LABELING AND DETECTION OF MARROW DERIVED MESENCHYMAL STROMAL CELLS USING MAGNETIC RESONANCE IMAGING

by

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A thesis submitted in conformity with the requirements for the degree of Master of Health Science
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University of Toronto

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Abstract

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Stem cell therapies hold great promise for diseases such as stroke, where few effective treatment options exist. Clinical translation of experimental stem cell therapies requires the ability to monitor delivery and behaviour of cells non-invasively \textit{in-vivo} with clinical imaging modalities such as MRI. This thesis presents the translation of established methods for labelling and imaging stem cells with specialized MRI systems to a more clinically relevant setting.

A methodology for harvesting and labelling a cell population containing stem cells with iron oxide for detection with a clinical MRI system is presented and single cell detection is demonstrated \textit{in-vitro}. The feasibility of detecting iron oxide labelled stem cells intravenously delivered in a rat model of stroke is tested. Results demonstrate that while MRI is highly sensitive to the presence and distribution of iron oxide containing cells \textit{in-vivo} the true origin of these cells remains ambiguous with the current methodology.
Dedication

To my family; past, present and future.
“Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.”
- Robert Frost
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# Contents

List of Tables ix

List of Figures x

1 MRI Monitoring of Stem Cell Therapies 1
   1.1 Introduction ....................................... 1
   1.1.1 Stem Cell Therapy, Stroke and Imaging ................... 2
   1.1.2 MRI Stem Cell Tracking .................................. 4
   1.2 Principles of Magnetic Resonance Imaging ...................... 6
   1.2.1 Brief Description of MRI Physics .......................... 6
   1.3 Image Contrast in MR ..................................... 12
   1.3.1 Gadolinium ........................................... 13
   1.3.2 Superparamagnetic Iron Oxide (SPIO) Particles ............ 15
   1.3.3 MR Pulse Sequences ................................... 16
   1.3.4 Signal to Noise Ratio, Contrast to Noise Ratio ............ 21
   1.4 Hypothesis and Thesis Overview ................................ 22
   1.4.1 Hypothesis .......................................... 22
   1.4.2 Thesis Overview ....................................... 22

2 **In-vitro** MRI of MPIO labeled rMSCs 24
   2.1 Introduction ............................................... 24
   2.2 Methods .................................................... 26
     2.2.1 Cell Sample Preparation .................................. 26
List of Tables

1.1 MRI Contrast Agents for Cellular Imaging ................. 5
1.2 MR Sequence Parameters ................................... 17
3.1 MRI Parameters ............................................. 55
# List of Figures

1.1 Spin Echo Sequence Concept ................................. 18

2.1 Confocal microscopy of MPIO-rMSCs. ........................... 34

2.2 Cellular Iron Uptake. ........................................... 35

2.3 Flow Cytometry of MPIO labeled rMSCs 24 Hours Post-Labeling. ... 37

2.4 Flow Cytometry of MPIO labeled rMSCs 120 Hours Post-Labeling. ... 38

2.5 Plating efficiency and proliferation of MPIO-rMSCs. ............... 40

2.6 *In-vitro* MRI of MPIO loaded rMSCs. .......................... 41

2.7 *In-vitro* MRI of single plane MPIO loaded rMSCs. ............... 43

2.8 *In-vitro* MRI and Correlative Microscopy of MPIO labeled rMSCs. .... 44

3.1 Experiment Design. ............................................. 53

3.2 Longitudinal MRI .............................................. 57

3.3 Comparison of Contrast from MRI Sequences ....................... 59

3.4 MRI and Corresponding Histology. ................................ 60

3.5 Immunohistochemistry ......................................... 61
Chapter 1

Magnetic Resonance Imaging as a Tool for Monitoring of Stem Cell Therapies

1.1 Introduction

This thesis presents the development and application of a methodology for effectively labelling rat bone marrow derived mesenchymal stromal cells (rMSCs, a cell population containing mesenchymal stem cells [1]) with micron sized superparamagnetic iron oxide (MPIO) particles to enable detection with a clinical MRI system. This introductory chapter begins with a review of the current state of the art for detecting and tracking stem cells focusing on the application of these techniques toward imaging injected stem cells in pre-clinical animal models of stroke. The following section provides a review of the essential concepts from MRI necessary to understand how MPIO contrast agents are detected and how they can be used for imaging cells. This chapter concludes with an outline of the thesis, objectives and hypotheses.
1.1.1 Stem Cell Therapy, Stroke and Imaging

Despite the advances in medical technology over the past century and the tremendous impact that medicine has made in some areas such as infectious disease, there remains a large number of injury-related and degenerative conditions where there is little if any effective treatment. Ongoing research in stem cell based therapies holds great promise for the emerging fields of tissue engineering and regenerative medicine where the goal is to develop novel treatment strategies to address these needs.

A stem cell is characterized as an unspecialized cell with the ability to maintain its unspecialized state or to differentiate into a more specialized cell type when dividing. These properties make stem cells of great interest in the treatment of degenerative or injury-related diseases where, ideally, stem cells could be used to replace or repair lost or damaged tissues. While the potential therapeutic applications of tissue engineering are seemingly endless, in regenerative medicine some of the areas of active research and development include: replacement of insulin secreting islet cells in diabetes [2], tissue restoration of damaged myocardium post infarction [3, 4] and repair of the central nervous system [5]. In the central nervous system specifically, stem cell therapies are being actively investigated in animal models of diseases such as spinal cord injury and stroke [6, 7, 8].

While the role of stem cells in stroke is not fully understood, motivation for stem cell implantation as a potential stroke therapy is supported upon a foundation of recent research. Studies have shown that the adult brain has native regenerative capacity [9, 10, 11, 12]. This is thought to be due to adult neural stem cells that have been identified in the subventricular zone of the lateral ventricle, and the subgranular zone of the hippocampal formation [13]. Adult neural stem cells supposedly migrate into the cerebral infarct area and regenerate some neurons, a hypothesis supported by the observation that some stroke patients regain lost function over time [10]. Studies within a rat stroke model have shown that injection of growth factors and erythropoietin encourage regeneration of neural tissue
and ultimately result in regained motor function [14, 15]. Since the potential exists for neural regeneration, the post stroke environment may be ideal for neurosupplementation using stem cells.

A recent review by Parr et al. [6] has summarized a number of studies showing therapeutic benefit of mesenchymal stem cell (MSC) therapy in rodent models of stroke. While initial use of MSCs focused on tissue engineering bone, cartilage, muscle, marrow stroma, tendon, fat, and other connective tissues [16] it has also been demonstrated that MSCs can be influenced to differentiate into neural cells \textit{in-vitro} [17]. Furthermore, it has been shown that MSCs release a plethora of bioactive, immunosuppressive molecules capable of creating a regenerative microenvironment potentially limiting the extent of injury and facilitating regeneration in damaged adult tissues [16, 18]. Therefore, MSCs could also play a neuroprotective role in stroke. As an additional benefit, MSCs can be easily harvested from the bone marrow, rapidly expanded \textit{in-vitro} and are believed to be immunopriveleged, potentially obviating the need for immunosuppresion following transplantation [16].

While these initial studies provide evidence for therapeutic benefit of MSC therapy, more research is required to better understand the regenerative mechanism. Moreover, to facilitate clinical translation the ability to monitor stem cell behaviour \textit{in-vivo} is critical when trying to establish both therapeutic benefit and patient safety. In humans non-invasive methods are the only viable option for implanted cell tracking. The modalities most suitable for \textit{in-vivo} cell tracking include single photon emission tomography (SPECT) [19], positron emission tomography (PET), bioluminescent imaging [20] and MRI [21, 22]. MRI has emerged as the predominant modality for cellular imaging due to superior temporal and spatial contrast, absence of ionizing radiation and the ability to detect even single cells under certain circumstances [23, 24]. Moreover, MRI is suitable for monitoring surrogate measures of treatment efficacy such as tissue regeneration. \textit{In-vivo} cell tracking using MRI is being actively pursued for a variety of clinical applications
of cell based therapies [7, 25, 26, 27].

### 1.1.2 MRI Stem Cell Tracking

A number of strategies have been developed in order to make cells detectable with MRI. All cell labelling strategies set out to create specific signal-altering characteristics allowing cells to be detected against the background. In addition to providing sufficient signal contrast, the ideal characteristics of a cellular contrast agent include: biocompatibility, lack of genetic modification or perturbation to the target cell, single-cell detection, the ability to quantify cell numbers at a given locus, minimum label dilution with cell division, minimum transfer to non-target cells, and the ability to image target cells over a period of months to years [26, 28]. These ideal characteristics may not all be explicitly necessary to effectively track a cell based therapy. The four major classes of contrast agents currently used for MR cell labelling include: Gadolinium (Gd) based compounds, superparamagnetic iron oxide (SPIO) nanoparticles, micron sized SPIO particles (MPIO) and MR reporter genes [7].

The properties of each cell labelling strategy including sensitivity, labelling methods and advantages/disadvantages are summarized in Table 1.1 adapted from Politi [7], Frangioni et al. [26] and Kraitchman et al [27]. Of these contrast agents, MPIO particles provide very high sensitivity with straightforward labelling methods - both of which are desirable for clinical applications. MPIO particles are available in various sizes up to 10 µm diameter and contain more than 60% magnetite by weight with a polymer coating rendering them biologically inert and suitable for long term detection. The conjugation of a fluorescent marker allows double labelling of cells for detection with both MR and fluorescent microscopy. Furthermore, fluorescent MPIOs have been used to effectively label and detect cells with both *in-vitro* and *in-vivo* MRI and have not been demonstrated to cause cell death or impaired differentiation capacity thus far [3, 23, 24, 29, 30, 31]. For the purposes of this thesis, fluorescent MPIO particles were chosen as the contrast
agent. In the next section our attention turns to the basic principles of MRI and how these cellular contrast agents can be detected.

<table>
<thead>
<tr>
<th>MR Contrast Agent</th>
<th>Gd-based</th>
<th>SPIO/MPIO</th>
<th>MR-reporter gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative MR Sensitivity</td>
<td>Low</td>
<td>High/Very High</td>
<td>Very Low</td>
</tr>
<tr>
<td>≈ 10⁵ cells</td>
<td>Single cells</td>
<td>Tissue Level?</td>
<td></td>
</tr>
<tr>
<td>Labelling Methods</td>
<td>Direct Non Specific (Incubation with contrast agents ± Transfection Agents)</td>
<td>Direct Non Specific (Magentofection/ Magnetoel troporation)</td>
<td>Gene Transfection (Viral vectors, Electroporation)</td>
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<tr>
<td>Indirect specific (receptor mediated)</td>
<td>Indirect specific (receptor mediated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advantages</td>
<td>Positive contrast detection</td>
<td>High sensitivity</td>
<td>No contrast dilution with cell division</td>
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<td></td>
<td>Less ambiguous quantification</td>
<td>Minimal biological effects</td>
<td>No exogenous contrast required</td>
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<td></td>
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<td>SPIOs are FDA approved</td>
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<td></td>
<td></td>
<td></td>
<td>MPIOs do not require use of TAs</td>
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<tr>
<td>Disadvantages</td>
<td>Low sensitivity</td>
<td>Negative contrast detection</td>
<td>Complicated labelling procedure</td>
</tr>
<tr>
<td></td>
<td>Possible toxicity</td>
<td>Endogenous sources of negative contrast confounded quantification</td>
<td>Very low sensitivity to date</td>
</tr>
<tr>
<td></td>
<td>Contrast dilution with cell division</td>
<td>Contrast dilution with cell division</td>
<td>MPIOs not FDA approved</td>
</tr>
</tbody>
</table>
1.2 Principles of Magnetic Resonance Imaging

This section provides a basic overview of the essential theory behind nuclear magnetic resonance needed to understand the origin of image contrast in the MR signal and how it can be manipulated by contrast agents in order to detect cells. In summary, the following steps are required to produce an MR image. The protons contained within the sample must be placed in a strong magnetic field tuning them to the resonance frequency. Energy is then transmitted into the sample via radio frequency (RF) pulses at the resonance frequency. Subsequently, the sample dissipates the absorbed energy through a process known as relaxation. Selective excitation of a pre-defined area to image within the sample is achieved using gradient coils to spatially modify the magnetic field. If enough information is collected during relaxation of the sample post excitation, an image can be produced that accurately reflects the sample’s proton environments.

There are several thorough descriptions of each aspect of this process tailored to the intended audience. For a more mathematical description the interested reader is referred to any of these more theoretical texts [32, 33]. For a more applied perspective on MRI the reader is referred to any of the following more clinically oriented texts [34, 35].

A brief section of MR physics is presented after which the focus shifts to the predominant contrast mechanisms in MR images. To conclude this section the pulse sequences used throughout this thesis are presented and the concepts of signal to noise and contrast to noise ratios (SNR and CNR respectively) are described as they provide a useful means of characterizing pulse sequence performance in terms of contrast agent detection.

