Cellular Imaging of Human Embryonic Stem Cells using a New Positive-Contrast Magnetic Resonance Contrast Agent

by

Andrei Venter

A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science
Institute of Biomaterials and Biomedical Engineering (IBBME)
University of Toronto

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Abstract

Human embryonic stem cells (hESCs) have the unique potential of developing into all the different cell types in our body and represent a clinically relevant cell source in the field of regenerative medicine. At present, we can inject cells and monitor their progress only in animals but not in humans (and this we do through sacrifice and histology to determine if the cells have survived or migrated). In human trials, these invasive methods cannot be applied, and information can be garnered only when tissue samples are retrieved, which is possible under rare circumstances. To address the need for a non-invasive method to monitor and guide cellular therapy in humans, we have explored a MRI based cell-tracking method that utilizes a positive-contrast manganese porphyrin agent, MnP-TPPS-NH₂, that has better sensitivity and safety than gadolinium-based compounds and is easy to scale up due to simplicity of synthesis. In this study, we investigate for the first time the efficacy of cell-permeable MnP-TPPS-NH₂ for labeling hESCs.

We have demonstrated a significant reduction in T1, leading to large increase in bright signal relative to the background. We have shown the agent has no adverse effects on cell survival and function and that the agent is internalized with higher concentrations inside the nucleus and along the membrane periphery.

We have presented a facile and easily scalable new method for sensitive MRI tracking of human embryonic stem cells to accelerate and enable future studies in the complex and interdisciplinary field of regenerative medicine.
I would like to thank Daniel Szulc for synthesizing and providing the contrast agent that made this research possible and also Inga Haedicke for synthesis of the apo-version of the agent.

I would like to thank Tamilla Sadikov for training me on how to culture human embryonic stem cells.

I would like to thank my fellow lab members and friends at the Translational Biology and Engineering program for providing such a fantastic work environment.

Mostly, I would like to thank my supervisor Dr. Hai-Ling Margaret Cheng for guiding me these past two years in my research and more.
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<tr>
<td>$B_0$</td>
<td>main static magnetic field</td>
</tr>
<tr>
<td>$B_1$</td>
<td>radiofrequency field</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Gd</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
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<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma atomic emission spectroscopy</td>
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<tr>
<td>$M_0$</td>
<td>Net magnetization vector</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>Mn</td>
<td>Manganese</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>T1</td>
<td>spin-lattice relaxation time</td>
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<td>spin-spin relaxation</td>
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Chapter 1

1.0 Introduction

1.1 What are stem cells

All life begins from a single cell. As cells divide, the daughter cells begin to specialize in function to form every organ and tissue in the human body. These original cells, which can divide indefinitely and become any other type of cell, are called stem cells. The most powerful of these cells are known as omnipotent or pluripotent stem cells, used to be thought to exist only in the very early stages of embryo development. Research has increasingly shown that there are populations of stem cells found in almost every tissue in the body, and that these cells are responsible for maintenance, repair, and regeneration subsequent to damage or cell death that occurs throughout someone's life. Furthermore, scientists are now capable of reverting regular cells that lack this differentiation ability back into stem cells, giving them the potential to grow new tissue. For these reasons, stem cells have been a hot topic in medicine for many years now. Researchers have hoped to harness their ability to proliferate indefinitely and
become any other cell type in the body and use them to repair any organ or tissue damaged either by disease or trauma.

1. 2 Regenerative medicine and cell therapy

Regenerative medicine is a promising field of research that many hope will help patients with significant organ and tissue damage caused by either disease or trauma. Although there have been many improvements to organ donation procedures and registries, there is still a vast shortage. The waitlist in the United States was over 95,000 in 2006 and has been steadily rising. There are many strategies being attempted to increase the number of donated organs, although it is unlikely that there will ever be anything close to a surplus. In many cases, such as myocardial infarction that damages part of the heart, a heart transplantation surgery is a huge expense to the healthcare system and carries significant risks to the patient. Furthermore, there exist other pathologies, such as spinal cord injury, that have no option for transplantation; yet, these may be cured by emerging cell therapies.

