5, 5.0 x 10^4, 1.0 x 10^4, 5.0 x 10^3 and 2.5 x 10^3 cells. Each sample was scanned using the 2D High Sensitivity/Isotropic mode. The scan time for each sample was ~ 3 minutes. Representative images for one set of samples for each nanoparticle are shown in Figure 2.9. Images collected with no sample (empty bed) and no sample bed (empty bore) are provided to show the background noise level. The lowest cell number that could be detected clearly under these imaging conditions was 1.0 x 10^4 for both MPIO and Vivotrax labeled cells. Images containing fewer cells were difficult to quantify as true signal was hard delineate from noise. The measured MPI signal for each cell number is shown in plots in Figure 2.10 and all data is presented in Table 2.5. To increase MPI sensitivity the number of signal averages was increased to 8 (~17 minutes) and the samples with the lowest cell number were scanned again. Scanning with 8 averages improved the detection of the cell sample compared to no averaging.
Figure 2.9: MPI images of MPIO (A) and Vivotrax (B) labeled cell samples with decreasing cell numbers. The white square indicates the position of the cell sample. The empty bed and empty bore scans show the background signal.
Figure 2.10: (A) Graphs showing the iron content measured from each cell sample scanned in Figure 2.9. The results show decreasing iron content with decreasing cell number and similar detection limits for MPIO and Vivotrax.

Table 2.5: Table showing the iron content measured from each cell sample scanned in Figure 2.9.
Iron content measurements for both the MPIO and Vivotrax labeled cells were similar. This was unexpected since cells labeled with MPIO typically have a higher iron load than Vivotrax. The PPB staining of Vivotrax labeled cells could shed some light on this result. The use of transfection agents to enhance labeling with Vivotrax appears to lead to more extracellular iron and iron clumping and this may lead to an overestimation of iron in a cell sample. Future work will examine the impact of labeling with transfection agents more closely.

This experiment was not meant to determine the minimum number of cells that could be detected, since this depends on imaging parameters and could be further improved with careful and systematic optimization of scan parameters. This experiment demonstrated how adjusting imaging parameters (i.e., increasing the number of averages) was able to improve MPI images where signal was originally difficult to detect.

2.3.4 MPIO Labeled Cell Viability After MPI

It is important to know if MPI affects cell viability. Since the technology is just emerging for cell tracking this had not been assessed before. The main concern is that the magnetic particles release energy as heat when exposed to alternating magnetic fields. The frequency (40 kHz) and excitation combination (20 mT) used by MPI are designed to be low enough not to increase temperature, however it is difficult to know how hot the surface temperature of a particle gets in comparison to tissue level. The other concern is that the alternating and moving fields cause the particles to physically rotate and this could potentially cause cellular damage.

Six cell samples were prepared. Three samples were scanned separately, and three samples served as control samples (no MPI). A trypan blue exclusion assay was first conducted on both the control samples and the samples that were going to be scanned by MPI. This was done by taking 10 μL of the cell solution and mixing it with 10 μL of the trypan blue dye. The mixture was pipetted into a cell counting chamber slide and inserted into the Countess™. Cell viability is reported as the percentage of stained cells. The labeled cancer cells then underwent a 2D high sensitivity MPI scan (about a 1 minute scan). The trypan blue exclusion assay was then repeated again on both the control and MPI scanned
samples. The samples had 90-93% cell viability before MPI scans and 88-90% cell viability after MPI scanning. Figure 2.11 shows a plot of the cell viability for all the control and MPI samples. There was no significant difference in % viability between MPI samples before and after being scanned. There was also no significant difference between control and MPI sample before as well as control and MPI samples after. There was however a significant difference in % viability between control before and after values.

Figure 2.11: Graph showing the percent viability between control and MPI scanned samples of MPIO labeled cells before and after MPI scans. The viability for control samples was 90-93% before and 88-89% after the experiment. The viability for samples scanned by MPI was 90-93% before and 89-90% after the experiment. There was no significant difference in viability before and after the experiment of MPI samples and no significant difference in viability when comparing control and MPI scanned samples. However, there was a significant difference in control % viability before and after.

2.3.5 Statistical Analysis

Simple linear regression was used to evaluate the relationship between total MPI signal and iron content and to create equations used for iron content quantification and the goodness of fit R² value. Repeatability and reproducibility data are presented as mean +/-
standard deviation; a 5% difference was deemed as good reproducibility. The data which compared measurements from 3 different MPI users used a repeated measures one-way ANOVA to determine statistical significance for each of the following: a) between samples from each user, b) between days for each user and c) iron values between all the users. Simple linear regression was performed on the cell detection data to determine the relationship between MPI signal and cell number represented by the $R^2$ value. A paired t-test was performed on the MPI viability data to determine statistical significance between control samples at the before and after time points. Another paired t-test was performed on the MPI samples before and after the MPI scan. Welch’s t-tests were done between all ‘before samples’ and all ‘after samples’ to determine statistical significance. These analyses were conducted using Prism software (8.0.2, GraphPad Inc.), where $p < 0.05$ was considered statistically significant.

2.4 Discussion

Relaxometry was performed on both MPIO and Vivotrax to provide information on the magnetic properties, and MPI performance, of these particles. This data provided values for resolution and sensitivity for each particle. The amplitude of the MPI signal for MPIO was higher than Vivotrax; the relative sensitivity was 1.5 for MPIO versus Vivotrax. The FWHM was larger for MPIO with a resolution 4.49 mm versus 1.69 mm for Vivotrax. This showed that MPIO could potentially be a suitable SPIO for MPI experiments. We continued to compare MPIO to Vivotrax in further experiments.

When we first started quantifying iron from MPI images we used a method that used a reference tube with a known amount of the SPIO, that was scanned alongside a cell sample or mouse in the same FOV. The signal from the subject of interest could then be compared to the signal from the reference tube to determine iron content. This was not always a reliable method as sometimes signal from the reference tube would interfere with the signal of interest because the iron content in the reference tube was greater than the iron in the sample. Trying to estimate how much iron is in the sample or subject being
imaged, so that the iron content in the reference tube was similar is challenging since it is usually not known, especially when scanning mice in vivo.

The use of a calibration line for quantifying iron content from MPI data was developed in our lab, in collaboration with Dr. Ashley Makela a former PhD student in the Foster lab, now using MPI as a postdoctoral fellow at Michigan State University. Calibration lines could be made after an imaging session based on the exact scanning parameters and a range of dilutions and produced fewer variable results. Calibration lines were made for MPIO and Vivotrax with different scan modes depending on the experiment. Samples of either Vivotrax or MPIO were diluted in PBS into aliquots before being scanned. This method was used to quantify all the iron content data in this thesis and is now used routinely in our lab for all other MPI experiments.