1.2.1 Brief Description of MRI Physics

Every proton and neutron within the nucleus of an atom possesses a property known as a nuclear spin. When combined, these nuclear spins within an atom impart a net nuclear spin angular momentum or magnetic spin to the nucleus. In atoms with an even mass
number (sum of the number of protons and neutrons) the magnetic spins oppose each other and results in an atom with a zero net magnetic spin. However, for atoms with an odd mass number the excess spin within the atom results in a net magnetic spin. Some examples of nuclei with a non zero magnetic spin include $^1\text{H}$, $^{13}\text{C}$, $^{23}\text{Na}$, $^{31}\text{P}$. Any nuclei with a non zero magnetic spin exhibits resonance and can theoretically be detected by nuclear magnetic resonance (NMR). However, given the abundance of hydrogen within all living things, it is the natural atom of choice for producing images with NMR, commonly referred to as magnetic resonance imaging (MRI). The interested reader is referred to [33] for a more thorough discussion of MRI including the necessary quantum mechanics. The remainder of this section focuses on the MR properties of the hydrogen atom.

It has been established that protons have a net spin angular momentum, which gives rise to a magnetic dipole moment ($\vec{\mu}$), called spin. When a hydrogen atom is placed within an external magnetic field (\(\vec{B}_o\)) two interesting things happen: the spin aligns itself either parallel or anti-parallel to the external field (quantum angular momentum \(\vec{J} = \pm \frac{1}{2}\)) and the spins precess around \(\vec{B}_o\), just as a spinning top precesses about a gravitational field. Each spin orientation, parallel (low energy) and anti-parallel (high energy) is separated by a difference in energy given by Equation 1.1, where \(\vec{B}_o\) is the external magnetic field, \(\hbar\) is the Dirac constant (reduced Plank’s constant) and \(\gamma\) is the gyromagnetic ratio, a constant known for each nuclear species (for \(^1\text{H}\), \(\gamma = 267.52 \times 10^6 \text{rads}^{-1}\text{T}^{-1}\)).

\[
\Delta E = \gamma \hbar B_o
\]  

(1.1)

To understand why the spins would precess around around \(\vec{B}_o\) we consider the torque that \(\vec{B}_o\) exerts on \(\vec{\mu}\). Here torque is expressed as the rate of change of the angular momentum \(\vec{J}\). Recalling that angular momentum is related to \(\vec{\mu}\) by the gyromagnetic ratio \((\vec{\mu} = \gamma \vec{J})\) we can express the behaviour of the spin \((\vec{\mu})\) placed in the presence of \(\vec{B}_o\) by Equation 1.2.
\[
\begin{align*}
\frac{\partial \vec{J}}{\partial t} &= \vec{\mu} \times \vec{B}_o \\
\frac{\partial \vec{\mu}}{\partial t} &= \gamma \vec{\mu} \times \vec{B}_o
\end{align*}
\]

Equation 1.2 is a differential equation which can be solved explicitly for the condition when \( \vec{B}_o \) is time invariant (i.e. \( \vec{B}_o \) is constant assumed here to be oriented along the longitudinal \( z \) axis). The general solution is expressed in Equation 1.3, which is a simple rotation matrix of the initial vector \( \vec{\mu}_0 \) clockwise about \( \vec{B}_o \) at the frequency given by \( \gamma B_o \). This frequency is the resonant frequency, commonly called the Larmor frequency \( (\omega_o = \gamma B_o) \) and is fundamental to MR experiments. The Larmor frequency for \( ^1H \) (42.5 MHz/T) at clinical field strengths (1 – 3 T) is 42.5 – 127.5 MHz.

\[
\vec{\mu}(t) = \begin{pmatrix}
\cos(\gamma B_o t) & \sin(\gamma B_o t) & 0 \\
-\sin(\gamma B_o t) & \cos(\gamma B_o t) & 0 \\
0 & 0 & 1
\end{pmatrix} \vec{\mu}(0)
\]

Thus far we have described the behaviour of a single hydrogen atom magnetic spin in the presence of an external magnetic field, however, we are interested in the behaviour of a much larger number of hydrogen atoms contained within our object to be imaged. The first consideration when taking a large number of hydrogen atoms is the relative number of spins in the parallel (low energy) versus anti-parallel (high energy) orientation as spins from each state oppose each other and reduce the net magnetization. Analytically, the ratio of spins in the lower energy state \( (n_+) \) to the higher energy state \( (n_-) \) is given by a Boltzmann distribution as given in Equation 1.4, where \( \Delta E \) is the energy difference given in Equation 1.1, \( T \) is absolute temperature in Kelvin and \( k \) is the Boltzmann constant \((1.381 \times 10^{-23} J K^{-1})\).

\[
\frac{n_-}{n_+} = e^{\Delta E/kT}
\]
At 1 Tesla there is approximately 3 excess spin aligned with $\vec{B}_o$ for every $10^6$ hydrogen atoms at room temperature [32]. While this is a relatively small amount, when considering the sheer number of hydrogen atoms in a single cubic centimeter of water ($\approx 12 \times 10^{23}$) there are sufficient excess spins to produces a detectable net magnetization when added together. It is important to note that although individual spins may have components in the transverse ($xy$) plane perpendicular to $\vec{B}_o$ (which by convention is oriented in the longitudinal $z$ axis) these spins are all randomly oriented out of phase and result in no net transverse magnetization, thus $\vec{M} = \sum \vec{\mu}$ is oriented along the $z$ axis. The bulk magnetization aligned with the external field $\vec{B}_o$ for a fixed volume is given by Equation 1.5.

$$M_o = \frac{\gamma^2 h^2 \rho \vec{B}_o}{4kT} \quad (1.5)$$

Here the proton density within the volume is given by $\rho$. Equation 1.5 illustrates the only modifiable parameters to increase the net magnetization of an object are decreasing $T$ and increasing $\vec{B}_o$. *In-vivo* tissues only tolerate slight changes in temperature thus we are left with increasing $\vec{B}_o$ and hence the motivation behind higher field MR scanners.

Recalling that the *Larmor* frequency of a proton is determined by the $\vec{B}_o$ field it experiences it becomes clear that any modification to field affecting a proton causes a change in its *Larmor* frequency. This simple fact is the basis of being able to selectively excite a region of interest such as an image slice inside a sample and consequently makes MR imaging possible. Equation 1.6 describes the external magnetic field of the MR scanner over space.

$$\vec{B}(x, y, z) = \vec{B}_o + \vec{B}_G \quad (1.6)$$

Through manipulation of $\vec{B}_G$ we can spatially vary the external magnetic field $\vec{B}$ throughout the sample in all three dimensions, and hence the Larmor frequency of the protons. By controlling the Larmor frequency throughout our sample, we can spatially se-
lect the section of our sample that we wish to image by applying an RF pulse of a defined frequency range (restricted bandwidth). A more thorough description of selective excitation and subsequent image reconstruction is outside of the scope of this introduction. The interested reader is referred to any number of excellent resources [33, 34, 35, 36].

**MR Signal Behaviour and Detection**

The net magnetization $\vec{M}$ cannot be effectively measured while oriented with $\vec{B}_o$ in the longitudinal $z$ axis. In order to detect $\vec{M}$ an RF pulse ($\vec{B}_1$) is applied to the sample containing $\vec{M}$. $\vec{B}_1$ is the magnetic component of the RF pulse (EM wave emitted from RF coils) aligned in the transverse plane perpendicular to the resting net magnetization $\vec{M}$. The force that $\vec{B}_1$ exerts creates a torque on $\vec{M}$ rotating it around $\vec{B}_1$. As a result, $\vec{M}$ will now have a component in the transverse plane which precesses at the Larmor frequency $\omega_o$ as similarly shown for individual spins in Equation 1.3 with the following result in Equation 1.7. The process of rotating $\vec{M}$ using an RF pulse is known as **RF excitation**. The same RF coils used to rotate $\vec{M}$ can now be used to detect this precessing magnetization in the transverse plane ($M_{xy}$) according to Faraday’s law of induction. The change in magnetic flux $\Phi$ through the coil induces an electromotive force (EMF), $\epsilon$, measured as a time varying voltage ($\epsilon = -\frac{\partial\Phi}{\partial t}$).

$$M_{xy}(t) = M_{xy}^0 e^{-i\omega_o t}$$ (1.7)

The EMF signal recorded is proportional to the initial transverse magnetization $M_{xy}^0$ of all spins within the sample volume and is given by Equation 1.8. $M_{xy}^0(x, y, z)$ is the initial transverse magnetization at time zero ($t = 0$) after RF excitation for any position $(x, y, z)$ within the sample volume.

$$\epsilon(t) \propto \int_x \int_y \int_z M_{xy}^0(x, y, z)e^{-i\omega_o t} dxdydz$$ (1.8)

Depending on the amount of energy used in the RF excitation pulse, the angle of
rotation can be precisely controlled. Rotating $\vec{M}$ by 90° completely into the transverse plane gives the maximum detectable signal. However, the absorbed energy used to rotate $\vec{M}$ is eventually dissipated as the transverse component $M_{xy}$ decays and $\vec{M}$ returns to the equilibrium position oriented along $z$, the longitudinal axis of the static field $B_0$. This signal decay corresponding to the dissipation of absorbed energy is referred to as relaxation. The decay of transverse magnetization is described by Equation 1.9 which is a first order differential equation. When solved for a 90° RF excitation pulse, a simple exponential decay results with $T_2$ corresponding to the time required for the net transverse magnetization to reduce to 36.8% of its initial value.

$$\frac{dM_{xy}}{dt} = -\frac{M_{xy}}{T_2}$$  \hspace{1cm} (1.9)

In practice, the transverse relaxation is also affected by magnetic field inhomogeneities and represents the overall transverse relaxation time constant comprised of both reversible and irreversible processes denoted $T_2^*$. The dephasing of spins due to interactions with dipolar fields (often caused by nearby spins) is a time dependent process and irreversible, this is defined as the pure $T_2$ relaxation effect. Dephasing due to interactions with externally applied static magnetic field gradients and field inhomogeneities is a reversible process which can be recovered. The contribution of reversible and irreversible effects to the overall $T_2^*$ is given by Equation 1.10, where $T_2'$ and $T_2$ are irreversible and reversible processes respectively.

$$\frac{1}{T_2^*} = \frac{1}{T_2'} + \frac{1}{T_2}$$  \hspace{1cm} (1.10)

Once the net magnetization $\vec{M}$ has been rotated into the transverse plane and the RF pulse has been removed, the longitudinal component of the net magnetization also begins to return to its previous alignment with $B_0$ by releasing the absorbed RF energy. The regrowth of the longitudinal magnetization is described by Equation 1.11. Here $T_1$, the spin-lattice relaxation, is defined as the amount of time required for the longitudinal...
magnetization to return to 63.2% of its initial value at thermal equilibrium.

\[
\frac{dM_z}{dt} = -\frac{M_z - M_o}{T_1}
\] (1.11)

The Bloch Equation is a phenomenological equation describing the overall behaviour of the bulk magnetization ($\vec{M}$) during relaxation and is given in Equation 1.12. $M_x$, $M_y$ and $M_z$ are the components of $\vec{M}$ along the unit vectors $\hat{i}$, $\hat{j}$ and $\hat{k}$ in the $x$, $y$ and $z$ axes respectively. $M_o$ is the bulk magnetization along $z$ at thermal equilibrium as defined by Equation 1.5.

\[
\frac{d\vec{M}}{dt} = \gamma \vec{M} \times \vec{B} - \frac{M_x \hat{i} + M_y \hat{j}}{T_2} - \frac{(M_z - M_o) \hat{k}}{T_1}
\] (1.12)

Incorporating $T^*_2$ relaxation effects into Equation 1.8 gives Equation 1.13. This time varying voltage signal is commonly referred to as a free induction decay (FID) and is the basic MR signal recorded.

\[
\epsilon(t) \propto \int_x \int_y \int_z M^o_{xy}(x, y, z)e^{-i\omega_0 t}e^{-\frac{t}{T^*_2}} dxdydz
\] (1.13)

### 1.3 Image Contrast in MR

The image contrast we observe in MR originates from the amplitude ($\propto M^o_{xy}$) and phase ($e^{-i\omega_0 t}$) measured in our MRI signal. Therefore, processes that affect the amplitude or phase of the measured MR signal are potential sources of image contrast. This property is one of the great strengths of MRI, as it is possible to manipulate the contrast in the image based upon the method of acquisition. This also complicates the interpretation of MR images as the contrast observed can potentially originate from a number of sources depending on how the image was acquired. Consequently, it is necessary to understand the sources of MR contrast and how to acquire images to represent the contrast of interest. In this section, endogenous and exogenous contrast mechanisms are briefly described.
While our discussion is limited to contrast mechanisms affecting the signal originating from the hydrogen protons in water, which are predominantly imaged in MRI, it is worthy to note that there have been many recent developments in gaining contrast through the imaging of other atoms including $^{13}\text{C}$ [37, 38, 39, 40], $^{19}\text{F}$ [41, 42, 43, 44, 45] and $^{23}\text{Na}$ [46, 47, 48, 49, 50, 51].

MRI provides high resolution regional contrast based on differences in tissue composition producing images of anatomical structures. The major characteristics of tissue composition that affect the MR signal are water content (proton density $\rho$) and net magnetization relaxation properties. The affect of $\rho$ on signal contrast is fairly intuitive with more protons per unit volume providing more MR signal all else being equal. On the other hand, net magnetization relaxation properties are composed of longitudinal relaxation time $T_1$, transverse relaxation time $T_2/T_2^*$ as described in the previous section. These are the major determinants of contrast between tissues in MRI. Tissues possess different $\rho$, $T_1$ and $T_2/T_2^*$ due to physical environment, water mobility and interactions with macromolecules and cell membranes. In general, an image pixel is brighter when there is a greater contribution to the MR signal acquired from the particular pixel region, whereas darker pixels correspond to a much smaller MR signal contribution. To further improve contrast, one can increase the strength of the MRI static field ($\vec{B}_0$), change acquisition parameters or use MR contrast agents.