For decades, doctors and researchers have been hoping to use the power of stem cells in medicine. Every organ presents its own set of unique challenges for cell implantation and regeneration, but many of these challenges are common to all cell therapies. There are many types of disease and trauma in which cell death or loss of cell function is the predominant issue and these are the ailments easiest to target initially. For example, one of the most promising therapies aims to inject pancreatic Beta cells to
produce insulin for patients suffering from Type 1 diabetes. To function, the implanted cells need only be in contact with a blood supply to produce insulin and release it into the body\textsuperscript{9}. Cell therapies for myocardial infarctions, to restore function to the heart, are much more complicated. These cells must not only form cardiomyocytes, but the cardiomyocytes must integrate electrically and mechanically with the native tissue and have the strength to pump out blood\textsuperscript{10}. There have been dozens of clinical and significantly more preclinical trials injecting cells into the damaged heart tissue in the hopes of restoring function\textsuperscript{11–13}. While some have shown small fractional increases in heart function, the majority have failed to show any real significant improvement\textsuperscript{14}.

1.2.1 Issues with cell therapies

As mentioned above, many different cells and applications of cell therapies have been brought to the level of clinical trial but have subsequently failed either due to complications or simply not meeting the milestones for restoration of function. Furthermore, even in applications where positive results are seen, the improvements are minimal and there remain many complications that have not been thoroughly investigated or solved\textsuperscript{15}. There are major issues with cell therapy that occur in the first few minutes, hours, and days of the procedure that have not been thoroughly investigate and optimized.

One such issue is that very soon after injection, a significant fraction of the implanted cells die within the first hour. This can be due to a variety of factors.
Turbulent forces experienced by the cell during the actual injection through the syringe likely are responsible for a large number of cell lysing. In moving tissues, such as the beating heart, the cells can be pumped away from the injection target area\textsuperscript{16}. Therefore, many cell injection protocols suspend the cells in hydrogels or scaffolds, to give structure and support to the cells and provide them with nutrients for enough time that they can hopefully integrate into the target tissue\textsuperscript{17}.

When large areas of tissue need to be repaired, such as in myocardial infarction, there is no consensus on the best location to inject the cells. The three major injection types are: intravenous, intracoronary, and intramyocardial\textsuperscript{18}. The intramyocardial sites range from the periphery of the damaged tissue to the center of the infarct, and multiple injections are attempted to evenly distribute the cells\textsuperscript{19}. The injection depth must also be considered.

Once the cells reach their target through one of the methods above, they must now survive and proliferate. The ischemic environment has poor blood and nutrient supply, and there may be inflammatory/immunogenic response that damages these implanted cells.

These are all issues that must be overcome and better characterized to understand the reasons behind the failure of so many cell therapy trials. To do so, the fate of the implanted cells must be known and it is necessary to track these cells. Knowing how and when the cells are dying would allow direct comparison amongst these methods to be made.
1.3 Current methods to track cells

Cell tracking is the use of labels to determine the location of a cell or population of cells over time and is used to find out if a population of injected cells is surviving and if so, how deep into the damaged area the injected cells are penetrating\(^2\). It would be very beneficial for any therapy to monitor cells for the first few days post-injection to see in real time how many cells are still alive and where they are. The current gold standard method for answering these questions is to fluorescently tag the cells, sacrifice the animal, and explant the target tissue to see where and how many implanted cells have survived\(^2\). With fluorescence imaging, we cannot know at what rate this is happening when usually explantation is done weeks later because it takes cells a long time to penetrate and divide. This brings forth the need for cell tracking and, ideally, a non-invasive, deep-tissue, and real-time method to observe the fate of the implanted cells.

The following sections give an overview of the current methods of cell tracking and their limitations.