Repeatability tests were conducted on the MPI system to determine how variable the data acquisition and analysis of data was. In experiment 1, samples were scanned six times without being removed from their position on the MPI bed. Those same samples were then taken out and placed back in the MPI bed, in between individual scans, for another round of six scans. Lastly, the samples were placed at the 4 cm mark on the MPI and scanned six times without being moved. Results showed that there is some variation in the measurements for all experiments. For assessing the precision of a new instrument, the manufacturers (in this case Magnetic Insight Inc.) consider 5% signal change to be the acceptable variation for a sample (100% Vivotrax) scanned repeatedly. Our data was not always within this range. The variability was less for the Vivotrax data compared to the MPIO data. This could be related to clumping of the larger MPIO particles. Recent data acquired by other students in the lab has indicated that vortexing of samples immediately prior to scanning changes the MPI signal. For MPIO the variability was lowest when the sample was at the 2 cm mark and not moved in between scans. The ‘true’ iron content for the Vivotrax sample (determined using manufacturer’s stated iron concentration) was 27.5 μg and the mean values measured for the three conditions were 28.5, 28.9 and 29.0, which are within 6% of the true value. For the MPIO sample, the iron content was 14 μg and the mean values for the three conditions were 17.4, 19.5 and 20.3, which are farther off.
Reproducibility was tested by having 3 different MPI users scan and analyze the same sample of \(2.5 \times 10^5\) cells that were MPIO-labeled. Results showed that even though these users were analyzing the same sample using the same quantification methods there was some variability in iron mass calculated. While user 1 and 2 results were not significantly different user 3’s results were significantly different from user 1 and 2. This is likely due to the fact that there is subjectivity when it comes to the amount of signal included in the ROI that is manually drawn by each individual user. Some may choose to include more signal than the other. This can also influence the values for MPI signal and area or volume which are used to calculate iron content.

Cell detection limits were also explored using MPIO or Vivotrax labeled cell samples ranging from \(2.5 \times 10^5\) to \(2.5 \times 10^3\) cells and scanned using the 2D high sensitivity isotopic mode. For both MPIO and Vivotrax, images of samples which contained fewer than \(1.0 \times 10^4\) cells showed increasing signal in the background and made it more challenging to detect and quantify the MPI signal. For the samples with the lowest cell number (\(2.5 \times 10^3\)) a 2D high sensitivity isotopic scan with 8 averages was also acquired to determine whether this approach would improve the signal and enhance detection. On the Momentum™ MPI system the projection images are acquired as 2D panels which are then stitched together to form the entire image. The overlap fraction is how much these panels overlap each other and are best thought of as a method of averaging. In this case a higher overlap fraction results in more signal averages and more signal for higher sensitivity; the trade-off is scan time. The scan time for images acquired with no averaging was 3 minutes, with 8 averages the scan time was 17 minutes. The relationship between signal averaging, via overlap fraction, and sensitivity has not been well studied.

These results showed how adding additional averages could be utilized to improve MPI image quality it comes with the trade-off of increased scan time from ~3 minutes with 1 average to ~17 minutes with 8 averages.
2.5 References


Chapter 3

3 Superparamagnetic microspheres can be used for magnetic particle imaging of cancer cells arrested in the mouse brain

The work in Chapter 3 was submitted as a manuscript to Magnetic Resonance in Medicine in September 2020. It has come back with requests for revisions and will be resubmitted by December 2020. For Chapter 3, some additional data has been added to the manuscript.

3.1 Introduction

Cellular MRI combines the ability to obtain high-resolution MRI data with the use of magnetic contrast agents for labeling specific cells, thereby enhancing their detectability. The most widely used cell labeling agents for cell tracking are magnetite (Fe₃O₄)-based super paramagnetic iron oxide (SPIO) nanoparticles. Commonly used iron oxide nanoparticles consist of a small (< 10 nm) iron oxide crystal core covered by a dextran coating bringing the total hydrodynamic size of the particles to approximately 20-50 nm (ultrasmall, USPIO) or 60-150 nm (standard, SPIO), respectively. Micron-sized iron oxide particles (MPIO) have also been used for preclinical cell tracking studies. MPIO are superparamagnetic microspheres with multiple small iron crystals (5-10 nm) distributed throughout a polymer matrix that are relatively large (0.9 – 1.63 μm hydrodynamic size). Of the SPIO used for MRI cell tracking, MPIO have the highest iron content per particle, approximately 1pg Fe/particle which is roughly equivalent to 1.5 million standard SPIO particles or 4 million USPIO. The presence of SPIOs causes a distortion in the magnetic field and leads to signal hypo-intensities in iron-sensitive images (T2- and T2*-weighted images are most often used). Areas containing SPIO-labeled cells appear as regions of low signal intensity (signal voids) on MRI images, creating negative contrast. Many different cell types have been pre-labeled with iron particles and tracked with MRI, including mesenchymal stem cells, progenitor cells, dendritic cells, cancer cells and
pancreatic islets. This technique is highly sensitive, permitting the imaging of single cells in vivo, under ideal conditions.

There are, however, several limitations of iron-based MRI cell tracking. The first is low specificity due to other low-signal regions in T2/T2* images, i.e., SPIO-labeled cells in the lung or in a region of hemorrhage cannot be detected. Although ultra-short echo time imaging methods have been developed for producing positive contrast from iron-labeled cells these too have similar problems with specificity. Second, quantification of iron-induced signal loss is complicated. Our group and others have shown that the degree of signal loss produced by SPIO-labeled cells is only linear at low iron concentrations. Typically, the degree of contrast (how black is it) or the volume of signal loss (how big a void is there) is measured from these images. Although some studies have used MR-based relaxometry to show a linear relationship between R2* values and SPIO-labeled cell concentration in samples the in vivo cell quantification by this method is more complicated. Overall, with iron-based MRI cell tracking there are issues with specificity and iron-labeled cell number cannot be accurately determined.

Magnetic Particle Imaging (MPI) is a new imaging modality that directly detects SPIOs. MPI cell tracking may address the limitations presented by SPIO-based cell tracking. First, the MPI signal is only generated when the magnetic moments of the SPIOs rotate; this change in magnetization is in response to the application of an excitation field and is localized only to the SPIO within a region devoid of a magnetic gradient. This results in positive “hot-spot” contrast that provides spatial localization without ambiguity. This is because there is no signal from within the subject as biological tissues neither generate nor attenuate MPI signals. Second, the MPI signal is linearly quantitative with iron concentration, and therefore the number of SPIO labeled cells can be directly calculated. The sensitivity of MPI derives from the direct detection of the electronic magnetization of SPIO, which is 10^8 times larger than the nuclear magnetization of protons seen in MRI. This translates to a theoretical MPI sensitivity in the hundreds of cells with current hardware and available SPIO. The highest cell detection sensitivity to date was reported to be 250 cells in vivo.
The ideal SPIOs for MPI are still not known. Both MPI sensitivity and resolution are closely related to the physical properties of a SPIO. The resolution of MPI is driven primarily by the interaction of the nanoparticle and the magnetic field gradient. Theoretical modeling predicts that resolution improves with increasing core size. However, this has not always been observed experimentally. The sensitivity of MPI depends on both nanoparticle and scanner specific factors. Nanoparticle factors include the strength of the nanoparticle magnetization (the greater the magnetization the greater the MPI signal and therefore higher sensitivity) and the efficiency of the nanoparticle cell labeling (more iron per cell leads to higher sensitivity).

Currently the most commonly used SPIO for MPI has been a commercially available agent, Vivotrax from Magnetic Insight Inc. (USA). Vivotrax is a Ferucarbotran with multi-core/aggregated particles and coated with carboxy-dextran. Vivotrax has been used in MPI studies of mice to detect mesenchymal stem cells\textsuperscript{24,25}, neural stem cells\textsuperscript{26}, neural progenitor cells\textsuperscript{27}, pancreatic islets\textsuperscript{28}, T-cells\textsuperscript{29}, and macrophages\textsuperscript{30,31}

Our lab has previously used MPIO to label metastatic cancer cells for detection in the mouse brain by MRI. In our previous studies MPIO-labeled cancer cells were administered via intra-cardiac injection which results in their arrest throughout the brain vasculature as individual cells or clusters of small numbers of cells. The high cellular iron loading created when labeling cells with MPIO (20-30 pg of iron/cell) permitted the detection of single cells in vivo by MRI\textsuperscript{10}. At this time there are no published reports of the use of MPIO-labeled cells for their in vivo detection by MPI. Therefore, the goal of this study was to evaluate if MPIO can be used for in vivo detection and quantification of cancer cells distributed in the mouse brain by MPI.