### 1.3.1 Gadolinium

The contrast agents predominantly used for cellular MRI include heavy metals of the lanthanide series such Gadolinium (Gd) and superparamagnetic iron oxides (SPIO). More recently, there has been interest in developing MR-reporter genes, which can produce a number of MRI detectable changes when expressed in the cell. The use of MR-reporter genes has two distinct advantages over currently used methods of cell labelling. Firstly, gene expression is only present in viable cells meaning detection of contrast also implies...
cell viability. Second, all cells originating from a cell with an MR-reporter gene also contain the gene, and therefore express the contrast. This circumvents the limitations of contrast agents that are diluted with ongoing cell division. Despite these advantages the use of MR-reporter genes is still in its infancy and is unsuitable for cell tracking due to lack of sensitivity at present [52].

Heavy metals from the lanthanide series such as Gd contain unpaired electrons, which create very strong magnetic dipole moments in the presence of an external magnetic field. Each unpaired electron creates a magnetic dipole moment over 600 times that of water protons. Water protons in close proximity to Gd experience a powerful interaction which promotes longitudinal relaxation and effectively shortens the $T_1$ (and to a lesser extent $T_2$ and $T^*_2$) causing hyperintensities on $T_1$-weighted images. Substances that tend to align their atomic magnetic dipoles with an external magnetic field, strengthen the local field and exhibit no net magnetism in the absence of the external magnetic field are referred to as paramagnetic [28]. Gd is toxic to cells in its ionic form, thus chelators such as diethylenetriamine penta-acetic acid (DTPA) are used to produce Gd-DTPA which is non-toxic. Gd-DTPA was the first MR contrast agent developed and has been used clinically for over 20 years to image tissues.

Gd-DTPA has not been widely adopted as an intracellular contrast agent for several reasons. Firstly, high intracellular concentrations of Gd-DTPA are necessary to produce sufficient contrast. Secondly, dechelated gadolinium is known to be toxic and detailed knowledge of the metabolic fate and excretion pathway of paramagnetics such as Gd-DTPA is currently not available, making clinical translation a challenge [27]. There is ongoing research investigating the development of customized Gd-based nanoparticles to overcome the limitations of sensitivity and possible cytotoxic effects [53, 54, 55]. Several applications of these particles for positive contrast of implanted stem cell tracking in stroke has been reported [54, 56, 57, 58].
1.3.2 Superparamagnetic Iron Oxide (SPIO) Particles

Superparamagnetic iron oxide (SPIO) particles are the predominant contrast agent for cellular imaging and has been in use for over a decade. The description of the physical and chemical properties of SPIO has been summarized from a comprehensive review by Thorek et al. [28]. SPIO particles are composed of single or multiple iron oxide cores embedded within a polymer matrix coating. The surface of this polymer can be modified to contain functional groups allowing the attachment of peptides or antibodies for targeted uptake. Fluorophores or radiotracers can also be conjugated to these particles to allow multi-modal imaging. Sizes of SPIOs range from monocristalline iron oxide nanoparticles (MIONs) \( \approx 3 \text{ nm} \) in diameter, ultra small iron oxide (USPIO) particles \( \approx 10 \text{ nm} \) and MPIOs (micron sized iron oxide particles) can be as large as \( 10 \mu \text{m} \) [28, 30].

Within these particles the iron atoms are arranged in the crystalline structure such that their magnetic spins interact and align parallel with each other creating magnetic domains which extend a certain distance within the crystal. At long range adjacent magnetic domains become anti-aligned. The transition between these two domains is called a Bloch wall, which becomes thermodynamically unfavourable at the nanometer scale (\( \approx 14 \text{ nm} \)) [28]. The result is single domain crystals, which are classified as superparamagnetic.

Superparamagnetic substances are characterized by the strong paramagnetic nature of the particles at this scale. Compared with paramagnetic substances such as Gd, SPIO also exhibits no magnetism when the external magnetic field is removed. However, SPIO has a much larger susceptibility due to alignment of the magnetic moments of the entire crystal with the applied field. Magnetic susceptibility \( \chi \) is defined as a material specific, unitless constant of proportionality between the external magnetic field and the degree of magnetization within the material. Materials with high magnetic susceptibility experience a large increase in magnetization within the material when placed in an external magnetic field. The net magnetization of SPIO is three orders of magnitude larger than that of paramagnetic substances and causes large disturbances in the local magnetic field.
through disrupting the phase of surrounding protons. These disturbances or field inhomogeneities cause disruption of phase coherence of water protons and have a potent effect on transverse relaxation, $T_2^*$. 

Thus, SPIO is imaged indirectly through its effect on longitudinal and transverse relaxation of the surrounding water protons. Although it has been shown that SPIO can generate detectable $T_1$ contrast, typically SPIO contrast is imaged with MRI by using sequences sensitive to transverse relaxation and susceptibility ($T_2$ and $T_2^*$ weighted imaging) [28]. For the purposes of this thesis $T_2$ sensitive sequences of interest include spin echo (SE) and balanced steady state free precession (b-SSFP) while $T_2^*$ sensitive sequences include spoiled gradient recalled echo (SPGR) and susceptibility weighted imaging (SWI).

### 1.3.3 MR Pulse Sequences

All MRI sequences are composed of a series of RF excitation pulses and subsequent recording of the resulting MR signal. An MRI sequence can be thought of as a general recipe for creating an image with basic set of ingredients. By varying the amounts of the different ingredients in the recipe the resulting image changes. The relevant ingredients for understanding the MR pulse sequences used in this thesis are summarized in Table 1.3.3.

#### Spin Echo (SE)

A spin echo (SE) sequence is initiated by an RF pulse rotating the net magnetization (flip angle, $\alpha$) by 90° into the transverse plane. Due to $T_2^*$ effects this transverse magnetization immediately begins to decay due to dephasing of the spins. If a 180° rotation RF is applied after a certain time $\tau$, the dephasing spins shift their phase by 180° reversing their direction of precession and therefore begin to rephase, becoming coherent at $2\tau$ as shown in Figure 1.1, panel A. This effect can be easily understood by a simple analogy. Three track runners (or spins) of varying speeds (or phase) start a race at $t = 0$ (coherence at
Table 1.2: MR Sequence Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flip Angle</td>
<td>degrees</td>
<td>Amount of rotation of the net magnetization into the transverse plane</td>
</tr>
<tr>
<td>Matrix</td>
<td>pixels</td>
<td>In plane resolution of the resulting image</td>
</tr>
<tr>
<td>Time to Repetition (TR)</td>
<td>milliseconds</td>
<td>Amount of time between RF pulse excitations</td>
</tr>
<tr>
<td>Time to Echo (TE)</td>
<td>milliseconds</td>
<td>Amount of time delay between RF excitation pulse and recording of the MR signal or echo</td>
</tr>
<tr>
<td>Number of Excitations (NEX)</td>
<td>number</td>
<td>Number of times the entire imaging experiment is repeated</td>
</tr>
<tr>
<td>Field of View (FOV)</td>
<td>millimeters</td>
<td>Dimensions of the volume to be imaged</td>
</tr>
<tr>
<td>Slice Thickness</td>
<td>millimeters</td>
<td>Thickness of the sample averaged into a single 2D image</td>
</tr>
<tr>
<td>Voxel Dimensions</td>
<td>millimeters</td>
<td>Smallest volume element from which MR signal is localized</td>
</tr>
</tbody>
</table>
Figure 1.1: Concept behind spin echo sequences. (A) Spin behaviour during SE pulse sequence. Fast spins (F) dephase more quickly than slow spins (S). The 180° phase shift causes S spins to be ahead of the F spins causing them to rephase and become coherent again at 2τ. (B) Track runner analogy of the spin echo concept. (C) RF pulse and FID signal diagram. The 180° refocusing pulse suppress the reversible transverse relaxation, T’₂. Figures adapted from Hashemi et al. [35]

excitation) and after a certain time (t = τ) are separated (dephased) by a certain distance. If the runners reverse direction at t = τ (180° phase shift) the distance between them decreases (rephase) until they all arrive back at the starting line at t = 2τ as depicted in Figure 1.1, panel B. The recorded MR signal in relation to the applied SE pulse sequence is shown in Figure 1.1, panel C. The amplitude of the two echoes is proportional to T₂ and the T’₂ effects have been effectively suppressed by the use of the 180° refocusing pulse.

Consequently, SE sequences can be used to produce images with T₂ weighted contrast. A long TR is typically used in SE to allow longitudinal magnetization to fully recover
before the next $90^\circ$ excitation pulse. This minimizes the influence of $T_1$ on the image contrast. Varying the TE of a spin echo sequence adjusts the amount of $T_2$ relaxation allowed to occur prior to signal acquisition. A SE sequence with a very short TE and long TR minimizes the contribution of $T_1$ and $T_2$ to the overall image contrast resulting in a proton density weighted image. On the other hand a long TR, long TE image minimizes the contribution of $T_1$ and proton density to the overall image contrast resulting in a $T_2$ weighted image.

**Spoiled Gradient Recalled Echo (SPGR)**

Spoiled Gradient Recalled Echo (SPGR) uses steady state free precession (SSFP) which is achieved during a train of RF pulses using a very short $T_R$ ($T_R \ll T_1, T_2$). The steady state is achieved when longitudinal and transverse magnetizations reach a dynamic equilibrium where the behaviour of the magnetization between excitation pulses is identical from pulse to pulse [32]. The theoretical foundations of SSFP were formally described by Carr [59] in 1958, however, only recently has SSFP imaging become feasible with advances in MR hardware and software. SPGR pulse sequences utilize the steady state for fast imaging achieved with successive RF pulses separated by a short $T_R$ with $\alpha$ typically much less than $90^\circ$. Magnetic field gradients refocus the magnetization and an echo is formed at TE, when the signal is acquired. The transverse magnetization after data collection is destroyed or spoiled prior to the next RF excitation pulse using unbalanced gradients and/or RF energy. Since the sequence is unbalanced, the net magnetization is not refocused and $T_2^*$ decay occurs making SPGR extremely sensitive to sources of susceptibility and field inhomogeneities such as SPIO. SPGR sequences with small $\alpha$ and short TE are more proton density weighted, while increasing $\alpha$ produces more $T_1$ weighting. Extending TE increases the amount of $T_2^*$ decay and hence adds $T_2^*$ weighting to the overall image contrast.
Balanced Steady State Free Precession (b-SSFP)

Recently, a number of SSFP based techniques including balanced SSFP (b-SSFP) have gained popularity for detecting SPIO labeled cells [23, 60]. b-SSFP is an implementation of SSFP where the residual magnetization at the end of each cycle is completely refocused into a single magnetization vector. The ability of b-SSFP to completely refocus the magnetization with each cycle results in extremely high signal to noise ratio (SNR) per unit time, sensitivity to field inhomogeneities and predominantly $T_2$ weighted contrast [61]. These features make b-SSFP ideal for angiographic and cardiac applications [61]. Unfortunately, b-SSFP sequences suffer from banding artifacts if the frequency range across the subject exceeds $\pm \frac{1}{2TR}$ which is especially problematic at higher field strengths [61, 62]. Therefore, it is difficult to implement b-SSFP without specialized strategies to compensate for these effects such as phase cycling or more powerful imaging gradients.

Susceptibility Weighted Imaging (SWI)

Susceptibility weighted imaging (SWI) is currently being applied to separate arteries from veins on MR angiography based upon differences in blood oxygenation that result in detectable phase differences [63, 64]. Deoxyhemoglobin is paramagnetic and exhibits greater susceptibility than oxyhemoglobin which is not. SWI is achieved by performing an SPGR acquisition with a specific TE and flow compensation to suppress phase changes due to chemical shifts from fat and flow respectively [63]. The low spatial frequency nature of spatial variations are used to reduce the background field effects [63]. Accounting for these factors leaves two sources of susceptibility: susceptibility field changes from paramagnetic deoxyhemoglobin and susceptibility field changes from the presence of a contrast agent, such as SPIO. In constructing the SWI image both magnitude and phase information from the acquired SPGR images is incorporated to produce the final image. Therefore, SWI is a very sensitive to sources of susceptibility and potentially useful to detect SPIO, although other sources of susceptibility such as the presence of hemorrhage...
could possibly confound interpretation.