1.3.1 Optical imaging

The current gold standard research method to see the fate of injected cells is to fluorescently label them to make implanted cells distinguishable from host tissue\(^1\).
Tissue is harvested at prescribed time points and histology is then performed. This allows precise viewing of cell morphology, viability, and the depths at which the cells have integrated into the host tissue. However, this method has a number of disadvantages, including cost, invasiveness, and lack of longitudinal data. This method can never be used clinically, and even in the pre-clinical setting, it cannot provide longitudinal data in a single subject. Each time point requires a different animal to be sacrificed. Not only is this expensive and time consuming, but every animal will react differently to treatment so there is no guarantee that using two animals with the same treatment will yield the same results.

Other optical imaging techniques, such as near infrared spectroscopy, that do not require explantation are limited by the penetration depth of light and poor resolution due to large amounts of photon scattering. These methods also cannot be used to track cells in deep tissue or organs.

1.3.2 Positron emission tomography and computed tomography PET/CT

Positron emission tomography is the most sensitive non-invasive imaging method currently available that is capable of imaging the smallest number of cells\textsuperscript{22}. Again, the cells must be labeled to distinguish them from native tissue, but to do so, the cells must be labelled with a carcinogenic radioisotope, and every PET/CT scan delivers a significant dose of radiation. Furthermore, due to the nature of radioactive decay when the isotope gives off a signal it can only be localized to within a few millimeters of the event preventing true biological representation of where the injected population of cells\textsuperscript{23}. PET also cannot take images of cells inside fast moving tissue such as the heart.
without large motion artifacts. To rectify the poor tissue contrast resolution of PET, it is often used in conjunction with CT, which further increases the radiation dose\textsuperscript{24}. No clinical trial could be approved that requires multiple PET/CT scans due to this large radiation dose on human patients. There is also the possibility that the radiation would have adverse effects on the cells implanted, preventing them from performing optimally.

1.3.3 Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) can be used to image soft tissue with fine spatial resolution non-invasively and without ionizing radiation\textsuperscript{25}. For these reasons, MRI is harmless and can be performed repeatedly on human patients without any concern. With the aid of contrast agents, MRI is also capable of tracking injected cells throughout the body\textsuperscript{26}. Positive contrast agents, which provide an increase in signal intensity, are the preferred method, since they provide more accurate spatial delineation. The most commonly used positive contrast agents today are gadolinium (Gd) based\textsuperscript{27}. Gd is not perfect: it can be toxic and is foreign to the body (we do not naturally have trace concentrations of Gd in our body)\textsuperscript{28}. An alternative to Gd agents are manganese (Mn) based agents that can provide the same or better contrast and lower toxicity. Manganese is naturally present in the body and any excess in low concentrations should theoretically be easily eliminated\textsuperscript{29}. The main drawbacks to MRI is that it cannot be used in patients that have metal implants or devices (e.g. pacemakers), poor contrast in hard
tissues, and complicated imaging sequences cannot be performed by most imaging technicians.

For these reasons, we believe a MRI cell-tracking method is the most promising, effective, and clinically translatable approach for non-invasive stem cell monitoring in vivo. To address the need for a non-invasive method to monitor and guide cellular therapy in humans, we have explored a MRI-based cell-tracking method that utilizes a manganese porphyrin agent, MnP-TPPS-NH2, that has better sensitivity and safety than gadolinium-based compounds and is easy to scale up due to simplicity of synthesis\textsuperscript{30–32}. In this study, we investigate for the first time the efficacy of cell-permeable MnPNH\textsubscript{2} for labeling cells. The following section provides background on the fundamentals of MRI and MRI contrast agents.