### 3.2 Methods

#### 3.2.1 Iron Oxide Nanoparticles Relaxometry

Two types of iron oxide nanoparticles were used in these studies: (i) Vivotrax (Magnetic Insight Inc, Alameda, CA, USA) and (ii) MPIO (Bangs Laboratories Inc,
Fishers, IN). The particle relaxometer module (RELAX) on the Momentum™ MPI system (Magnetic Insight Inc., Alameda, California) was used to characterize MPIO and Vivotrax (30 mg Fe for each). In this mode, the localizer gradient field is switched off and a negative magnetic field is turned on and then switched to a positive field (and vice versa). As a result, iron nanoparticles are driven from a negative magnetic saturation to positive (positive scan) and vice versa (negative scan). This measures the point spread function (PSF) of the nanoparticles and allows for measurements of the MPI signal per iron content (sensitivity) and full-width half-maximum (FWHM; spatial resolution). A narrower tracer response indicates superior spatial resolution and a greater signal intensity per unit iron indicates superior sensitivity.

3.2.2 Calibration Line Preparation

Calibration lines were generated for use in quantifying iron content in brains after iron-labeled cell injection. To construct each line, samples of each nanoparticle were scanned in the same mode as images being analyzed (In vivo = 3D isotropic, ex vivo = 2D default). Samples of either Vivotrax or MPIO were diluted in PBS into 100%, 75%, 50%, 37.5%, 25%, 10%, 7.5%, 5%, 3.75%, 2.5% aliquots. 1μL of each dilution was then pipetted into capillary tubing and spaced out 2 cm from each other on the MPI sample bed to be imaged, 5 samples at a time. The field of view (FOV) was 12 x 6 x 6 cm.

3.2.3 Cell Culture and Labeling

Two cancer cell types with similar growth characteristics were used in these studies: (i) human MDA-231-BR brain metastatic breast cancer cell line and (ii) murine 4T1BR5 metastatic breast cancer cell line. Both cell lines were maintained at 37°C and 5% CO₂ in DMEM (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin streptomycin antibiotic. Cells were passaged every 2-3 days. For cell labeling with MPIO, adherent cells were incubated with 25 μg Fe/mL MPIO beads for 24 hours. Cells were washed three times with Hanks balanced salt solution (HBSS) and then trypsinized with 0.25% Trypsin-EDTA. The cells were then collected and washed another three times with HBSS to remove unincorporated MPIO before cell injection and in vitro evaluation. For cell labeling with
Vivotrax, cells were grown for 2-4 days until they reach 80-90% confluency. A labeling mixture was prepared using 2 falcon tubes of 2.5 mL of serum free DMEM. In tube A, 60 μL of stock protamine sulfate was added and vortexed to mix. In tube B, 90 μL of Vivotrax was added and vortexed to mix. 20 μL of stock heparin was then added to tube B and then vortexed to mix. Old media was removed from cells and rinse once with HBSS. Tubes A and B were combined and vortexed. This labeling mix was added to the flask and incubated at 37°C, 5% CO2 for 2-4 hours. 5 mL of complete DMEM was added to the flask after the 2-4 hours and incubated at 37°C, 5% CO2 overnight. On day 2 cells were washed 3 times with HBSS and then trypsinized with 0.25% Trypsin-EDTA. The cells were then collected and washed another three times with HBSS to remove unincorporated Vivotrax before cell injection and in vitro evaluation. This approach for cell labeling is what we routinely do for labeling cells with ferumoxytol nanoparticles (i.e., Feraheme) and was used in this study for cell labeling with Vivotrax because cells could not be labeled efficiently with simple co-incubation, as they could be with MPIO. In both scenarios, cell viability after labeling was assessed using the trypan blue exclusion assay and labeling efficiency was assessed by Perl’s Prussian Blue (PPB) staining by counting the number of positively stained cells in 3 random 40x magnification fields under the microscope.

3.2.4 Mouse Model

Female nude (nu/nu) (6-8 weeks; Charles River Canada or USA) or NSG (NOD/SCID/IL1r−/−) mice were obtained and cared for in accordance with the standards of the Canadian Council on Animal Care, under an approved protocol by the Animal Use Subcommittee of Western University’s Council on Animal Care and Use Committee. Mice were anesthetized with isoflurane administered at 2% in oxygen and iron-labeled cancer cells were injected intracardially into the left ventricle of the heart under ultrasound guidance using a Vevo 2100 ultrasound system (Visual Sonics Inc.). The delivery of cells to the brain after intracardiac injection is related to the cardiac output, with only 15-20% of cardiac output reaching the brain. Based on this, we estimate that a similar percentage of iron-labeled cells will be initially delivered to the brain with a technically accurate injection. In our previous studies which have used intracardiac injection of iron-labeled cells to study brain metastasis, MRI is performed on the same day of the injection to verify
delivery of cells to the brain. We have demonstrated that the number of signal voids, caused by iron-labeled cells, increases with the number of cells injected. When considering cell detection sensitivity for MPI it is worth noting that cells injected this way will be distributed throughout the entire brain, not in a similar region all together.

Experiment 1 (ex vivo MPI): Mice received an intracardiac injection of either $2.5 \times 10^5$ (n=2, ~50,000 cells in brain) or $5 \times 10^5$ (n=2, ~100,000 cells in brain) MPIO-labeled 231BR cells in 0.1 mL of HBSS. One mouse from each group was imaged with MRI in vivo (as described below) on the day of the injection (day 0) and then the mice were euthanized, and the fixed mouse heads were shipped to Michigan State University (East Lansing, MI) to be imaged with MPI on a Momentum™ MPI system by co-author AM. At this time, we did not have an MPI system installed at Robarts Research Institute. The goal of this preliminary experiment was to determine if MPIO-labeled cells could be detected in this mouse brain model system. MPI data was acquired with default 2D scans using a 4 x 6 x 6 cm FOV and a 5.7 T/m gradient.

Experiment 2 (in vivo MPI): Mice received an intracardiac injection of either $2.5 \times 10^5$ (n=3, ~50,000 cells in brain) or $5 \times 10^4$ (n=3, ~10,000 cells in brain) MPIO-labeled 231BR cells in 0.1 mL of HBSS. These mice were imaged in vivo on the day of the injection (day 0) with MRI to verify the success of the cell delivery to the brain and then MPI on a Momentum™ MPI system at Robarts Research Institute. 3D images were acquired using a 3 T/m gradient, 35 projections and a FOV 12 x 6 x 6 cm, with a total scan time ~1 hour per mouse (the 3D high sensitivity mode on the Momentum™ system).

Experiment 3: Mice received an intracardiac injection of $5 \times 10^4$ Vivotrax- or MPIO-labeled 4T1BR5 cells (n=4 per group, ~10,000 cells in brain). Mice were imaged in vivo on the day of the injection (day 0) with MRI to verify the cell injection and MPI, as in experiment 2.

MPI images were analyzed utilizing Horos imaging software. (Horos is a free and open source code software program that is distributed free of charge under the LGPL license at Horosproject.org and sponsored by Nimble Co LLC d/b/a Purview in Annapolis, MD USA). Images were displayed in full dynamic range and total MPI signal was...
calculated. Areas of interest from 3D images were manually outlined, slice by slice, creating a 3D volume. In 2D images, the areas of interest were manually outlined in a single slice. The mean signal from these ROI’s were then multiplied by the ROI volume/area to determine the total MPI signal. For the nanoparticle samples the total MPI signal (y) was plotted against iron content (x) to derive the calibration lines. This relationship was used to quantify iron content (x) in mouse brains. Where the total MPI signal was substituted for y, (m) is the slope of the line and (b) is the y-intercept in y = m x + b. All MPI images were analyzed in the same way to ensure consistency.