1.3.4 Signal to Noise Ratio, Contrast to Noise Ratio

Signal to Noise Ratio, SNR

One of the fundamental measures of MRI image quality is the signal to noise ratio (SNR). This is defined mathematically by Equation 1.14. \( S \), is the signal amplitude and \( \sigma_{\text{noise}} \) is the standard deviation of the noise.

\[
SNR = \frac{S}{\sigma_{\text{noise}}} \quad (1.14)
\]

SNR is dependent on tissue parameters (proton density \( \rho \), \( T_1 \), \( T_2 \)), acquisition parameters (sequence, voxel volume \( V \), readout time \( t_{ro} \)) and the MR system used to create the measurements (static field strength \( B_o \) and receiver coil properties). The \( t_{ro} \) is a product of NEX, phase encodes and time per acquisition. While tissue parameters are fixed for a given sequence, the other factors contribute to the overall SNR as follows:

\[
SNR \propto B_o V \sqrt{t_{ro}} \quad (1.15)
\]

Contrast to Noise Ratio, CNR

Another important measure of MR image quality of particular interest in cellular MRI is the contrast to noise ratio (CNR), which characterizes the ability to detect a signal change due to labeled cells against a homogenous background. The CNR is defined in Equation 1.16 as the difference in signal between the region of interest, in this case \( S_{\text{cell}} \) and \( S_{\text{background}} \) divided by the standard deviation of the noise in the image \( \sigma_{\text{noise}} \). Hence, MR sequences that maximize the difference between \( S_{\text{cell}} \) and \( S_{\text{background}} \) generates the highest CNR and provides the best cell detection.
Chapter 1. MRI Monitoring of Stem Cell Therapies

\[ CNR = \frac{S_{\text{background}} - S_{\text{cell}}}{\sigma_{\text{noise}}} \]  \hspace{1cm} (1.16)

1.4 Hypothesis and Thesis Overview

1.4.1 Hypothesis

It is the intent of this thesis to test the following hypothesis:

1. Rat marrow derived mesenchymal stromal cells (rMSCs) can be effectively labeled with micron sized superparamagnetic iron oxide particles (MPIOs) with minimal negative biological effects and detected with a clinical 3 Tesla MRI system using conventional imaging sequences and simple surface coils.

Furthermore, this thesis will test the feasibility and explore the limitations of detecting intravenously delivered MPIO labeled rMSCs with a 3 Tesla clinical MRI system using conventional imaging sequences in a permanent focal ischemia stroke model in rats.

1.4.2 Thesis Overview

The principle goal of this work was to translate the current tools for labelling and imaging stem cells used with specialized hardware and experimental MRI systems to a more clinically relevant setting. Specifically, this thesis focuses on labelling an rMSC population containing mesenchymal stem cells with MPIO. All MRI was performed using a 3 Tesla clinical MRI system with a simple RF surface coil and conventional imaging sequences.

In Chapter 2, the ability to detect single MPIO labeled rMSCs using a clinical MRI system is presented. In Chapter 3, the feasibility of applying these methods to detect intravenously delivered MPIO labeled rMSCs in-vivo in a rat stroke model is tested.

Well established methods from the literature have been implemented to harvest, grow and label rMSCs with MPIO. Similarly, routine tests to determine basic cell function have
been used to assess cell viability, plating efficiency, growth rate and labelling efficiency. \textit{In-vitro} imaging of MPIO labeled rMSCs was performed using SPGR MRI sequences which are highly sensitive to the presence of iron. Optical validation studies to verify single cell detection followed directly from the foundational work of Chris Heyn [65].

The established rat stroke model employing permanent focal devascularization performed in the lab of Greg Stanisz was used for the detection feasibility study. Following the experimental treatment strategy in this stroke model developed by Cindi Morshead initiating growth factor supplementation, intravenous injection of MPIO-rMSCs was performed 3 days post stroke. The protocol for combined immunohistochemical and Prussian blue staining to facilitate epifluorescence and confocal microscopy was adapted from Andrea Vaags.

My contributions to this work included: cellular work including harvest, plating and maintenance, biological function assays, cell phantom preparations and MR imaging, data analysis and microscopy, animal handling, MR imaging sessions and intravenous injections, animal sacrifice and perfusions, tissue specimen fixation, sectioning and immunohistochemical, fluorescent and prussian blue staining for histology. All stroke procedures were performed by Amy Hoyles. This project was completed under the supervision of Greg Stanisz and David Mikulis.
Chapter 2

In-vitro magnetic resonance imaging of micron sized superparamagnetic iron oxide labeled rat bone marrow derived mesenchymal stromal cells

2.1 Introduction

Non-invasive monitoring of stem cell therapies is essential in establishing both the treatment efficacy and safety of clinical applications. Imaging modalities capable of stem cell detection and tracking in-vivo include optical imaging, PET, SPECT and MRI. MRI is very useful for stem cell therapy monitoring in that it combines high contrast and 3D spatial resolution with the ability to detect small numbers of cells.

Currently, MRI is being used to monitor a variety of experimental stem cell therapies targeting various diseases including myocardial infarction and stroke [20, 26, 66, 67]. The most commonly used method of detecting delivered stem cells with MRI involves labelling stem cells with superparamagnetic iron oxide (SPIO) based contrast agents.
which produce negative contrast on $T_2/T_2^*$ weighted MRI sequences [27, 58, 67, 68, 69]. The majority of studies investigating the delivery and tracking of SPIO labeled stem cells in the treatment of stroke have used specialized hardware or high field small animal MRI systems. Before these methods of stem cell labelling and detection can be applied to the treatment of patients they need to be translated to a more clinically relevant MRI system.

This chapter describes the necessary materials and methods used for harvesting, growing and labelling rat derived bone marrow mesenchymal stromal cells (rMSCs) with micron sized SPIO particles (MPIOs). MPIO uptake, localization and labelling efficiency was determined. Cell viability, proliferation and plating efficiency were assessed. Detection of MPIO labeled rMSCs (MPIO-rMSCs) with \textit{in-vitro} MRI was demonstrated with optical validation. The focus of these experiments was to develop a protocol for labelling and detection of rMSCs with a clinical MRI for future \textit{in-vivo} cell-tracking studies in animal models of stroke.

The choice of rMSCs was based on various studies showing therapeutic benefit in promoting central nervous system repair within animal models [6, 70, 71, 72, 73, 74, 75, 76, 77] and the potential clinical applications within the rapidly growing fields of tissue engineering and regenerative medicine [5, 16, 18]. Furthermore, MSCs are relatively easy to harvest and grow \textit{in-vitro} and others have successfully labeled such cells with SPIO based MR contrast agents [27, 67, 78, 79, 80, 81, 82, 83, 84, 85, 3, 86]. The SPIO contrast agent selected was a 0.9 $\mu$m diameter SPIO Bang’s particle (MPIO) composed of iron oxide immersed in a divinyl benzene polymer conjugated to a green fluorophore, which has also been previously shown to effectively label stem cells for detection with MRI [3, 23, 29, 31].
2.2 Methods

2.2.1 Cell Sample Preparation

Cell Harvest and Culture

rMSCs were obtained and cultured as described previously by Javazon et al. [1]. Briefly, rMSCs were harvested from bone marrow aspirates obtained from the tibias and femurs of male Long-Evans rats (Charles River Laboratories) 60 days post gestation. A 21-gauge needle was inserted into each bone shaft and flushing was performed to collect the whole bone marrow with 3 mL of complete $\alpha$-modified Eagle’s medium ($\alpha$MEM, Gibco, 12571-063) containing 20% fetal bovine serum (FBS, Gibco, 12319-018), 2 mM L-Glutamine, 100 U/mL penicillin (Gibco, 15140-122), 100 $\mu$g/mL streptomycin (Gibco, 15140-122) and 25 ng/mL Amphotericin B (Sigma, A2942). Once all of the bones were flushed, 9 mL of cold red blood cell lysis buffer (Stem Cell Technologies, 07800) was added, the suspension was then incubated at 37°C for 6 minutes and centrifuged at 450 g for 7 minutes. Cells were washed two more times by resuspension in fresh complete media and subsequent centrifugation. Following the third resuspension the cells were filtered through a 70 $\mu$m nylon filter (BD Falcon, 352350) to remove any bone fragments or clots. Filtered cells were then plated onto a 75 cm$^2$ tissue culture flask. The cells were grown in complete $\alpha$MEM at 37°C and 5% CO$_2$. After three days the medium was replaced leaving only the adherant cells in culture. The cells were then expanded to 90% confluence. This population was defined as passage zero (P0).

Passaging of rMSCs

Cells at P0 were washed with phosphate buffered saline (PBS, Sigma, D8537) without Ca$^{2+}$ and Mg$^{2+}$ and then detached by incubation in 0.25% trypsin 0.1% ethylenediaminetetraacetic acid (trypsin-EDTA, Gibco, 25300-054) for 5-10 minutes at 37°C. Fresh complete medium was added to deactivate the trypsin and cells were centrifuged for 7
minutes at 450g. Cells were counted using a hemocytometer and then plated into 175 cm$^2$ tissue culture flasks at initial densities of 500-1000 cells/cm$^2$ (Passage 1 - P1). Cells were refed by replacing the complete medium every 3-4 days. Once cultures reached confluence the cells were passaged again as described above. The procedure was repeated up to five times. All experiments were performed with cells at P3-P5.

**Labeling of rMSCs with MPIO Particles**

Prior to labelling, cells were plated into a 6 well plate (Corning Costar, 3516) at approximately 500,000 cells per well. Cells were allowed to adhere for 12 hours. The medium was replaced with fresh complete medium and the MPIO contrast agent was added. These MPIO particles (MPIOs) are composed of a divinyl benzene polymer with a mean diameter of 0.9 $\mu$m immersed with 62% magnetite ($\text{Fe}_3\text{O}_4$) by weight and a Dragon Green fluorophore with 480 nm peak absorption and 520 nm peak emission spectra (Bangs Labs, MC05F). MPIO doses of 2.4, 4.8, 9.6, 18.8 and 37.8 $\mu$g iron/cm$^2$ were used for labelling experiments. The cells were incubated with MPIOs for 6, 24 or 48 hours to allow endocytosis to occur after which they were washed three times while still adherent inside the wells with PBS to remove free contrast agent. After initial washing the cells were detached with trypsin-EDTA and washed three or four more times by repitive centrifugation (100g, 5 minutes) and resuspension in PBS. Prior to the final centrifugation the cells were counted using a hemocytometer and the final suspension of cells was adjusted to the desired concentration in PBS or complete media as the intended experiment required.

**2.2.2 Cellular Iron Quantification and Localization**

**Colorimetric Iron Assay**

The mean iron dose per cell was determined by a colorimetric iron assay as described by Bernas et al. [31]. After labelling, cells were resuspended in PBS at a concentration of $2 \times 10^6$ cells/mL. Using a 96 well plate (Corning Costar, 3596) approximately 200,000
cells at each loading dose (0, 2.4, 4.8, 9.6, 18.8 and 37.8 µg iron/cm².) were plated into individual wells in addition to PBS alone and a series of serial dilutions of known iron concentration (Bangs particles suspended in PBS) from 64µg/mL - 1µg/mL for a calibration curve. All samples were plated in triplicate. The plate was then placed in an oven overnight at 80°C to dessicate the wells and denature any proteins. The following day concentrated (5 M) hydrochloric acid was added to each well, the plate was sealed and returned to the oven at 60°C for five hours. Finally, potassium ferrocyanide was added to each well and the plate was incubated for 15 minutes at 37°C. The iron in the well formed a complex which absorbs light at 650 nm. To quantify the total iron per well the plate was read using an ELISA plate reader (Biotek Powerwave x340-1 Microplate Reader). The absorption data from the standard dilutions was used to determine the total iron per well which was averaged to determine the mean iron load per cell.

Confocal Microscopy

In order to localize the contrast agent within the cells and qualitatively assess labelling efficiency and homogeneity, confocal microscopy was performed on a Zeiss LSM 510. MPIO particles were visualized with confocal microscopy due to the conjugated Dragon green fluorophore (480 nm absorption, 520 nm emission). To allow fluorescent visualization of the intracellular space MPIO-rMSCs and rMSCs were labeled with carbocyanine dyes [DiI (540 nm,580 nm) or DiD (640 nm,680 nm) cell labelling solutions (Molecular Probes, Invitrogen, V-22889)]. DiI was used to label MPIO-rMSCs (DiI-MPIO-rMSCs) and DiD was used to label control rMSCs (DiD-rMSCs) such that the cell populations could be differentiated when imaged together. DiI-MPIO-rMSCs and DiD-rMSCs cell populations were mixed together in suspension, allowed to adhere to a sterile microscope slide coverslip and fixed with 4% paraformaldehyde (PFA) in PBS. After subsequent washing, coverslips were mounted onto microscope slides with a fluorescent mounting medium (Dako Fluorescent Mounting Medium, Dakocytomation).
2.2.3 Contrast Uptake Efficiency and Biological Effects on Cell Function

**Fluorescence Activated Cell Sorter (FACS) Analysis**

Serial fluorescence activated cell sorter (FACS) analysis was performed (BD Biosciences, FACSCalibur) once (n=1) to assess labelling efficiency and viability on MPIO-rMSCs and control rMSCs plated at high plating density (HPD), near confluence or at low plating density (LPD) over a five day period. Aliquots of cells were collected at 24, 48, 72, 96 and 120 hours and analyzed. To differentiate between live and dead cells, propidium iodide (PI) was added at 5 µg/mL. Cell samples were analyzed for size (forward scatter), granularity (side scatter), PI−/+ (live/dead) and green fluorescence from MPIO+/− (labeled/unlabeled). Dead cells were PI+ as the disrupted cell membrane allowed PI into the cell whereas live cells are PI− with intact cell membranes. The thresholds for live versus dead and MPIO labeled versus unlabeled cells (≈ 20 and ≈ 200 respectively) were determined using test and control samples prior to the longitudinal experiment and gate values were held consistent throughout. Solutions were adjusted to a concentration of 10^6 cells/mL prior to analysis and 10,000 event counts were used to characterize each sample.