1.4 MRI: basic principles and applications in regenerative medicine

1.4.1 Overview

Subatomic particles have an inherent fundamental property called spin. An atom will have a net spin if it has an odd number of protons, or neutrons, or both\textsuperscript{26}. It is this “net atomic spin” that is detected by MRI. There are many isotopes with a net atomic spin found naturally in the body. The most abundant is unpaired hydrogen nuclei $^1\text{H}$, or
simply unpaired protons. In a classical sense, this spin implies that each hydrogen atom behaves like a small magnet. Typically, due to the random motion of atoms, these magnetic moments are all randomly oriented and cancel each other out. However, in the presence of an external magnetic field, similar to a compass needle pointing north in the presence of the Earth's magnetic field, the magnetic moments of these atoms are no longer random. Instead, they will preferentially align in the direction of the external magnetic field and produce a net magnetization (sum of all magnetic moments) in the direction of the external field. In the body, the unpaired protons we are interested in detecting are those residing in water, as our body is comprised of 60-70% water. Therefore, MRI can be thought of as imaging the water protons in our body. However, water is found in vastly different environments. Some are essentially free floating, such as in blood or cerebral spinal fluid. Others are tightly bound in bone and other hard tissues. The majority of water is found in soft tissue, such as liver and brain. Based on the environment in which water resides in our body, the water protons respond differently to magnetic fields. They are excited away from equilibrium state at different rates, and their decay back to equilibrium occurs at different rates.

This is the basis of NMR/MRI. Complex sequences of radiofrequency pulses are sent through the body and the atoms in various tissues respond differently. These differences can then be reconstructed into images of the different tissues with high resolution and contrast. Herein lies both the main advantage and disadvantage of MRI over the other imaging modalities. These magnetic excitation sequences can be precisely tuned to tease out the specific information one is after. Different pulses can be designed to create
images that enhance contrast in blood or hide the signal from fat tissue; other pulses can be timed with the beating of the heart to show high resolution images of moving tissue. The disadvantage is that due to this immense amount of control, imaging can be very complicated and time consuming. Consequently, there are only a few common imaging sequences used in clinical practice. Anything more complex requires a trained MRI physicist.

The following sections provide a more rigorous explanation of how signal and contrast is generated in MRI. The truest explanation requires an understanding of quantum mechanical principles and a very strong mathematical background, but it is possible to get a working understanding using classical quantum mechanics. This is what I have attempted to do, to create a simple explanation not relying on too much mathematical derivation though some is unavoidable.

1.4.2 Signal generation in an MRI image

This section explains how the signal in one single pixel of an MRI image is generated. Expanding upon the previous section, hydrogen atoms in an external magnetic field do not simply point in the direction of the field. Rather, they spin like a top, with the point of the top rotating around the direction of the external magnetic field which we will denote $B_0$ shown in Figure 1.1. This rotation around the axis aligned with the external magnetic field is known as precession. In any given area, there are uncountable millions of hydrogen atoms. These atoms precess around $B_0$ such that their magnetic moments
add together into an average magnetic moment denoted $M_0$. In other words, there is a net magnetization vector from the hydrogen atoms in our body from simply sitting in a strong external magnetic field. However, in this state, no image can be produced, because we need to get the spins to move away from the axis $B_0$ in order to generate electromagnetic fluctuations that we can detect and convert into an image. To move the spins off-axis, we apply radiofrequency excitation to the hydrogen atoms. This excitation is a second pulse denoted $B_1$, which is $90^\circ$ perpendicular to $B_0$ and drives the net magnetization into the XY plane, the plane transverse to $B_0$. Measurement is performed after the $B_1$ pulse is turned off when the excited atoms relax, or return, to their equilibrium state. Two predominant mechanisms underlying relaxation give rise to the two main sources of tissue contrast in MRI, namely T1 and T2.
**Figure 1.1:** Schematic showing: A) the precession of a single hydrogen atom about the magnetization vector $B_0$, B) in a given area the hydrogen atoms all precess but not in phase, C) the net magnetization of all the hydrogen atoms represented by vector $M_0$ points along $B_0$.

**T1**

The net magnetization itself moves from the XY plane back to the z-axis at an exponential rate governed by the rate constant $T1$. This rate constant is known as longitudinal relaxation time and varies in different tissues. Figure 1.2 shows a schematic of $T1$ contrast.

**Figure 1.2:** Schematic showing how A) the net magnetization is excited to rotate into the XY plane by a second magnetic field $B_1$. B) Once $B_1$ is turned off the excitation moves back towards $B_0$, and C) the exponential function that represents the recovery of the net magnetization back to its equilibrium state governed by the rate constant $T1$. 