3.2.5 Magnetic Resonance Imaging

In vivo proton (1H) MRI for all mice was performed at 3 Tesla using a GE MR750 system equipped with an insertable gradient coil and a solenoidal mouse head radiofrequency coil. While imaging, mice were anesthetized with 2% isoflurane in oxygen. A 3D balanced steady state free precession pulse sequence was used. Image resolution was 200 x 200 x 200 μm and sequence parameters were as follows: FOV = 1.5 cm x 1.5 cm, matrix = 150 x 150, flip angle = 35°, receiver bandwidth = +/-41.67 kHz, repetition time/echo time (TR/TE) = 4.2/2.1 ms, 2 signal averages and 8 phase cycles resulting in a scan time of ~30 minutes.

MRI data was also visualized and analyzed using Horos imaging software. For experiment 1 brain images were assessed for the presence of signal voids attributable to iron-labeled cells arrested in the mouse brain vasculature after the intracardiac injection. Iron-labeled cells in the brain were quantified by determining the percentage of black pixels by drawing a region of interest (ROI) around the whole brain and setting a threshold value based on the mean signal intensity value of a signal void ± 2 standard deviations. The total number of black pixels below this threshold value was obtained from the entire brain volume signal intensity histogram. The number of black pixels was divided by total number of pixels to calculate the percentage of black pixels.

3.2.6 Statistical Analysis

Data are presented as mean +/- standard deviation in experiment 2 to compare the amount of iron content measured in the brains of mice injected with 2.5 x 10⁵ cells and the
brains of mice injected with $5.0 \times 10^4$ cells. Simple linear regression was used to evaluate
the relationship between total MPI signal and iron content to create equations used for iron
content quantification and the goodness of fit $R^2$ value. These analyses were conducted
using Prism software (8.0.2, GraphPad Inc.), where $p < 0.05$ was considered statistically
significant.

3.3 Results

3.3.1 Evaluation of Vivotrax and MPIO Performance by Relaxometry and MPI

The relaxometer mode on the MPI system was used to compare the performance of
MPIO to Vivotrax. This data is shown in Chapter 2 in Figure 2.2. The amplitude of the
MPI signal for MPIO was slightly higher than Vivotrax; the relative sensitivity was 1.5 for
MPIO versus 1.0 for Vivotrax. The FWHM was wider for MPIO; the resolution of MPIO
was more than 2.5x that of Vivotrax; 4.5 versus 1.7 mm for a 6.1 T/m gradient.

Figure 3.1 shows an example of the images of MPIO and Vivotrax samples
measured to generate the calibration lines. Ten samples were scanned for each SPIO, 5
samples were scanned at a time. This allowed for proper spacing between samples so that
the signals did not overlap. The images are displayed in full dynamic range so all samples
are visible, which is why the samples with the lowest 5 iron contents appear to have signal
as strong as the highest 5 iron contents.

There was a strong linear relationship between iron content and MPI signal
(arbitrary units, A.U.) for both MPIO ($R^2 = 0.9498$, $P < .0001$) and Vivotrax ($R^2 = 0.9938,$
$P < .0001$). The equation of the line was: MPI Signal = 24.32 * (Iron Content) + 2.507 for
MPIO and MPI Signal = 20.57* (Iron Content) + 0.8914 for Vivotrax. Using this
relationship, iron content could be determined for a given MPI signal in subsequent
imaging experiments.
Figure 3.1: (A) 3D low resolution MPI images of a series of diluted samples of MPIO (B) and Vivotrax (C) used to create the calibration lines shown in (D) and (E). Images are scaled so that all samples are visible. The relationship between iron content and MPI signal is linear for both nanoparticles. Equations derived from the calibrations are used for calculating iron content in MPI brain images.

3.3.2 Imaging

PPB staining confirmed the successful labeling of cancer cells with either MPIO or Vivotrax (shown previously in Figure 2.6). Labeling efficiency was greater than 90% for MPIO and 70% for Vivotrax. Labeling with either nanoparticle did not change cell viability; > 95% before and after labeling. Figure 3.2 A shows in vivo MRI of mice injected
with $5 \times 10^5$ cells or Figure 3.2 B with $2.5 \times 10^5$. Ex vivo MPI images from experiment 1 for all 4 mice injected with either $5 \times 10^5$ (Fig 3.2 C & E; n=2) or $2.5 \times 10^5$ (Fig 3.2 D & F; n=2) MPIO-labeled 231BR cells. One mouse from each group was imaged in vivo with MRI. MR images showed the characteristic signal voids throughout the brain (3.2 A & B) representative of iron-labeled cells arrested in the brain on the day of the intra-cardiac injection, consistent with numerous previous MRI studies conducted in our lab. MPIO-labeled 231BR cells were detected by MPI in all fixed brains. MRI and MPI images are both presented in the coronal plane. Quantification of the MRI and MPI data is shown in Table 3.1. The signal voids were quantified by measuring the percentage of black pixels in the whole brain. These values were 1.04% for the $2.5 \times 10^5$ cell injection and 2.77% for the $5 \times 10^5$ cell injection. The iron content measured by MPI ranged from 2.39 to 3.32 $\mu$g of iron per brain. The iron content measured in mouse 3 was similar to that of mouse 1 and 2 which received a higher number of cells. In the images reference tubes can be seen which can be used for quantification and co-registration purposes. However, for this analysis we used the calibration lines for quantification instead. This preliminary experiment did not use sufficient animal numbers to perform a statistical analysis of the data but did demonstrate the ability to detect MPIO-labeled cells arrested in the mouse brain. In experiment 2 MPI was performed in vivo and fewer cells were injected to learn more about MPI cellular detection sensitivity.
Figure 3.2: In vivo MRI of brains in mice injected with $5.0 \times 10^5$ (A) or $2.5 \times 10^5$ (B) 231BR MPIO labeled cells. White arrows point to discrete signal voids generated by MPIO labeled cells arrested in the brain vasculature. The white ‘R’ stands for reference tube. Ex vivo MPI of brains from the same mice injected with $5.0 \times 10^5$ cells in (C) and (E) or $2.5 \times 10^5$ cells in (D) and (F).
Table 3.1: Summary of data for each mouse in experiment 1. MRI on the day of the cell injection (Day 0) used to verify cell injections was quantified for one mouse from each group by measuring the percentage of black pixels above a defined threshold. Quantification of MPI signal and iron content in the brain.