**Colony Forming Unit Assay**

To determine the effect of MPIO particle loading on plating efficiency a colony forming unit assay was performed. Cells labeled at ≈ 44 and ≈ 54 pg iron/cell and unlabeled controls were plated in 100 mm diameter petri dishes in 10 mL of complete medium at densities of 250, 500 and 1000 cells per plate and incubated for 10 days at 37°C and 5% CO₂. Subsequently, the medium was removed and the plates were stained with 5 mL methylene blue solution (3g methylene blue in 1 L 50% ethanol in distilled water) for 10 minutes after which the methylene blue was removed and the plates were rinsed with
Chapter 2. *In-vitro* MRI of MPIO labeled rMSCs

water and allowed to dry. An initial survey of the stained plates was used to determine the threshold size of a macroscopic colony, which was then applied to enumerate the total colonies formed in each of the plates at the various densities and MPIO particle loading levels. Each condition was tested in triplicate and the experiment was repeated 3 times (n=3).

**Cell Metabolism and Proliferation**

Post labelling cell metabolism and proliferation was assessed using an methyl tetrazolium salt assay (MTT assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Invitrogen, V13154). After 24 hours incubation with MPIO and subsequent washing as previously described, MPIO-rMSCs and rMSC controls were suspended into complete medium without phenol red and 10% fetal bovine serum (FBS) and plated into 96 well plates at 10,000 cells per well in triplicate. The assay was run over a 6 day period and repeated in triplicate. On each day of the assay the 10 µL of MTT solution was added to each microwell and the cells were incubated for four hours during which the metabolic activity of the mitochondria converted the MTT into purple formazan crystals. After the incubation period the media was removed from the well and 100 µL of an anionic solvent, sodium dodecyl sulfate in dilute hydrochloric acid (SDS-HCl), was added and the plate was returned to the incubator overnight to allow the SDS-HCl to dissolve the formazan crystals rendering the well a shade of purple proportional to the initial amount of formazan crystals and hence metabolic activity of the cells. The produced color intensity was quantified using a 96-well plate reader (Biotek Powerwave x340-1 Microplate Reader) by reading absorbance at 570nm and subtracting off background absorbance at 650nm. To determine whether MPIO particle loaded cells were less metabolically active than unlabeled cells the MTT assay was performed on fixed cell numbers (10,000 30,000 and 60,000) for medium (≈ 44 pg/cell) and high (≈ 54 pg/cell) loading levels as well as unlabeled controls over 5 days [87].
Growth Assay

Post labelling cell growth rate was assessed over seven days using an *in-vitro* growth assay. Labeled and unlabeled cells were plated in triplicate into seven identical six well plates at 10,000 cells per well in 2 mL of complete medium. On each day of the experiment one plate was used for counting. Briefly, adherent cells were removed from each well of the six well plate by incubation with 250 µL trypsin-EDTA for 5 minutes and subsequently quenched with 750 µL complete medium. After centrifugation at 450g for five minutes cells were resuspended at a density of 10 – 100 × 10^4 cells/mL to facilitate counting. Samples from each well were counted in triplicate using a hemocytometer. The experiment was repeated 3 times (n=3).

2.2.4 Cellular MRI and Correlative Fluorescence Microscopy

Cell Sample Preparation

Gel phantoms were used for imaging MPIO loaded rMSCs in bulk (greater than 1000) and at the single cell level. Cells were suspended in 4% gelatin in sterile PBS at 37°C and serial dilution gel phantoms were created with 10^6, 5 × 10^5, 10^5, 5 × 10^4, 10^4, 5 × 10^3 and 10^3 cells non-uniformly suspended in 500 µL total volume in 6 × 50 mm borosilicate glass NMR tubes (VWR, 6820-068). Samples were cooled and stored in a refrigerator at 4°C to allow solidification. For fewer than 1000 cells, cells were plated onto a single plane gelatin (gel) sandwich using the microwells (6 mm diameter) of a 96 well plate (Corning, 3596). To create a flat meniscus on which to place the cells 50µL of 8% PBS-gel was added to each well and allowed to solidify. Then approximately 5µL of 2% PBS-gel was carefully added so as to fill and flatten the hardened meniscus. The desired amount of cells suspended in 2% gel-PBS was then added and allowed to adhere onto the hardened gel surface. Finally, the remainder of the well was filled with 2% PBS-gel resulting in a single layer of cells sandwiched between layers of 8% and 2% PBS-gel.
Chapter 2. *In-vitro* MRI of MPIO labeled rMSCs

**MR Experiments and Optical Validation**

All imaging was performed on a clinical 3 Tesla GE Signa MRI scanner (GE, Milwaukee, USA). All specimens were placed directly onto the center of a 3 × 5 cm receive only rectangular surface coil and aligned with the scanner isocenter. Imaging gel phantoms was achieved using two dimensional spoiled gradient recalled echo (2D-SPGR) sequence. The SPGR parameters were: 20° flip angle, 30 × 30 × 1 mm FOV, 1 mm slice thickness, 512 × 512 matrix and 15.63 kHz receive bandwidth. The number of excitations (NEX), time to repetition (TR) and time to echo (TE) ranged from 8 – 70 NEX, 57 – 111 ms and 11 – 34 ms respectively, depending on the sample. For the NMR tube phantoms and single plane phantoms these parameters were: TE/TR 34/111 or 24/57 ms with 24 NEX and TE/TR 24/57 ms with 70 NEX respectively. MR images from single plane phantoms were compared to phase contrast and epifluorescence microscopy performed using a Leica DM RB Fluorescent Microscope with Cool Snap Digital Camera.

**2.3 Results**

**2.3.1 MPIO Particle Uptake and Localization Characteristics**

**MPIO Localization**

Localization of the MPIO particles post labelling and washing was achieved with confocal microscopy. Total particle uptake was heterogeneous throughout the population as shown in a representative sample at high magnification in Figure 2.1. Panel A shows DiI-MPIO-rMSCs and untreated control DiD-rMSCs labeled with DiI(red) and DiD(blue) endosomal fluorescent markers respectively. Panel B shows green fluorescence from the MPIO particles present within the mixed cell population. Panel C shows co-localization of endosomal DiI(red) and MPIO particles (green) revealing a cytoplasmic, granular distribution of MPIO particles only within the DiI labeled cells that were incubated with
MPIO. The untreated DiD labeled control rMSCs do not fluoresce green. In panel D, Nomarski optics reveal the outlines of the rMSCs with the MPIO particles clustered in perinuclear organelles. Each incubation concentration and plating density clearly showed a perinuclear, cytoplasmic granular distribution of MPIO particles in the rMSC population.

**MPIO Particle Uptake**

MPIO particle uptake by rMSCs was observed to be a function of MPIO concentration and incubation time. Figure 2.2 shows the relationship of average iron per cell versus iron incubation concentration per unit area. The average iron content per cell plateaued after 24 hours at an incubation concentration of 18.8 µg/cm² resulting in approximately 54 pg iron/cell. There was no observable difference in average iron loading between 24 and 48 hour incubation times. Decreasing the incubation time to 6 hours resulted in decreased average iron uptake, approximately half (27 pg iron/cell) of the 24 hour value at the same incubation concentration (18.8 µg/cm²).

**2.3.2 Labeling Efficiency and Biological Effects of MPIO Particle Loading**

**Labeling Efficiency**

Fluorescence activated cell sorting (FACS) analysis by flow cytometry observed 89% of MPIO-rMSCs were alive and labeled after 24 hour incubation with MPIO particles at 18.8 µg iron/cm². Figure 2.3 summarizes the results of the flow cytometry analysis of MPIO-rMSCs versus unlabeled control rMSCs. In Figure 2.3 panels A and B are the size (forward scatter) versus granularity (side scatter) plots showing increased side scatter observed in labeled (B) versus unlabeled cells (A) respectively. Panels C and D are PI versus MPIO green fluorescence plots with quadrants defining the gating thresholds
Figure 2.1: Confocal microscopy performed on DiI-MPIO-rMSCs and DiD-rMSCs. (A) Multi-channel confocal acquisition showing fluorescent endosomal labelling of the MPIO treated (red) and untreated (blue) rMSCs with lipophilic carbocyanine dyes: DiI (red) and DiD (blue). (B) Single channel confocal acquisition of green fluorescence originating from MPIO particles. (C) Multi-channel co-localization of endosomal DiI (red) and MPIO particles (green) revealing a cytoplasmic, granular distribution of MPIO particles within the MPIO treated cells whereas untreated control rMSCs show no green fluorescence. (D) Nomarski optics (differential interference) revealing the outlines of the adherent rMSCs and MPIO particles clustered in perinuclear organelles. A subconfluent population of rMSCs were exposed to MPIO particles for 24 hours while identically plated control rMSCs were not. rMSCs incubated with MPIO particles were labeled with an endosomal fluorescent marker, DiI (red) while untreated control rMSCs were labeled with DiD (blue) so as to differentiate the populations after being mixed together and simultaneously imaged.
Figure 2.2: Average iron uptake per cell is shown as a function of iron incubation concentration for three different incubation times. Error bars are representative of the standard deviation of 3 samples per data point. After 6, 24 and 48 hours of incubation with MPIO particles, cells were washed, counted and loaded into a 96 well microplate. Treatment with hydrochloric acid and potassium ferrocyanide produces a blue pigment proportional to the amount of ferric iron, quantified by absorbance at 650 nm on a microplate reader. Error bars represent standard deviation of 6 samples at each loading. Experiment was repeated in triplicate and typical results are shown.
for sub-populations of live labeled (cells with MPIO, LL), live unlabeled (cells without MPIO, LU), dead labeled (DL) and dead unlabeled (DU)]. Panel E is the histogram of MPIO green fluorescence distribution for the live cells within the MPIO-rMSCs and control rMSC populations.

FACS analysis after 120 hours incubation showed 78% MPIO-rMSCs at high plating density (MPIO-rMSCs-HPD) were live and labeled versus 29% of MPIO-rMSCs at low plating density (MPIO-rMSCs-LPD). Figure 2.4 summarizes the results of flow cytometry analysis for MPIO-rMSCs-HPD, MPIO-rMSCs-LPD and unlabeled control rMSCs. Panels A,B and C contain FSC-SSC plots showing densely plated cells (B) retain more granularity as compared to cells plated at low density (C) and unlabeled controls (A). Panels D,E and F are MPIO fluorescence versus PI plots showing the subpopulations of live/dead, labeled/unlabeled cells in unlabeled controls (D) and labeled cells plated at high (E) and low (F) density. There were no significant differences in viability between labeled and unlabeled cells over the course of the experiment regardless of the incubation concentration of MPIO used for labelling [9.8 or 18.8 µg iron/cm² (ANOVA, p= 0.61)]. The distribution of MPIO green fluorescence after 120 hours incubation is shown in panel G. The distribution of live-labeled cells plated at HPD (black) is furthest to the right and contains more green fluorescence compared with cells plated at LPD (gray) versus live-unlabeled cells (white). Panels H and I show confocal microscopy of the cell suspensions from the HPD (B,E) and LPD (C,F) conditions respectively. Cells in suspension were allowed to adhere to a slide coverslip and were stained endosomally with DiI (red). Panel H shows more green fluorescence (MPIO particles) retained within HPD cells as compared with those at LPD (panel G). An LPD cell with strong green fluorescence is indicated with a white arrow.
Figure 2.3: Flow cytometry of MPIO labeled rMSCs 24 Hours Post-Labeling. Labeled and unlabeled rMSCs were analyzed for size (forward scatter), granularity (side scatter), viability (PI$^{-/+}$) and MPIO green fluorescence. (A,B) Size versus granularity plots showing increased granularity in labeled A, versus unlabeled cells B after 24 hours incubation. (C,D) PI versus MPIO green fluorescence plot showing sub-populations [live labeled with MPIO (LL), live unlabeled (LU), dead labeled (DL) and dead unlabeled (DU)] within the cell populations from A,B. (E) Histogram characterizing the MPIO green fluorescence distribution gated to include the live control rMSCs (white) versus live MPIO-rMSCs (black).
Figure 2.4: Flow cytometry of MPIO labeled rMSCs 120 Hours Post-Labeling. MPIO-rMSCs and unlabeled control rMSCs were analyzed for size (forward scatter), granularity (side scatter), viability (PI\(^{-+}\)) and MPIO green fluorescence. (A,B,C) Size versus granularity plots of labeled and unlabeled cells at 120 hours in culture post labelling. High plating density (HPD) MPIO-rMSCs (B) are more granular when compared with low plating density (LPD) MPIO-rMSCs (C) or unlabeled control rMSCs (A). (D,E,F) PI versus MPIO plots showing 78% MPIO-rMSCs at high plating density (MPIO-rMSCs-HPD) were live and labeled versus 29% of MPIO-rMSCs at low plating density (MPIO-rMSCs-LPD). (G) Histogram showing the distribution of MPIO green fluorescence in live cells from MPIO-rMSCs-HPD (black) and MPIO-rMSCs-LPD (gray) versus control rMSCs (white). (H,I) Confocal microscopy of HPD and LPD cell suspensions after mounting and DiI staining (red). More MPIO green fluorescence is observed in the HPD versus LPD cells in general. An LPD cell with strong green fluorescence is indicated with a white arrow.
Plating Efficiency and Proliferation

To assess the effect of loading rMSCs with MPIO particles on plating efficiency and proliferation, colony forming unit (CFU), methyl tetrazonium salt (MTT) and in-vitro growth assays were performed. The CFU assay revealed no significant difference (ANOVA, p= 0.813) on the plating efficiency (colonies formed as a percentage of cells plated) of MPIO-rMSCs versus unlabeled control rMSCs as shown in Figure 2.5, panel A. The MTT assay on controlled cell numbers with various MPIO loading levels shows no significant difference (ANOVA, p= 0.77, 0.34 and 0.50) in the metabolic activity of labeled versus unlabeled cells for 10,000, 30,000 and 60,000 cells as shown in Figure 2.5, panel B. In panel C, the serial MTT assay shows significantly less metabolic activity in labeled versus unlabeled cells at 24, 48, 72 and 96 hour time points (ANOVA, p=0.001, 0.004, 0.000 and 0.026), which later diminished, see Figure 2.5, panel C. The in-vitro growth assay also shows significantly slower growth in MPIO-rMSCs versus versus control rMSCs from 48 to 120 hours, see Figure 2.5, panel D. Doubling time for the first 72 hours in culture was estimated at 22 and 48 hours for control rMSCs and MPIO-rMSCs respectively. From 72-120 hours in culture the doubling times were estimated at 22 and 30 hours for control rMSCs and MPIO-rMSCs respectively. The plateau in both the MTT and growth curve assays correspond to cells reaching confluence. All error bars represent standard deviations of at least 3 samples at each data point. All experiments were repeated at least in triplicate and representative results are shown.