$M = M_0(1 - e^{-t/T1})$
T2

Not only are the protons aligned in the direction of $B_1$ following excitation, they also precess in phase. When excited, these molecules precess in phase with one another around the Z axis. Once $B_1$ is turned off, these molecules slowly lose their phase alignment and slowly cease to rotate in synchrony. This is what is known as dephasing, spin-spin relaxation, or T2 relaxation.

A summary of the differences between T1 and T2 as well as a comparison image of the brain cross-section can be found in the following figure.

**Figure 1.3:** Comparison of T1 and T2 weighted MRI contrast in the human brain (obtained from copyright free Wikipedia Creative Commons). A chart
summarizing which biological tissues and compounds appear bright and dark in each imaging modality

1.4.3 Quantifying T1 and T2

In any given MRI image, different tissues will appear brighter or darker based on their T1 and T2 relaxivities. The images are not absolute; they are only relative and the actual relaxivity values cannot be measured from them. These values can be quantified using a more complex manipulation of magnetic excitation pulses and readouts. The most common method, and also the standard method, to quantify T1 values is by using a repeated inversion-recovery sequence. Instead of exciting the atoms 90°, as shown in Figure 1.2, they are fully flipped 180°. The different tissues will begin to relax back to align with Bo at different rates governed by their T1 relaxation time constant. After a certain time, called the inversion time, a 90° pulse is sent to rotate recovered longitudinal magnetization back into the XY plane for signal detection. When the inversion time is optimized, the tissues will have all been separated based on their T1 relaxation rates from the 180° inversion pulse. Based on these differences, an exponential fitting curve can be used to calculate each materials’ individual T1. This is also known as T1 mapping. An analogous procedure based on a different pulse sequence
is used to quantify T2. A much more mathematical rigorous and detailed explanation of the physics and derivations behind magnetic resonance imaging is shown by Plewes et al.\textsuperscript{33}

1.4.4 Contrast agents

Even though MRI is the most versatile medical imaging modality in regard to contrast generation and manipulation, in most regenerative medicine applications this is not sufficient. If the therapy calls for the regeneration of the target tissue, the cells that are going to be injected are likely to be incredibly similar, if not the same, as the native cells. There will be no source of difference in proton density, T1, or T2 contrast. It is, therefore, necessary to introduce an artificial source of contrast, and the simplest and most common method to do so is an exogenous contrast agent. Recalling from the last section, the two main types of MRI contrast are T1 and T2, and, likewise, the most common MRI contrast agents intensify either T1 or T2 signal. The important thing to note is that in an image, T1 contrast agents make the targeted area brighter, and T2 contrast agents makes the affected area darker. The mechanisms behind T1 and T2 contrast are different, but most agents are designed to exert a stronger effect on one contrast channel than the other. Rarely will an agent discreetly operate on only one and usually will have an effect on both T1 and T2 relaxation. One thing of note in MRI is that the contrast agents themselves are not visible as in other imaging modalities, but rather the effects of the contrast agents on the surrounding water molecules is what is actually
visualized in the images. A brief overview of the advantages and disadvantages of the two most common types of MRI contrast agents is given below. For a more complete explanation, consult Contrast Agents in Magnetic Resonance by Muller\textsuperscript{34}.

**T2 Contrast Agents**

There is only really one type of T2 contrast agent and these are superparamagnetic iron oxide nanoparticles. These are various differently structured molecules that contain a core of iron. These nanoparticles can range in size from a few nanometers to several micrometers depending on the application\textsuperscript{35}. Since iron is the strongest magnetic molecule, these agents create strong local magnetic fields. These nanoparticles contain cores of many oriented iron molecules which align together and act, on the molecular scale, as one large magnet. This large magnetic field strongly interacts with the water molecules around it causing them to rapidly dephase following excitation, and it is this effect that is responsible for their efficacy as a sensitive contrast agent\textsuperscript{34}. T2 contrast agents are also called dark contrast agents, because they make the image around them darker.