<table>
<thead>
<tr>
<th>Cell Number</th>
<th>Mouse</th>
<th>MRI (% Black Pixels)</th>
<th>MPI (Iron Content μg)</th>
<th>MPI Signal (A.U.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 000</td>
<td>1</td>
<td>2.77%</td>
<td>3.32</td>
<td>559.56</td>
</tr>
<tr>
<td>500 000</td>
<td>2</td>
<td>-</td>
<td>3.09</td>
<td>521.96</td>
</tr>
<tr>
<td>250 000</td>
<td>3</td>
<td>1.04%</td>
<td>3.16</td>
<td>533.28</td>
</tr>
<tr>
<td>250 000</td>
<td>4</td>
<td>-</td>
<td>2.39</td>
<td>388.83</td>
</tr>
</tbody>
</table>

In experiment 2, mice were injected with either $2.5 \times 10^5$ (n=3) or $5 \times 10^4$ (n=3) MPIO-labeled 231BR cells and were imaged in vivo with MPI. MPI signal was detected in all mouse brains shown in Figure 3.3 where full body MPI scans of mice injected with $2.5 \times 10^5$ or $5 \times 10^4$ MPIO-labeled cells can be seen. The average MPI signal and iron content measured in the brains of mice that were injected with $2.5 \times 10^5$ cells (1.25, 1.65, and 1.31 μg Fe; mean = 1.4 μg) was approximately four times greater than in brains injected with $5 \times 10^4$ cells (0.62, 0.25, and 0.22 μg Fe; mean = 0.37 μg).
In experiment 3 mice were injected with \(5 \times 10^4\) MPIO-labeled 4T1BR5 cells (n=4) or \(5 \times 10^4\) Vivotrax-labeled 4T1BR5 cells (n=4) and imaged in vivo with MRI and MPI. Day 0 MRI showed distinct signal voids in mouse brains injected with MPIO labeled cells, consistent with previous experiments. Vivotrax labeled cells appeared as very faint signal voids in MRI which were more difficult to detect and not distinct enough to permit quantification. No MPI signal was detected in the brains of mice injected with \(5 \times 10^4\) Vivotrax-labeled 4T1BR5 cells (Figure 3.4). MPI signal was detected in all mice injected with \(5 \times 10^4\) MPIO-labeled cells.
Figure 3.4: (A) Representative in vivo MRI of a brain from a mouse injected with $5.0 \times 10^4$ MPIO labeled 4T1BR5 cells and the corresponding 3D high resolution in vivo MPI (B). (C) Representative in vivo MRI of a brain from a mouse injected with $5.0 \times 10^4$ Vivotrax labeled 4T1BR5 cells. White arrows on MR images point to signal voids caused by iron-labeled cells. Very few voids were visible in mice injected with Vivotrax labeled cells.

Figure 3.5 A shows the full body MPI scans of mice injected with $5 \times 10^4$ MPIO-labeled cells. The signal can be visualized in all 4 mouse brains. Figure 3.5 B shows a representative full body MPI scan of a mouse injected with $5 \times 10^4$ Vivotrax labeled cells. There is no quantifiable signal seen in this image. The iron content measured from MPI of the brains of mice which were injected with $5 \times 10^4$ MPIO-labeled 4T1BR5 cells was 0.21, 0.33, 0.36 and 0.22 μg Fe, which was not significantly different from that from the experiment 2 mice which were injected with the same number of MPIO-labeled 231BR cells; average brain iron content was 0.28 μg for 4T1BR5 cells and 0.37 μg for 231BR cells.
**Figure 3.5:** (A) Full body MPI scans of mice receiving $5.0 \times 10^4$ MPIO labeled 4T1BR5 cell injections. Optical image indicates brain location and liver/gut signal. In the bottom right of images is a reference tube. (B) Full body MPI scans of mice receiving $5.0 \times 10^4$ Vivotrax labeled 4T1BR5 cells where brain signal was not able to be detected.
Figure 3.6 illustrates how adjusting the signal intensity in the images and changing the FOV affects visibility of the brain signal. Figure 3.6A shows the same 3D whole mouse image as shown in Figure 3.5 (first panel). In B the same image is shown after the signal intensity has been adjusted to show the maximum signal intensity in the brain region. This is what is typically done when quantifying the MPI signal; this enhances the ability to see regions of low MPI signal but also increases background signal.

In Figure 3.6 C we tested whether we could use a smaller FOV centered on the brain to improve signal detection. Here the FOV was reduced from 12 x 6 x 6 cm to 4 x 6 x 6 cm. At the time, the investigators at Magnetic Insight had not tested this and were unsure if this approach would work. As shown, this image has a large region of zero signal in the FOV and was not quantifiable. We later learned that the reconstruction algorithm makes the assumption that the voxels along the outside edges of the FOV have no iron in them and sets the signal to zero and this leads to image distortion and artifact. Although iron is not expected to be along the edges of the FOV in this example it is possible that MPIO labeled cells are located in the heart, lungs or liver and that this signal extends to the edge of the FOV. This issue is still not well studied. In D we further reduced the size of the FOV to 3 x 6 x 6 cm and the same artifact did not appear. The MPI signal in this image appears smoother (compared to Figure 3.5). This may be, in part, because we used the high sensitivity scan mode which trades some resolution for sensitivity. The iron content measured for this mouse brain was 0.57 μg, compared to 0.33 μg measured from the whole body image of the same mouse. This issue requires more study.
Figure 3.6: (A) is the same whole body 3D image of the same mouse as in Fig 3.5, first panel. This is showing the image displayed without adjusting the signal intensity. (B) The same image has been window/leveled to enhance the signal in the mouse brain for quantification. In (C) a smaller FOV 4 x 6 x 6 cm was used, and the scan mode was changed to high sensitivity to test whether this improved visualization of brain signal. This image contains artifact attributed to non-zero signal at edges of the FOV. (D) The FOV was further reduced to 3 x 6 x 6 cm. This image does not contain the same artifact. Oval indicates brain region.

3.4 Discussion

This is the first study to demonstrate that MPIO-labeled cells can be detected and quantified in vivo by MPI. MPI spatial resolution and sensitivity are heavily influenced by the physical properties of the nanoparticle, such as the effective core size, relaxation time and size distribution. One way to improve resolution is to increase the (effective) magnetic core size of the particles. Tay et al. have found that improved resolution with increasing magnetic core size follows the predictions up to approximately 25 nm when the effects of SPIO rotational times become significant. Sensitivity depends, among other factors, on the strength of the nanoparticle magnetization. The magnetization can be increased by
enlarging the iron core diameter, as the strength of the MPI signal increases by the third power of the iron core diameter.\textsuperscript{32}

In the early days of MPI, commercially available SPIOs used for MRI were evaluated and Resovist (which is the same as Vivotrax) showed a good MPI performance. Although widely used, Vivotrax is now not considered optimal for MPI because it has a bimodal size distribution, predominantly containing small cores $\sim 5$ nm in diameter with a small fraction (30\%) of multi-core aggregates with an effective size of 24 nm.\textsuperscript{33} The individual cores are too small to magnetize significantly and so the MPI signal only comes from the clustered multi-core structures. One approach being taken to improve MPI sensitivity is to design similar particles but with a bigger fraction of the aggregates. Eberbeck et al. have designed Nanomag-MIP particles which are similar to Vivotrax, they are composed of individual cores between 3-8 nm along with multi-core aggregates with an effective core size of 19 nm.\textsuperscript{34} However, for Nanomag-MIP 80\% of the nanoparticles are of the larger size and the MPI signal is two times larger than that of Resovist. Yoshida et al. showed that fractionation of Resovist can improve the MPI signal by 2.5 times.\textsuperscript{35} Another approach is the synthesis of homogeneously distributed single-core SPIOs with a dedicated iron core diameter for ideal MPI characteristics. Ferguson et al. observed increasing MPI signal with increasing magnetic core size (14-27 nm) for monodisperse single core particles and up to three times greater signal intensity per unit iron compared to multi-core Vivotrax.\textsuperscript{33}

MPIO are quite different from the nanoparticles typically used for MRI and MPI. MPIO consist of multiple small cores ($\sim$5-10 nm)\textsuperscript{36} embedded in polystyrene matrix; because of the clustered nature of the iron cores they can be regarded as having one very large superparamagnetic core. The 0.9 $\mu$m sized MPIO we have used have a broad size distribution, the specified range is 0.5-2 $\mu$m, however all of these particles would be expected to contribute to the MPI signal. MPIO also contain much more iron per particle than SPIO.\textsuperscript{3}

Figure 3.1 shows that the MPI signal generated by the MPIO has reduced resolution compared to Vivotrax, consistent with theory. The low resolution is apparent in MPI images of MPIO-labeled cells in the mouse brain which show one large area of signal.
Unlike, with MRI, it is not possible to determine where within the brain the cells are located from the MPI images. Figure 1 shows that the MPI signal for MPIO is greater than that for Vivotrax for equal iron concentrations. This suggests that the net change in magnetization of the MPIO is greater than the net magnetization change of the same mass of Vivotrax, since the MPI signal results from the change in magnetization of an excited particle. The high MPI signal produced by MPIO and the high intracellular iron content with MPIO labeling both contribute to our increased ability to detect MPIO-labelled cells distributed throughout the mouse brain.