2.3.3 In-vitro MRI and Comparison with Optical Microscopy

Bulk Phantoms

NMR tube phantoms containing highly labeled MPIO-rMSCs (≈ 54pg/cell) and control rMSCs uniformly distributed at a concentration of $10^5$ cells/mL were imaged using a 2D-SPGR sequence with TR/TE of 111/34 ms, 24 NEX, $30 \times 30$ mm FOV, 1 mm
Figure 2.5: Colony forming unit (CFU), methyl tetrazonium salt (MTT) and *in-vitro* growth assays were performed to assess the effect of MPIO loading on plating efficiency and proliferation. (A) Plating efficiency as assessed by a colony forming unit (CFU) assay shows no significant difference in plating efficiency in MPIO loaded versus unloaded cells (ANOVA, p= 0.813). (B) An MTT assay performed on controlled numbers of MPIO-rMSCs versus unloaded cells showing no significant difference in metabolic activity (ANOVA, p= 0.77, 0.34 and 0.50). (C) A longitudinal MTT assay showing significantly less metabolic activity in MPIO loaded cells at 24, 48, 72 and 96 hours (ANOVA, p=0.001, 0.004, 0.000 and 0.026). (D) A growth curve assay performed over 7 days shows substantially slower proliferation observed from 48 to 120 hours in MPIO loaded rMSCs versus unlabeled cells. All error bars represent standard deviations of at least 3 samples at each data point. All experiments were repeated at least in triplicate and representative results are shown.
Figure 2.6: *In-vitro* MRI of MPIO loaded rMSCs. (A) 2D-SPGR acquisition showing signal voids throughout the phantom containing MPIO-rMSCs at $10^5$ cells/mL (left) versus control rMSCs (right) at the same concentration. (B) 2D-SPGR acquisition of non-uniform dilutions (right to left) of MPIO labeled rMSCs revealing heterogeneous structure and distribution of signal voids against the homogeneous background. MPIO-rMSCs and control rMSCs were suspended in 500 $\mu$L of PBS-gel in 6 mm glass NMR tubes for scanning. SPGR parameters were $20^\circ$ flip angle, 24 NEX, $30 \times 30$ mm FOV, 1 mm slice thickness, $512 \times 512$ matrix. TR/TE was 111/34 ms and 57/24 ms for the images shown in panels A and B respectively.

Slice thickness and $512 \times 512$ matrix as shown in Figure 2.6, Panel A. Signal voids were observed throughout the MPIO-rMSCs phantom on $T_2^*$ weighted SPGR images. Similar imaging (TR/TE 57/24 ms) was performed on serial dilutions of non-uniformly distributed, MPIO-rMSCs. Distinct patterns of signal voids were present in SPGR images of phantoms down to $5 \times 10^3$ cells suspended in 500$\mu$L. Figure 2.6, Panel B exemplifies the ability to image the structure of the non-uniform distribution of signal voids caused by MPIO-rMSCs against a homogeneous background on an SPGR sequence.
Single Plane Phantoms

Figure 2.7 shows imaging of single plane phantoms performed using 2D-SPGR sequence with a TR/TE of 57/24 ms, 30 × 30 mm FOV, 1 mm slice thickness and 512 × 512 matrix giving voxel dimensions of 60 × 60 µm. Nine wells in a three by three well corner of a 96 well plate were used to produce the single plane phantoms. The number of cells decreases by column from left to right at 1000, 500 and 250 cells whereas the iron loading varies by row from top to bottom: unlabeled control, ≈ 44 pg iron/cell, ≈ 54 pg iron/cell. The plane containing the cells was localized by performing an 8 NEX scan with 2 mm slice thickness orthogonal to the microwells as shown in Figure 2.7, panel A. The plane containing the cells contains dark signal voids sandwiched between bright layers of PBS-gel. Figure 2.7, panel B shows signal voids distributed along the surface of phantoms with MPIO-rMSCs containing ≈ 54/44 pg iron/cell. The calculated SNR at 70 NEX and 1 mm slice thickness as calculated with Equation 1.14 was ≈ 30. CNR (see Equation 1.15) for isolated signal voids was ≈ 23/20 for phantoms with ≈ 54/44 pg iron/cell respectively.

Figure 2.8, panel A shows MR images from one of the wells of the single plane phantoms showing discrete signal voids. Panel B shows the corresponding phase contrast image of the same microwell. The chosen region of interest is magnified showing five signal voids in Panel C. Phase contrast and epifluorescence microscopy of the same region containing five cells are shown in Panel D and E respectively. Both phase contrast and epifluorescence images show MPIO loaded cells, which fluoresce green, colocalizing with the signal voids detected with MRI.

2.4 Discussion

In-vitro single cell detection of MPIO-rMSCs was achieved for the first time using a clinical 3T MR scanner with conventional SPGR scanning protocols and a simple surface coil.
Figure 2.7: *In-vitro* MRI of single plane MPIO loaded rMSCs. Single plane phantoms of MPIO labeled rMSCs were created in a three by three corner of a 96 well plate. (A) Scan plane localization using 2D-SPGR, TR/TE 57/24 ms, 8 NEX and 30 × 30 mm FOV orthogonal to the microwells. (B) 2D-SPGR acquisition of the cellular plane with TR/TE: 57/24 ms, 70 NEX, 30 × 30 mm FOV, 1 mm slice thickness and 512 × 512 matrix giving voxel dimensions of 60 × 60 µm in plane. Calculated SNR (see Equation 1.14) at 70 NEX was 30. Estimated CNR (see Equation 1.15) for isolated signal voids for phantoms with ≈ 54/44 pg iron/cell was ≈ 23/20.
Figure 2.8: *In-vitro* MRI and Corresponding Optical Microscopy of MPIO-rMSCs. (A) MRI from a single microwell showing signal voids in the presence of MPIO-rMSCs on a single plane. (B) Phase contrast microscopy of the same microwell from A showing distribution of cells. (C) Magnification of the region of interest from the MRI in A showing five signal voids in a distinct pattern. (D) Phase contrast microscopy of the region of interest showing five cells in the same distinctive pattern. (E) Epifluorescence microscopy showing five distinct regions of MPIO particles fluorescing green which co-localize to the same distinctive pattern from both the cells in D and the signal voids from C.
The MPIO loading was found to be highly efficient and without serious adverse effects on rMSCs based on assessment of viability, proliferation, metabolism and plating efficiency. MPIOs have been used to label several different types of cells including glioma cells, T cells, macrophages, hepatocytes and stem cells [23, 24, 29, 31, 88, 89, 90]. Furthermore, single cell detection of MPIO labeled cells with *in-vitro* MRI has been previously demonstrated [29, 30, 60, 83, 91, 92]. However, these studies used either specialized high field MR systems or customized hardware such as gradient inserts or send/receive coils. This is the first demonstration of single cell detection of MPIO labeled rMSCs using a clinical 3T MRI system, conventional SPGR imaging sequences and simple receive only surface coils. The methodology developed in this work implements clinically translatable tools to achieve sensitive detection of iron loaded stem cells and herein lies its greatest strength.

The other major contribution of this work lies in the characterization of MPIO particle uptake and investigation of labelling localization and efficiency, metabolic effects, plating efficiency and proliferation of MPIO-rMSCs. In this study loading levels of > 50 pg of iron per cell were achieved, corresponding to > 100 MPIO particles within a single cell, as quantified by colorimetric iron assay and qualitative observation by confocal fluorescence microscopy. Others have achieved similar iron loading levels of 10-100 pg iron/cell or more labelling various cell types with MPIOs including MSCs [30, 31, 93, 94]. The colocalization of green fluorescence and fluorescent cell marker DiI from confocal microscopy (see Figure 2.1, panel C) in addition to the increased granularity and green fluorescence as detected by flow cytometry (see Figure 2.3) strongly support that the MPIO particles were localized intracellularly in perinuclear cytoplasmic organelles. The mechanism of MPIO entry into MSCs by phagocytosis/endocytosis has been demonstrated previously by transmission electron microscopy [29].

Cell labelling with MPIO was highly efficient and did not require the use of transfection agents to achieve high levels of iron loading, thus simplifying the labelling process [21, 83, 95]. Analysis of MPIO-rMSCs by flow cytometry showed efficacious and non-
toxic labelling of cells with approximately 94% survival rate post labelling with 95% of viable cells being labeled. Several reports in the literature show that cells appear to have a high tolerance to MPIO particles with typical post labelling survival rates as high as 95% [24, 29] in agreement with our experience.

FACS analysis of MPIO-rMSCs maintained in culture post labelling showed loss of intracellular MPIO over time (see Figure 2.4). The labeled proportion of MPIO-rMSCs decreased to 78% at 120 hours from 89% at 24 hours (see Figure 2.3, Panel E). In comparison, the labeled proportion of cells plated at low density was only 29% at 120 hours (see Figure 2.3, Panel E). These findings indicate that when labeled cells are plated at high density and have limited room to proliferate the majority of cells retain enough contrast agent to be considered labeled. Conversely, when labeled cells are plated at lower density and are not limited in their proliferation, the amount of intracellular contrast agent rapidly decreases, likely being divided between the daughter cells.

Alternatively, the loss of contrast could be due, in part, by cells actively ejecting the contrast agent or apoptosis of labeled cells releasing the contrast agent into the culture media. Free MPIO in the culture media could be ingested by other cells or deposited into extracellular structures. This could be further investigated by analyzing the culture media for the presence of free MPIO particles. In addition, confocal microscopy of MPIO labeled cells in culture at various time points additionally labeled with Bromodeoxyuridine (BrdU), labelling actively dividing cells, could be used to determine which sub-populations of cells are actively dividing and to what extent these cells retain the contrast agent. Similar observations from FACS analysis of MPIO labeled glioma cells and monocytes/macrophages have been reported [29, 89]. The flow cytometry data supports the expectation of increased retention of intracellular contrast agent when cells are not rapidly dividing.

While we have shown, like many others [29, 89] that the intracellular MPIO particles do not seem to have any cytotoxic effects or cause any metabolic impairment we have also
observed an impairment in the rate of proliferation of MPIO loaded rMSCs (see Figure 2.5) both by the MTT assay and in-vitro growth curve assay. While, MTT assays are commonly used to determine effects on proliferation of iron loaded cells [29, 31, 3] it is important to recognize that this assay is a measure of metabolism. Increased metabolism could be due to increases in cell number or changes in cell metabolism while in culture. Therefore, with an MTT assay it is not possible to preclude the possibility that some cells may be dying and additional metabolism from other cells can mask this affect. The results of the growth curve assay suggest that impaired proliferation is likely the dominant effect.

The estimated doubling time for control-rMSCs and MPIO-rMSCs (≈ 54pg iron/cell) was approximately 22 and 48 hours respectively. MPIO-rMSCs later regained a proliferative doubling time of approximately 30 hours. The slower rate of proliferation might be due, in part, to difficulty in reorganizing the organelles and cytoskeleton in the presence of > 100 micron sized particles when trying to divide. The observation that the proliferation rate of the MPIO loaded cells increases at later time points may be due to cells being able to divide rapidly once dilution of the intracellular contrast agent reaches a certain level. A further investigation using live confocal microscopy of dividing cells with and without MPIO loading would provide evidence to substantiate these claims.

The cell death assays used showed that the majority of labeled cells were viable in culture. These idealized culture conditions will not be present once cells are delivered in-vivo therefore this assessment of viability is somewhat limited. It may be beneficial to compare the response of MPIO-rMSCs to controls rMSCs in a non-ideal environment such as in-vitro conditions of ischemia or reduced nutrients. While it has also been shown that MPIO particles do not affect the ability of MSCs to differentiate into chondrocytes, adipocytes and osteocytes [29], to fully determine the effect of intracellular MPIO particles on cell function assessments of protein levels and gene expression would be beneficial.
In-vitro MRI revealed easily detectable signal voids against a homogeneous background that are attributed to aggregates of cells as well as single cells loaded with MPIO particles. Using the gold standard of optical validation, individual cells can be attributed to discrete signal voids. However, even in the idealized single plane phantom, a single signal void may represent two or more cells in close proximity. Therefore, quantification of cells present within signal voids on SPGR sequences is a formidable challenge.