Until now, T2 agents have been the most researched and most commonly implemented in cell labeling due to their incredibly high sensitivity\textsuperscript{36}. This sensitivity ensures that labelled cells will be visible in smaller numbers and after dilution from multiple cell divisions. There are, however, many different issues with negative contrast agents that can introduce confounding results. Referring back to Figure 1.3, there are many sources of dark signal present in the body naturally, which could be confused for contrast agent signal. Iron oxide nanoparticles create blooming artifacts due to iron's
extremely strong magnetic response\textsuperscript{37}. Blooming artifacts present as large black voids much larger in area than the actual extent of the iron molecules creating the negative contrast, thereby obliterating signal in adjacent tissue. This size discrepancy between what is seen in the image and what exists prevents quantitative measurements and precise localization. Iron agents are also absorbed by the body’s macrophages, preventing washout and supporting signal retention even after the target cells have died\textsuperscript{38}. This is particularly troublesome if one needs high specificity, meaning the assurance that dark contrast stems only from labeled cells. Iron oxide nanoparticles used to be FDA approved for clinical use but were taken off the market around 10 years ago. There have been no clear reports as to what adverse effects led to this; they are still available for use in some European countries.

**T1 Contrast Agents**

T1 contrast agents are based on the paramagnetic behavior of the lanthanide elements. These elements have unpaired electrons, giving them a net nuclear spin, but without the presence of an external magnetic field have no magnetic effect of their own. Once exposed to a magnetic field, however, they align with it contributing a signal that can be many hundreds of time greater than that of a hydrogen atom.

Recall that T1 contrast is due to the rate at which the longitudinal magnetization following an excitation pulse recovers. As more and more molecules reorient themselves through random motion and return to equilibrium through alignment with the main magnetic field, signal recovers and, as such, a T1 contrast agent must be a molecule that speeds up this recovery process. Speeding up this process significantly will cause the
affected area to have a very different relaxation rate to the surrounding tissue, which manifests as an increased bright contrast in the image. Instead of being large magnetic molecules that have a strong magnetic signal affecting many molecules around them like a T2 contrast agent, T1 contrast agents tend to be small molecules that directly act on a few hydrogen atoms at a time. In contrast to T2 agents, T1 agents make the image brighter.

The most common T1 contrast agents are based on the molecule gadolinium, because at 7, it has the largest number of unpaired electrons. These agents, as their name implies, use the paramagnetic molecule gadolinium as their base. Gadolinium-based contrast agents are the only ones currently approved for clinical use in North America. Gadolinium, however, is not present naturally in the body and there is much concern over the accumulation of this metal due to its widespread use clinically and also through exposure in groundwater\textsuperscript{39,40}. There are many reports coming out in the past few years that show that there is residual gadolinium, which is known to be toxic found in the brain, liver, and kidneys\textsuperscript{41}. A link between Gd-containing contrast agents and nephrogenic systemic fibrosis, in which hardened skin and plaques form in the skin and, in severe cases, on internal organs has been made; it is now not recommended to prescribe Gd-agents to patients with a glomerular filtration rate under 60 ml/min\textsuperscript{42}.

It is necessary, going forward, to find a contrast agent that does not suffer from toxicity issues. A promising alternative to Gd agents are manganese based agents which can provide the same or better contrast and lower toxicity\textsuperscript{41,43}. Manganese is naturally present in the body and excessive amount in low concentrations can be properly
eliminated\textsuperscript{29}. A subclass of manganese contrast agents known as manganese porphyrins are of particular interest. These porphyrin structures are incredibly thermodynamically stable so there is very little chance of any significant amount of manganese being released\textsuperscript{31}. Manganese porphyrins do not only clear renally but are broken down in the liver. This will release free manganese into the body but since the total amount of manganese is low it should clear through the body's natural manganese clearance pathways\textsuperscript{44,45}. The majority of free manganese in the blood is converted to bile in the liver and removed through biliary secretion\textsuperscript{46}. It has also been shown that increased oral intake of manganese does not result increased urinary manganese excretion\textsuperscript{47}. Oral doses of administered manganese chloride was found to have a clearance half-life of between 10-37 days in humans\textsuperscript{48}. These agents also have the added benefit of having a higher sensitivity than the current clinical gadolinium agents, and this is due to inner sphere and outer sphere reactions of the contrast agents with nearby water molecules\textsuperscript{49}.