We were able to detect and measure MPI signal in the brains of mice injected with as few as 5.0 x 10^4 MPIO-labeled cells; an intra-cardiac injection of 5 x 10^4 cells is estimated to deliver 10,000 cells to the mouse brain. We did not detect MPI signal in the brain when 5.0 x 10^4 Vivotrax-labeled cells were injected. This is, in part, due to the fact that cell labeling with MPIO is more efficient than for Vivotrax (~90% versus 70% of cells labeled) and the amount of iron per cell is significantly greater for MPIO than Vivotrax labeling (20-30 pg/cell versus 10-15 pg/cell). Our MRI data for Vivotrax cells in the brain supports this; signal voids were considerable fainter and harder to detect in MR images of mouse brains which received Vivotrax-labeled cells. Furthermore, the MPI signal generated from MPIO is higher than Vivotrax. While Vivotrax is currently the most common SPIO used for MPI, our results show that MPIO-labeled cells can be detected and quantified in this mouse brain model more readily than Vivotrax-labeled cells. It is worth noting that MPIO are inert, nonbiodegradable particles only suitable for preclinical experimental studies.

MPI provided information which was not attainable with MRI. With knowledge of the amount of iron per cell achieved with labeling the cell number could be estimated. Our routine measurements of mean iron/cell after labeling the cancer cell lines used in this study with MPIO are consistently in the range of 20-30 pg of iron/cell, using inductively-coupled mass spectrometry (ICP-MS). As described above, with an estimate of 20% of injected cells arresting in the brain the intra-cardiac injections of 5.0 x 10^5, 2.5 x 10^5 and 5 x 10^4 cells will deliver approximately 100,000, 50,000 and 10,000 cells to the brain, respectively. Using the higher value (30 pg) for MPIO labeling of cells, for example, this amounts to
approximately 3.0, 1.5 and 0.3 μg of iron in the brain due to iron-labeled cell burden. As shown in Table 3.2, our data falls within these approximations.

There are a number of factors which make it impossible to calculate true cell number in our study. First, the value for iron/cell that we measure from ICP-MS or MPI is an average value, some cells will contain more iron, some less. Second, the amount of iron/cell achieved with each cell labeling experiment has a range of values, even for the same labeling protocol, depending on precise timing and culture conditions. Third, the number of cells delivered to the brain by intra-cardiac injection is an estimation. We used an estimate of 20% of cardiac output to the brain resulting in 20% of cells delivered; published values are between 5-25%.

Intra-cardiac injections are also technically challenging and, while we have significant expertise, not every injection is likely to deliver the exact same number of cells. Lastly, there may be an upper limit to the number of cells that arrest and then persist in the brain. For these last two reasons, there are unlikely to be double the number of cells arrested in the brain with double the number injected. These factors are also likely the reason why different values for iron content in the brain were measured for mice injected with the same number of MPIO-labeled cells, but in different experimental groups (i.e., mice that received 2.5 x 10^5 MPIO-labeled cells in experiment 1 and 2). Considering all these caveats our values for iron content measured by MPI are in good agreement with the rough estimate of iron expected in the brain. In conclusion, we have demonstrated that MPIO-labeled cells can be detected and quantified in vivo in a model where cells are dispersed throughout the mouse brain.
Table 3.2: Summary of all experiments, showing the number of cells injected intra-cardiac, the estimated number of cells arrested in the brain, the estimated amount of iron per cell, the estimated amount of iron in the brain and the measured iron content in the brain and the measured iron content in the brain from MPI images.

<table>
<thead>
<tr>
<th>Number of Cells Injected (IC)</th>
<th>Estimate of Number of Cells Arrested in Brain</th>
<th>Estimate of Iron per cell (μg)</th>
<th>Estimate of Iron in the Brain (μg)</th>
<th>MPI Measurement of iron in brain (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 000</td>
<td>10 000</td>
<td>30 pg</td>
<td>0.3 μg</td>
<td>0.2 - 0.7 μg</td>
</tr>
<tr>
<td>250 000</td>
<td>50 000</td>
<td>30 pg</td>
<td>1.5 μg</td>
<td>1.3 – 3.2 μg</td>
</tr>
<tr>
<td>500 000</td>
<td>100 000</td>
<td>30 pg</td>
<td>3 μg</td>
<td>3.1 - 3.3 μg</td>
</tr>
</tbody>
</table>

3.5 References


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31. Sehl OC, Makela A V., Hamilton AM, Foster PJ. Trimodal Cell Tracking In Vivo:


Chapter 4

4 Conclusions, Limitations, and Future Work

This Chapter summarizes all the findings in the experiments in this thesis.
Limitations and future work.

4.1 Discussion and Conclusions

4.1.1 Chapter 2 Summary and Discussion

Relaxometry data was collected for MPIO and Vivotrax to compare the sensitivity and resolution of each particle. The amplitude of the MPI signal for MPIO was higher than Vivotrax; the relative sensitivity was 1.5 for MPIO versus Vivotrax. However, Vivotrax outperformed MPIO in resolution. The calculated resolution for MPIO was 4.49 mm versus 1.69 mm for Vivotrax. For our application where the goal was to estimate the number of cancer cells in the mouse brain sensitivity was most important and therefore MPIO was used for subsequent mouse studies.

The generation of calibration lines was a method we developed to quantify the iron content in MPI images acquired in this chapter and in chapter 3. Calibration lines were made for MPIO and Vivotrax for each scan mode used to image samples or mice. A series of samples of each SPIO were diluted in PBS into aliquots to be scanned. These samples were then measured using area/volume multiplied by mean signal to give us our total MPI signal value. Total MPI signal was then plotted against the known iron content of each dilution sample. Total MPI signal and iron content are linearly related so we use the slope of that line to help us calculate the amount of iron content in our experiments. Calibration lines provide a more accurate means of quantification compared to using reference tubes. This is because reference tubes are images with the experimental sample and therefore the iron content in these reference tubes needs to have similar iron content to what is in the experimental sample. Sometimes this is not always known, especially in vivo. If the amount of iron in the reference tube is too much it can overwhelm the signal you are
actually trying to detect. If it is too low, it can be overwhelmed by the signal you are trying to detect and then total MPI signal cannot be calculated from your reference tube.