Nevertheless, the signal void pattern observed in the single plane phantom was representative of the planar distribution of cells within the slice (see Figures 2.7 and 2.8). Distinct patterns of signal voids were also observed in the non-uniform cell dilution phantoms as shown in Figure 2.6, panel B. Unlike the single plane phantoms, the cells are distributed throughout the thickness of the imaging plane and multiple cells can potentially occupy a single voxel. The ability to detect these patterns of signal voids demonstrates the possibility of imaging the structural distribution of iron loaded cells in-vivo within the context of surrounding anatomy.

In summary, detection of single MPIO-rMSCs has been achieved in-vitro with a clinical MRI system. Cellular MPIO uptake was characterized as both efficient and non-toxic. In addition, MPIO loading was found to produce no substantial negative biological effects other than impaired rate of proliferation. The feasibility of applying this methodology for detection of MPIO-rMSCs delivered in-vivo will be explored in Chapter 3.
Chapter 3

Feasibility of detecting MPIO labeled rMSCs in-vivo

3.1 Introduction

Over the past few decades great progress has been made in the prevention, diagnosis and treatment of stroke [96]. This progress was made possible in no small part by extensive research using animal stroke models, which has deepened our understanding of the pathophysiology behind cerebral ischemia, as well as the advancement of imaging techniques to localize and determine the threat and extent of cerebral damage [97]. Despite these improvements stroke is still associated with high mortality and morbidity and an effective therapy is still desperately needed. Recently, there has been a plethora of experimental therapeutics that have shown great promise in animal models, but ultimately fail to make the transition from bench to bedside [96, 98, 99]. Identifying the contributing factors has been the focus of considerable attention in the medical community, including the Stroke Therapy Academic Industry Roundtable (STAIR) [100, 101, 102].

Many recommendations have been made by the STAIR committee to assist investigators in the design of studies to develop novel stroke therapies from pre-clinical animal
models to phase III clinical trials. Specifically, the STAIR-II committee suggests the
design of early clinical trials requires careful consideration of: therapy administration
route, dose range and duration, time from stroke onset to treatment, distribution to
proposed site of action and evidence of therapeutic activity by clinical and/or surrogate
markers. While these recommendations focus on the development of novel drug based
stroke therapies, the same guiding principles can be used to assist in the development
of stem cell based therapies now actively being investigated for the treatment of several
diseases including stroke [5, 18].

The delivery of exogenous stem cells is currently being investigated in animal mod-
els as a treatment for stroke. Mesenchymal stem cells (MSCs) are of particular interest
due to their ready availability from bone marrow. The initial use of MSCs focused on
applications in tissue engineering. However, the unanticipated observation that MSCs
produce a myriad of bioactive, immunosuppressive molecules capable of creating a re-
generative microenvironment in damaged adult tissues [16] has opened the possibility
of using MSCs to treat stroke. It has also been shown that MSCs can be influenced
to differentiate into neural cells in-vitro [17]. Therfore, MSCs could potentially provide
both neuroprotection and neuroseuplementation therapy in stroke. The potential mecha-
nisms of action of these cells include: integration into host neuronal circuitry, reduced
death of host cells, host plasticity stimulation, increased neovascularization, attenuation
of inflammation and recruitment of endogenous progenitors [5].

Further motivation is provided by a recent review by Parr et al. [6] summarizing a
number of studies showing therapeutic benefit of mesenchymal stem cell (MSC) therapy
in small animal models of stroke. These studies have demonstrated functional recovery
post stroke with MSCs delivered via intravenous, intrarterial or intracerebral injection.
While intrarterial and intracerebral injections maximize the likelihood of MSCs reaching
the target tissue, they are also the most invasive methods of delivery and pose tangible
risks to the patient. Thus, an intravenous injection route is potentially the safest route
of administration provided therapeutic benefit can be demonstrated [70, 71, 72, 74, 75, 76, 103]. A recent clinical trial intravenously injecting autologous MSCs into five stroke patients has demonstrated safety and some degree of benefit up to a year post stroke [104].

While the aforementioned studies have shown that MSCs can provide therapeutic benefit in stroke within the rat animal model there is a general lack of understanding as to their precise mechanism of action. Recalling the STAIR-II committee recommendations, optimizing MSC therapy for stroke will require assessment of: administration route, dose range and duration, time from stroke onset to treatment, distribution to proposed site of action and evidence of therapeutic activity. Combining magnetic resonance imaging (MRI), a well established modality for the clinical assessment of stroke, with the recent innovations in MRI based cell tracking provides a powerful tool for the development of MSC therapy for stroke.

MRI based imaging of iron labeled stem cell migration in the presence of stroke has been previously reported by a number of groups [8, 78, 105]. Hoehn et al. demonstrated the ability to track embryonic stem cells injected into the contralateral hemisphere and were observed to migrate towards the stroke lesion [105]. Walczak et al. have very recently reported the ability to track intrarterially injected MSCs persisting in the stroke affected hemisphere [78]. While these studies used administration routes that are both invasive and pose significant risks to the patient including additional stroke, they also used high magnetic field specialized small animal MRI scanners. These factors will pose a formidable challenge when considering transitioning to clinical trials. Therefore, one aspect of our feasibility study is to translate the currently established methods of iron labeled stem cell delivery and tracking to a minimally invasive and more clinically relevant context.

The other aspect of this feasibility study is to explore the limitations of established methodology for detecting iron-loaded cells in the presence of stroke. Several studies ex-
ploring cell tracking with MRI use combinations of labeling cells with fluorescent tracking molecules combined with MR contrast agents [24, 23, 78, 56]. Once contrast on MRI can be colocalized with fluorescence it is often concluded that cell tracking has been achieved. Recent studies have shown that transplanted MSCs labeled with fluorescent tracking dyes transfer those dyes to host cells after rejection and were detected up to 12 weeks post implantation [106]. In addition, it has been shown that iron oxide labelled cells cannot be differentiated from hemorrhage in the myocardium [107]. Macrophages can also be labelled with iron-oxide \textit{in-vivo} and subsequently detected with MRI [108, 109, 110].

In Chapter 2, we presented the ability to harvest, grow and label rat bone marrow derived mesenchymal stromal cells (rMSCs, a cell population containing mesenchymal stem cells) with micron sized superparamagentic iron oxide particles (MPIO) without significant negative biological effects aside from decreased proliferation. We then demonstrated the ability to detect these MPIO-rMSCs \textit{in-vitro} using a clinical MRI system with conventional imaging sequences. In the present chapter we report our initial experience testing the feasibility of detecting intravenously injected MPIO-rMSCs at the site of injury in a rat stroke model of permanent focal devascularization using a clinical MRI system. Specifically, we will compare whether the injection of MPIO-rMSCs can be differentiated from injection of free MPIO particles.

### 3.2 Methods

#### 3.2.1 Experimental Design

The study design was as follows. Each animal received a baseline MRI session prior to the stroke procedure performed on Day 0. A post stroke MRI session was performed on Day 1. On Day 3 each animal received an intravenous injection according to the treatment group. The treatment groups were: unlabeled rMSCs (n=2), DiI labeled rMSCs (n=2), MPIO labeled rMSCs (n=2), DiI and MPIO labeled rMSCs (n=2) and MPIO alone in
PBS (n=3). Two more MRI sessions were performed on Day 7 and 14. On Day 15 animals were sacrificed, organs of interest were removed and histopathology was performed. The experimental timeline is summarized in Figure 3.1.

3.2.2 Stroke Model

Male Long Evans rats (n=11) 60-90 days post gestation at the start of the experiment were used as subjects. Animals were housed and maintained in the Sunnybrook Research Vivarium according to the regulations set forth by the Canadian Council on Animal Care. On the day of stroke (defined as Day 0) surgery animals were deeply anesthetized with isofluorane and given ketoprofen for analgesia. Following the methodology of Kolb et al. [14] a rectangular hole was drilled into the frontal and parietal bones over the right hemisphere running +3/−4 mm (anterior/posterior) to Bregma and 1.5/4.5 mm lateral to midline. Once the dura was removed, the pia and cortical surface were detached of the supplying vaculature by mechanically rubbing the surface with sterile, saline soaked cotton swabs. The model of focal, permanent devascularization (stroke) is described in more detail elsewhere [111]. The rectangular hole in the skull was closed using bone wax secured with contact cement. The animals were monitored post surgery for any complications and returned to group housing after 24 hours.
3.2.3 rMSC Preparation and Delivery

Cells were harvested, grown and labeled with MPIO particles to achieve $\approx 54$ pg iron/cell as described in the methods of Chapter 2. Cells were additionally labeled with DiI (Molecular Probes, Invitrogen, V-22889), filtered into a cell strainer flow cytometry tube (BD Bioscience, 352235) and kept on ice prior to injection. Cells were resuspended by vortexing prior to loading into a 1 mL syringe (BD Bioscience, 309628). Animals were treated three days post stroke with either: $\approx 3 \times 10^6$ MPIO loaded rMSCs (around 54 pg iron/cell), $\approx 3 \times 10^6$ rMSCs without MPIO or MPIO suspended in sterile PBS (around 160 $\mu$g iron). Injections were performed via an intravenous 24 gauge catheter inserted in the tail vein. The total injected volume for each animal did not exceed 0.7 mL.

3.2.4 Longitudinal MRI Studies

Serial MR imaging sessions were performed on each animal while anesthetized with isofluorane and monitored via pulse oximetry, each session lasting not longer than 2 hours. Each scanning session consisted of a FSE with two echo times (proton weighted and $T_2$ weighted), $T_2^*$ weighted SPGR and a modified SPGR for susceptibility weighted imaging (SWI). Imaging was performed on day $-7$, day +1, day +7 and day +14. Scanning parameters are summarized in Table 3.1.

3.2.5 Histopathology and Correlative Microscopy

Animal Sacrifice and Tissue Perfusion

At the study conclusion animals were sacrificed by lethal dose of sodium pentobarbital intraperitoneal injection followed by transcardial perfusion with 100 mL of sterile PBS followed by 100 mL of 4% Paraformaldehyde (PFA) in PBS. The brain and spleen were removed and stored overnight in 4%PFA-PBS. The following day tissue samples were placed into 20% sucrose-4%PFA-PBS and stored at 2-4°C until the tissues sank in the
fluid. The tissues were then stored in 20% sucrose PBS at 2-4°C prior to sectioning.

Cryosectioning, Immunohistochemistry and Histological Staining

Sectioning was performed on a Leica Cryostat CM 3050S. Samples were frozen and mounted using OCT (Tissue-Tex, Indiana, USA) and sectioned at 10 µm. Sectioned samples were mounted onto microscope slides (VWR, 48311-703) and stored at −20°C prior to staining. Primary antibodies for staining microglia and macrophages were unconjugated CD11b/c (OX42 equivalent) primary mouse anti-rat antibody, 1/200 dilution (Abcam, 1211) and unconjugated CD68 (ED1 equivalent) antibody 1/100 (AbD Serotec, MCA341R).

Fluorescent secondary staining of the primary antibody was performed using a Cy5 (649 nm excitation, 670 nm emission) conjugated IgG goat anti-mouse secondary antibody 1/500 dilution (Jackson ImmunoResearch, 115-175-003). Prussian blue (PB) staining was performed for detection of ferric iron through treatment with hydrochloric acid and potassium ferrocyanide which produced a bright blue pigment visible with bright field and appears black with Nomarski optics (differential interference contrast, DIC).
Finally, a fluorescent nuclear stain was performed using 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) at 1/50,000 dilution in PBS (Sigma, D9542). Once staining was completed slides were mounted with coverslips using an aqueous fluorescent mounting medium (Fluorsave, Calbiochem).

Multi-channel epifluorescent microscopy was performed using a Zeiss Axiovert 200M. A multi-channel acquisition was acquired to simultaneously image each section for Prussian blue staining (black on Nomarski), DAPI nuclear stain (blue) and Cy5 labeled CD11b/c or CD68.

3.3 Results

3.3.1 Longitudinal MRI

Longitudinal MRI scanning sessions provide temporal monitoring over the course of the study. Figure 3.2 summarizes SPGR imaging sequences for representative animals from each treatment group. Substantial negative contrast is observed within and surrounding the ischemic region on day 7 following treatment on day 3 in the MPIO-rMSCs and MPIO alone treated groups when compared with the animal treated with rMSCs-alone. The negative contrast observed on day 7 persists and increases at day 14 in the same MPIO-rMSCs and MPIO alone treated groups.