Repeatability of the MPI measurements was assessed to determine the amount of variability there was when acquiring MPI images and quantifying data. In experiment 1, samples were scanned six times without being removed from their position on the MPI bed. Those same samples were then taken out and placed back in the MPI bed, in between individual scans, for another round of six scans. Lastly, the samples were placed at the 4 cm mark on the MPI and scanned six times without being moved. The reason for moving samples to different locations was based on our observations of changes in the location of background signal due to noise. These results showed that there is some variation in the measurements for all experiments. The ‘true’ iron content for the Vivotrax sample (determined using manufacturer’s stated iron concentration) was 27.5 μg and the mean values measured for the three conditions were 28.5, 28.9 and 29.0, which are within 6% of the value given by the manufacturer Magnetic Insight Inc. For the MPIO sample, the iron content was 14 μg and the mean values for the three conditions were 17.4, 19.5 and 20.3, which are farther off. This is most likely due to the fact that since MPIO particles are so large they have a tendency to quickly settle at the bottom of the tube when samples were loaded in. This essentially creates an even larger particle as all the MPIO particles interact when they are in close proximity to each other. This can affect how the particle reacts in the MPI system as larger particles experience more Brownian relaxation creating a slower loss in magnetization which negatively affects the resolution quality. This most likely lead to a larger volume being calculated when measuring the MPIO sample ROIs in the MPI scans which is used to calculated iron content.

Reproducibility was tested by having the same sample scanned and quantified by 3 different MPI users. Our results showed that even though these users were analyzing the same sample using the same quantification methods and calibration line there was some variability in iron mass calculated. User 1 and 2 results were not significantly different from one another. However, the results for user 3 were significantly different from user 1 and 2. This is likely because of the subjectivity involved with manually drawing ROIs around the MPI signal. In this case we determined that user 3 was drawing larger ROIs
than user 1 and 2. This larger volume influences the calculation of total MPI signal which is subsequently used to calculate iron content.

The detection of cell samples was explored using a range of cell numbers, from $2.5 \times 10^3$ to $2.5 \times 10^5$ cells, labeled with either MPIO or Vivotrax. Images were acquired with the 2D high sensitivity isotopic scan mode and no signal averaging. For both MPIO and Vivotrax, samples that contained less than $1.0 \times 10^4$ cells showed high background signal (lower SNR) and were not clearly visualized. In these images the MPI signal was difficult to quantify since the signal of interest could not be discriminated from the background noise. For the lower $2.5 \times 10^3$ cell samples, increased averaging (8) allowed for detection and quantification of the MPI signal, although with the trade-off of increased scan time. Using a 3D scan could have also been a way to improve the amount of signal visualized in MPI images.

4.1.2 Chapter 3 Summary and Discussion

In Chapter 3 we studied whether MPIO could be used for labeling cells to be detected by MPI. Our prior work using MRI to track MPIO-labeled cancer cells in the brain showed very high sensitivity, down to single cell under ideal conditions, and allowed us to monitor both proliferative and non-proliferative cell populations. However, we could not determine cell number from our MRI data. We were able to detect and quantify the MPI signal in mice which received intra-cardiac injections of different numbers of cells, amounting to detection limits of approximately 25,000, 50,000 or 100,000 cells in the mouse brain. These are estimates based on what is known about how many cells are expected to arrest in the brain vasculature after intra-cardiac injection. To calculate the exact cell number was not possible considering this estimate and factor related to MPIO cell labeling. We used an estimate of 20 μg of iron per cell based on prior measurements made in the Foster lab using the same MPIO and the same cell type. However, the iron loading does vary from experiment to experiment, even in the same lab under the same conditions, and this number is an average value, some cells contain more and less iron.

Still, our values for iron content measured from in vivo mouse MPI were 0.2 to 0.7 μg for mice injected with 50,000 cells where the estimated iron content in the brain was 0.3 μg; 1.3 to 3.2 μg for mice injected with 250,000 cells where the estimated iron content
in the brain was 1.5 μg; and 3.0 – 3.3 μg for mice injected with 500,000 cells where the estimated iron content in the brain was 3.0 μg. MPI provided quantitative information which could not be extracted from MRI data.

There are currently two other papers that have used MPIO labeled cells for MPI. Mallett et al. fed mice 3 different types of diet that contained varying amounts of iron to mimic livers with different endogenous iron loadings. MPIO-labeled cancer cells were later delivered to the liver via intrasplenic injection. The mice were then imaged in vivo using MRI. In images with high iron loading the MPIO-labeled cells could not be detected. This is because all types of iron generate signal loss in MR images. Livers were harvested and imaged ex vivo by MPI afterward and MPIO-labeled cells could be detected in all livers, demonstrating a role for MPI cell tracking in models of excessive liver iron or iron overload.¹ In a preprint by Parkins et al. (where I am a co-author and acquired and analyzed the MPI data) a primary tumour was generated by injecting unlabeled 231-BR cancer cells into the mouse mammary fat pad. After 41 days of growth MPIO-labeled MDA-MB-231BR-eGFP cells were injected intracardially into the left ventricle of the mouse heart. Mice were imaged using MPI 3 days after this injection and the images showed that MPIO-labeled cells could be detected in the established MDA-MB-231 primary tumour.² The work in this thesis is novel in that it is the first show that MPIO-labeled cancer cells dispersed in the mouse brain can be detected and quantified using in vivo MPI.

4.2 Challenges and Limitations

4.2.1 Late Arrival of MPI

I began my graduate work in January 2019. The Momentum™ MPI system was originally supposed to be installed in April 2019. Unfortunately, the system was damaged during delivery from Alameda, California, USA to London, ON, Canada and had to be shipped back. A new system was installed in August 2019. This resulted in a delay of 4 months on planned experiments. Once the system was installed, we wasted no time in making sure the system was calibrated and ready for use.
4.2.2 Contamination

Contamination of the MPI system occurs easily due to iron that can be in multiple different products that are common in a lab. For example, dust, paper towels, certain tapes, scissor cuts can all introduce residual iron which if present in the bore of the system or on the sample beds, can produce unwanted signal which the manufacturer refers to as contamination. When there is contamination the background signal is higher than normal. We now routinely collect an empty bed scan before starting experiments. Figure 4.1 shows an example of an empty bed scan where the background is clear from contamination and a bed that is considered contaminated.

Contamination will always be an issue; however, it can be minimized. We have installed a plastic shield to cover the entrance to the bore of the MPI system. This shield stays on at all times, except when scans are in progress. Second, we make sure that the MPI sample bed is wiped and sanitized with 70% ethanol before and after each scan. We also run empty bore and empty bed scans before imaging samples to ensure there is no serious contamination in the bore. We also keep the MPI room clean by continuously wiping down surfaces and floors to minimize dust build up. Recently, we had a more serious contamination inside the bore of the MPI system and have now installed a compressed air hose in the MPI lab to regularly blow out any source of contamination that may settle inside the bore.

Figure 4.1: (A) Mouse feed in 50ml falcon tube. (B) 2D high sensitivity MPI scan of mouse feed overlaid on top of optical image of mouse feed.
4.2.3 Unwanted Sources of Signal

There is also an issue with detecting sources of unwanted signal. Sometimes MPI signal is detected in the gut of mice due to the presence of iron in mouse feed. Figure 4.2 shows a 50 ml tube full of the mouse chow scanned by MPI which produced signal. This signal is typically low intensity but can become a problem when trying to quantify signal that is of similar iron concentration such as a low cell number or when using an SPIO with lower iron content. It becomes especially difficult when the signal of interest is in close proximity to the unwanted mouse gut signal. MPI of a control, uninjected mouse is shown in Figure 4.3. A strong signal is observed in the region of the abdomen, likely due to mouse feed. We have also observed MPI signal at the site of injection of cells, from dried blood in the form of hemosiderin from needle pricks (not shown).

Figure 4.2: Naïve mouse scanned using a 2D high sensitivity MPI scan where signal can be seen coming from the gut from mouse feed.

Figure 4.3: (A) Uncontaminated MPI bed. (B) Contaminated MPI bed.
4.2.4 COVID-19 Pandemic

As we all know COVID-19 has created challenges for everyone. During the pandemic when our labs at Robarts were shut down from mid-March until June, I was unable to conduct any experiments to complete work for this thesis. The in vivo work in Chapter 3 was most affected by this shutdown. However, despite the world shutting down for most of 2020 I was able to complete as many experiments as I could and write this thesis in time for submission.

4.2.5 Limitations of MPI

MPI itself has several limitations, some which are common to other imaging modalities and some that are unique to MPI, and others that we are just learning about as our lab works on developing and advancing this emerging imaging modality.

The resolution of MPI is considered low in relation to MRI, and similar to micro-PET. Resolution is considered the main limitation of MPI but it is also a feature of MPI that is expected to improve dramatically over time as advances in MPIO tailored nanoparticles are made. Also, similar to PET imaging, MPI images provide no anatomical information. Anatomical information has to be obtained through additional separate scans from either CT or MRI and then co-registered to learn exactly where a signal is originating from. Magnetic Insight Inc. recently made available a dual modality MPI/CT system that allows the CT image to be obtained without moving the subject. The MPI system at Robarts is equipped with a camera that takes a photo of the subject in the FOV and this provide a rough anatomical reference for users when overlayed with an MPI image. Like MRI, MPI is unable to differentiate between live or dead iron labeled cells. When iron labeled cells die, they may release the iron label which can be taken up by phagocytic cells such as macrophages. This issue is known as bystander cell uptake and can lead to misinterpretation of the signal as the cells of interest.

It is not possible to use a small, focused region of interest in MPI. For MRI, we are used to choosing a FOV that fits around our region of interest and determines the voxel size and influences the scan time. In MPI, the reconstruction algorithm makes the assumption that the voxels along the outside edges of the FOV have no iron in them and
sets the signal to zero. When the edge of the FOV goes through the mouse (as in Chapter 3, Figure 3.6 C) there will be image distortion and negative signal, this can be major or minor and depending on the quantity and volume of iron at the edge of the FOV but results in data that is not quantifiable. This is an example of an issue that was unfamiliar to even the scientists at Magnetic Insight Inc. and one of the many challenges associated with implementing a new imaging modality for cell tracking. In our lab we regard these as welcomed challenges and are excited about doing what we can to advance this technology.

4.2.6 Limitations of Study

Limitations of the experiments in this thesis are listed here:

1. During the experiments in 2.3.4 MPIO Labeled Cell Viability After MPI, the cell samples were scanned using the 2D high sensitivity scan mode which takes ~ 2 minutes and uses a weak gradient (3 T/m). This may not affect cell viability as much as a longer scan or a stronger gradient would.

2. Labeling cells with Vivotrax leads to some extracellular iron and some iron on the cell surface. We didn’t fully understand the extent of this at the time our experiments were conducted. This excess iron will lead to more MPI signal and an overestimation of the iron content measured from the images.

3. The number of animals used in all of the in vivo experiments in Chapter 3 should be increased.

4. At the time of these studies, we did not have access to ICP-MS which we now use to measure iron mass per cell (used in conversion of iron content measured by MPI to cell number). Therefore, the estimates of cell number from in vivo MPI images of the mouse brains in Chapter 3, experiments 2 and 3 used values which were measured in the past in the Foster lab for MPIO and the same cell type.

Future work, which could address these limitations is described below.
4.3 Future Work

4.3.1 Next Steps

Next steps for this project would be to add additional mice to all the in vivo experiments conducted in Chapter 3. Mouse group sizes for experiments 2 and 3 were determined using an *a priori* power calculation using expected effect sizes based on previous studies that used MRI to detect differences in the number of signal voids for mice injected with different numbers of cells. A study with an effect size of 1.08 and power of 80% required 8 mice/group to test the association at 5% using two-tailed test.

Since we were able to detect MPI signal with as few as $5 \times 10^4$ MPIO-labeled cells injected intra-cardiac ($\sim 10,000$ cells estimated in brain) it would be interesting to reduce the number of cells injected until we are unable to detect MPI signal. One paper estimates that MPI should be able to detect 200 Vivotrax labeled cells.$^3$ This estimate was based on the SNR of an ex vivo cell sample. A study which used a SPIO designed and synthesized specifically for MPI has demonstrated the detection of 250 cells injected in a bolus intramuscularly a mouse.$^4$

Access to an advanced user interface was granted to the Foster lab by Magnetic Insight Inc. in summer 2020. This allows users to adjust more imaging parameters and optimize MPI acquisitions. Having this ability could increase imaging sensitivity allowing for lower cell numbers to be detected in vivo. The in vivo work done in Chapter 3 at the start of using MPI was all scanned used basic imaging parameters. Due to COVID-19, I did not have the time to repeat these experiments to test options in the advanced interface.

Ultimately, we are interested in tracking MPIO-labeled cells over time in the mouse brain as cells proliferate and detecting and quantifying the number of cells that persist as non-proliferative MPIO-retaining cells. This work is beyond the scope of my thesis. My results show that it is possible to detect and quantify MPIO-labeled cells in this model and these techniques, possibly along with advanced imaging parameters, may allow for this additional work by a new student.
As described above, the original experiment to test whether MPI affected cell viability was done using a 2D high sensitivity scan which is not only a short scan but also a scan that uses the weakest gradient strength (3 T/m). The viability test should be repeated using an MPI scan that is longer and uses a stronger gradient (i.e., 3D isotropic scan). This may provide more realistic information on cell viability especially as in vivo work will almost always be done using some sort of 3D scan.

Unwanted signal from the gut was an issue that was intermittent and bothersome, and this has been observed by several other users. In the future we plan to purchase an iron-free mouse chow which we can switch mice over to a week prior to imaging. We hope this will eliminate this problem. Another solution may be fasting mice 24 hours prior to scanning to ensure elimination of food by-products in the gut.

4.4 References


Appendices

Appendix A: TEM images of (A) MPIO and (B) Vivotrax. Images provided by the manufacturers.
Curriculum Vitae, Kierstin Melo

EDUCATION

01/19 -12/20 Master’s Science: Medical Biophysics (expected)
Western University, London, Ontario, Canada
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Thesis: Detection and Quantification of Cells using Magnetic Particle Imaging and Magnetic Microspheres

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SCHOLARSHIPS AND AWARDS

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01/19- present Western Graduate Research Scholarship
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Amount: $1 500 per month

RESEARCH EXPERIENCE

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Supervisor: Dr. Paula Foster

06/17-08/17 Summer Research Student
Western University
Supervisor: Dr. Paula Foster
PUBLICATIONS


REGIONAL CONFERENCES


Melo KP, Makela AV, Hamilton AM, Foster PJ. “Comparing detection limits of Magnetic Particle Imaging (MPI) to Magnetic Resonance Imaging (MRI) using super paramagnetic iron oxide nanoparticles in a breast cancer metastasis model”. MPI eSymposia 2020. (Poster Presentation)


INTERNATIONAL CONFERENCES

Melo KP, Makela AV, Hamilton AM, Foster PJ. “Micron-sized iron particles for the detection of cancer cells by magnetic particle imaging”. World Molecular Imaging Conference 2020. (10 Minute Talk)

Melo KP, Makela AV, Hamilton AM, Foster PJ. “Comparing MRI and MPI for cell tracking and quantification” Great Lakes Advanced Molecular Imaging Course 2019. (Poster Presentation)