3.3.2 MRI Sequence Comparison

The different tissue contrast provided by each of the MRI sequences is shown in Figure 3.3. The same representative animals from each treatment group and image slices shown in Figure 3.2 from day 14 are shown in conjunction with the FSE and SWI images of the same slices. The SWI images show the most negative contrast within the ischemic region, followed by the SPGR images. In animals treated with MPIO-rMSCs and MPIO alone, proton and $T_2$ weighted images from the FSE show delineation between areas of negative
Figure 3.2: Longitudinal MRI showing images acquired with 3DSPGR for representative animals from each treatment group. Following treatment on day 3, MPIO-rMSCs and MPIO alone animals show negative contrast appearing within and surrounding the ischemic region when compared to rMSCs-alone on day 7. Negative contrast persists and increases in these animals at day 14.
Chapter 3. Detecting MPIO-rMSCs in-vivo

contrast and hyperintensities within the ischemic region. Conversely, animals receiving rMSCs-alone only showed hyperintensities within the ischemic region. The FSE images also provide sufficient contrast to identify anatomical structures such as the ventricles and corpus callosum.

3.3.3 MRI and Histopathology

Histopathology revealed the extent and composition of the tissue within and surrounding the ischemic region. In Figure 3.4 MRI of the lesion site from the sequences summarized in Figure 3.3 is compared with bright field microscopy of histological sections. Anatomical structure is represented by proton and T$_2$ weighted images, with hyperintensities corresponding to areas of lost tissue replaced by fluid visible on histology. In the animals treated with rMSCs-alone, little negative contrast is present in T$_2$, T$_*^2$ and proton weighted images. The SWI image from the same animal contains an area of negative contrast at the outer surface of the brain toward the lesion edge that tested positive for cells containing ferric iron on Prussian Blue (PB) staining. No positive PB staining was detected in the lesion periphery in the animals treated with rMSCs-alone.

In both MPIO-rMSCs and MPIO alone groups, negative contrast and positive PB staining is present in the area containing the lesion and in the lesion periphery close to the ventricle and corpus callosum. Negative contrast is most pronounced in SWI and T$_*^2$ weighted images but also present on proton and T$_2$ weighted images. Qualitative comparison of the areas of negative contrast on MRI to the PB staining pattern reveals negative contrast on T2 and proton weighted images is more specific to areas with positive PB staining. High magnification epifluorescence microscopy shows PB positive areas (black on Nomarski) closely associated with cell nuclei (bright blue) in all treatment groups (see bottom of Figure 3.4). Immunohistochemistry staining for cell markers expressed on microglia and macrophages (CD11b/c, CD68) showed the majority of iron positive cells are CD68 positive in both the lesion site and periphery in Figure 3.5.
Figure 3.3: Comparison of MRI imaging sequence contrast on day 14. SPGR, SWI and FSE images for the same slice within representative animals from each treatment group. SWI images provide the most negative contrast within the ischemic region of the MPIO-rMSCs and MPIO-alone treated animals followed by SPGR. Proton and $T_2$ weighted images show delineation between areas of negative contrast and hyperintensities within the ischemic region of MPIO-rMSCs and MPIO-alone treated animals. Conversely, animals receiving rMSCs-alone only showed hyperintensities within the ischemic region. FSE images reveal anatomical structures such as the ventricles and corpus callosum.
Figure 3.4: MRI of lesion site for representative animals from rMSCs-alone, MPIO-rMSCs and MPIO-alone treatment groups with corresponding histology. Areas of negative contrast on MR images show Prussian Blue positive staining for the presence of iron. DAPI staining reveals location of cell nuclei relative to iron deposits (black on Nomarski).
Figure 3.5: Immunohistochemistry and fluorescent microscopy of iron containing cells in the lesion site and periphery. Prussian Blue positive staining for the presence of iron appears black on Nomarski optics. DAPI staining in blue reveals location of cell nuclei relative to black iron deposits. Staining for microglia and macrophages (CD11b/c,CD68) in cyan and fuscia respectively. Scale bars represent 30 µm.
3.4 Discussion

This chapter presents, to our knowledge, the first report of a comparison between intravenous delivery of MPIO-rMSCs and free MPIO particles showing similar detection of negative contrast on MRI due to the presence of iron in the periphery of a stroke lesion with a clinical MRI system and conventional imaging sequences. These results raise into question the ability to detect the intravenous delivery of iron labeled cells in the presence of stroke unambiguously.

This detection was achieved by using MR sequences sensitive to the presence of MPIO by means of localized changes in $T_2$, $T_2^*$ and susceptibility. Our initial experience suggests that this mechanism is independent of whether the MPIO is delivered alone or contained within rMSCs. However, evidence of partial tissue regeneration in the stroke lesion was only observed in animals receiving MPIO-rMSCs and rMSCs. Histology confirmed the presence of iron and suggests the iron is localized within cells. While others have previously shown the ability to detect the delivery and migration of iron oxide within stem cells using MRI in the presence of stroke, these studies have used more invasive intrarterial and intracerbral injection methods and specialized high field experimental MRI systems [78, 105]. We have demonstrated non invasive monitoring and detectable changes in response to an intravenous injection with a clinical field strength MRI system without specialized hardware. Our methodology provides a clinically relevant tool for further investigation of stem cell therapy for stroke.

These observations raise a number of questions for further consideration. Iron containing cells were found within the stroke lesion of all treatment groups including the rMSCs-alone treated group although to a much lesser extent. These endogenous iron deposits could be due to hemosiderin resulting from digestion of damaged blood cells and hemorrhage within the stroke [112]. It has already been shown that hemorrhage within myocardial infarction can interfere with tracking iron oxide labeled cells [107]. Although others have demonstrated that MPIO injected directly into infarcted myocardium is
rapidly cleared and does not persist [3], our observations suggest the delivery route and
target site may also play a role in retention of MPIO.

In the MPIO-rMSCs and MPIO-alone animals PB staining in the stroke lesion was
much stronger than in the rMSCs-alone animals suggesting at least part of the iron
detected was from the exogenously delivered MPIO. The possibility exists that MPIO-
rMSCs released their MPIO contents through cell lysis caused by damage upon injection
due to shear forces or cell death post injection. It is estimated that the vast majority
(> 95%) of intravenously injected cells become trapped in the lungs, liver and spleen
and the released MPIO is likely taken up by the reticuloendothelial system [65]. Despite
the limited survival of these cells, studies have shown that small numbers of MSCs can
cross the blood brain barrier and localize to the stroke lesion after intravenous injection
[5, 113].

The iron containing cells in the surrounding tissue within the stroke lesion also exhibit
significant autofluorescence in the green and red channels occupied by DiI and MPIO flu-
orescence. This autofluorescence combined with edge effects (autofluorescent scar tissue
boundaries) confound the ability to identify the source of the iron unambiguously by
means of fluorescence of MPIO and DiI alone. Transmission electron microscopy may be
able to determine what extent of this intracellular iron is MPIO more conclusively based
on the large particle size (≈ 0.9µm).

While the iron we observe appears to be cellular given the close proximity to cell
nuclei, it is not clear as to which cells are now labelled with iron. In the MPIO-rMSCs
and MPIO-alone animals iron containing cells were also detected in the periphery of the
stroke lesion close to small vessels along the corpus collosum and near the ventricle. Given
the similar morphology and distribution of these cells in both treatment groups, these
cells could potentially be macrophages/microglia that have ingested MPIOs released into
the vasculature and have subsequently started to migrate toward the stroke. Immuno-
histochemical staining showed that the majority of iron positive cells were CD68 positive
which supports this claim. Furthermore, several groups have reported the ability to label macrophages *in-vivo* with iron oxide [90, 114, 108, 115, 116, 117].

The majority of iron positive cells were also positive for CD68, however, there were also a few iron positive cells that were CD68 negative. Therefore, we cannot conclude whether some of the iron containing cells in the stroke lesion are in fact MPIO-rMSCs at present and this is one of the major limitations of this study. Further limitations include limited numbers of animals in each treatment group (n=3 or 4 per group) and lack of a cell specific marker that demonstrates viability for the injected cells. This limitation can be circumvented through the use of bone marrow from an animal expressing green fluorescent protein (GFP) in all cells similar to Coyne et al. [106]. This would allow detection of viable cells arriving at the lesion site and could be combined with staining for other phenotypic markers to provide insight into transplanted cell fate. One study found 4% of intravenously injected MSCs in the brain with 60% of these cells in the ischemic boundary zone on day 14 post stroke [103]. Phenotypically, less than 5% of the MSCs found in the brain expressed some neuronal and angiogenic markers [103].

Many studies using iron oxide and fluorescent dyes to label cells for detection with MRI and optical validation have relied upon PB staining combined with fluorescence microscopy to verify the presence of transplanted cells [3, 23, 24, 78]. Our observations suggest that these methods of histology are insufficient to conclusively determine the identity of iron containing cells when studying areas of ischemia, hemorrhage and necrosis such as stroke. Furthermore, it has also been shown that stem cells transplanted into a normal rat brain were rejected by an inflammatory response and transferred their fluorescent labels to host neurons and glia [106].

Therefore, one must exercise caution in the interpretation of PB staining and fluorescence as verification of the presence of transplanted cells, especially in the brain. These iron containing cells may simply be phagocytic cells such as macrophages/microglia which have typically been ruled out or confirmed fairly easily with immunohistochemistry
(IHC). Some studies now suggest that in the pathologic brain, such as that post-stroke, markers such as CD11b/c and CD68 may not be entirely specific for macrophages/microglia further complicating the issue [118]. This finding calls into question the interpretation of similar staining to identify macrophages/microglia which is commonly practiced in the field of iron based MRI cell tracking.

Unfortunately, most stem cells can only be uniquely characterized by a panel of cell differentiation (CD) markers which is a formidable task in IHC of tissue sections. A highly specific test for the presence of transplanted cells is clearly needed. Consequently, many groups have started to investigate MR reporter genes that will allow detection with non-invasive imaging which implies viability and unambiguous detection of transplanted cells at the time of sacrifice [52].

Interestingly, only some of the animals treated with rMSCs showed some evidence of what might be tissue regeneration. The best evidence of this was observed in the MPIO-rMSCs treated animal showed in Figure 3.4. None of the MPIO-alone treated animals showed similar evidence of tissue regrowth. The timing of treatment delivery is likely a contributing factor and will need to be further investigated. The ideal treatment window for MSC therapy may be after the acute phase of ischemia has subsided. A preliminary experiment where MPIO-rMSCs were injected at two weeks post stroke resulted in the most tissue regeneration of all treated animals to date. Shen et al. [72] found that functional recovery can occur when delivering MSCs as long as 1 month after the initial stroke episode. The time of sacrifice chosen for this study may have also been premature, as the lesion site was still evolving from day 7 on day 14. Further studies varying the timing of treatment and allowing more time for recovery and observation will be necessary in order to fully assess this treatment strategy. Behavioural tests for functional recovery should also be added to complement the assessment of tissue loss and regrowth from MRI and histology.

The feasibility of detecting groups of iron containing cells within our current stroke
model and imaging methods has been established. However, our results have shown that intravenous delivery of MPIO-rMSCs cannot be differentiated from the injection of MPIO alone in our current stroke model. Furthermore, this study underlines several sources of ambiguity within investigations of MR based cell tracking in the presence of stroke. As strategies to overcome some of the current limitations of iron oxide based cell labelling and tracking are further developed we will be able to understand the mechanisms at play more clearly.
Chapter 4

Thesis Summary and Conclusion

4.1 Thesis Summary

Chapter 2 demonstrated the ability to detect single MPIO labeled rMSCs in-vitro using a clinical MRI system. The uptake of MPIO particles was characterized and the effects of MPIO labeling on viability, proliferation and plating efficiency were determined. High levels of iron loading were observed to significantly decrease the rate of proliferation of MPIO-rMSCs in culture. Chapter 3 tested the feasibility and identified some of the limitations of applying the methodology developed in chapter 2 toward detection of intravenously delivered MPIO labeled rMSCs in-vivo in a rat stroke model with longitudinal monitoring using MRI.

4.2 Conclusion

The principle goal of this work was to translate the current tools for labeling and imaging stem cells used with specialized hardware and experimental MRI systems to a more clinically relevant setting. Specifically, a population of rat marrow derived mesenchymal stromal cells (rMSCs) were effectively labeled with micron sized iron oxide particles (MPIOs) and detected in-vitro with a 3 Tesla clinical MRI system with standard sur-
face coils and conventional imaging sequences. Therefore, this goal has been effectively achieved and *in-vitro* detection of single cells has been confirmed.

The results of *in-vivo* studies have demonstrated that detection of iron containing cells accumulating within the stroke lesion and periphery is possible using our current methodology but remains ambiguous. While the majority of iron containing cells are CD68 positive suggesting these cells may be macrophages/microglia, the recent findings that CD68 may not be entirely specific to macrophages/microglia in the post-stroke environment [118] further complicates identification of these cells. Therefore, despite our best efforts we cannot claim the identity of these iron containing cells with certainty. Clearly, there is a need for more specific detection methods such as the use of reporter genes [52].

The first conclusion to be drawn is one of caution when interpreting signal voids on MRI when using an iron based cellular contrast in the treatment of damaged tissues where confounding factors such as immune cells are active and hemorrhage could be present. The same consideration needs to be taken toward histological analysis of the corresponding tissue specimens of interest where endogenous iron deposits, autofluorescence and transfer of fluorescent labels to host cells need to be accounted for.

The agreement between the pattern of signal voids on MRI and histological staining for iron demonstrate that it is possible to obtain information regarding the evolving gross distribution of iron labelled cells within the context of the surrounding anatomy providing insight into cell behaviour and migration. However, further studies addressing the limitations of these methods is necessary prior to investigation of potential clinical applications.
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