1.5 Our agent MnP- TPPS-NH\textsubscript{2}

Due to the above considerations -- the many sources of dark signals acting as potential confounds in T2 agents, and the toxicity of current T1 agents -- we selected a manganese based T1 agent as the best candidate for future cell labeling and cell tracking experiments. There are other considerations beyond simply addressing which contrast agent provides the highest contrast. One must also consider the feasibility and ease of
use. Often porphyrin chemistry is complex and offers a low yield which is why it may not currently be widely implemented\textsuperscript{41}.

In the past, we recently developed a very efficient cell trappable manganese-porphyrin contrast agent\textsuperscript{49,50}. We showed that this agent was highly effective at labeling and tracking mouse embryonic stem cells\textsuperscript{50}. However, the synthesis of the agent was time consuming and difficult to scale up. Large amounts of the agent must be readily available for cell therapy as often billions of cells are injected for each trial. Most labs that are interested in creating a cell therapy for regenerative medicine do not have trained chemists on their team ready to synthesize complex molecules; therefore, the agent itself must be either commercially available or easy to synthesize. Furthermore, cell therapies are already very complex and a very large amount of time and energy is spent preparing the cells for implantation. These researchers would not want to incorporate a very complex labelling method into their already complex protocols. So, the contrast agent should be very easy to add to the cells. Therefore, a contrast agent that is simple to synthesize, easy to use, and cheap to manufacture is a candidate more likely to be used.

This leads to our choice for the best candidate for a contrast agent for cell tracking. Our contrast agent, henceforth denoted MnP-TTPS-NH\textsubscript{2}, is a manganese porphyrin agent with chemical structure shown in Figure 1.5 The manganese atom responsible for the paramagnetic contrast enhancement is tightly bound in the center of the porphyrin ring. Manganese porphyrins have shown to have higher relaxivity compare to the clinical gadolinium agents\textsuperscript{49}. The amine group makes our agent lipophilic and capable of passively diffusing through the cell membrane. While this agent is not
available for clinical use and a full toxilogical study is required before it could be used in humans, it can be used pre-clinically to optimize cell therapies before they reach human trials.
**Figure 1.4:** Chemical structure of our manganese porphyrin agent for T1 contrast cell tracking. The manganese is stably bound in the center of the molecule and the amine group makes the molecule cell permeable.

### 1.6 Thesis motivation and summary

The principle aim of this work was to develop a non-invasive, non-ionizing, gadolinium-free positive contrast MRI method to track cells intended for regenerative purpose. This method was designed to be easy to use so that it can be readily applied to any cell therapy protocol simply by the addition of our contrast agent to media of the cells as they expand in vitro prior to implantation.

Chapter 2 shows the optimization of our protocol in human embryonic stem cells. Our agent, MnP-TPPS-NH₂, was used at different concentrations and labeling intervals to quantify the T1 relaxivity and predict the amount of contrast that would be generated. The agent was also tested for its effects on the viability and function of the human embryonic stem cells; no adverse effects were noted. Furthermore, we demonstrate the localization of the intracellular distribution of our agent using fluorescence microscopy.
The final chapters show the supplementary experiments completed during development of the project that were not included in the final publication.
Chapter 2

2.0 – Simple and effective tracking of human embryonic stem cells using a manganese based positive T1 contrast MRI agent
Simple and effective tracking of human embryonic stem cells using a manganese based positive T1 contrast MRI agent

Andrei Venter,1,2 Daniel A. Szulc,1,2 Inga E. Haedicke,1,2 Hai-Ling Margaret Cheng1,2,3,4,5

1Institute of Biomaterials and Biomedical Engineering, University of Toronto
2 Translational Biology and Engineering Program, Ted Rogers Centre for Heart Research
3 The Edward S. Rogers Sr. Department of Electrical and Computer Engineering, University of Toronto
4 Heart & Stroke/Richard Lewar Centre of Excellence for Cardiovascular Research
5 Ontario Institute for Regenerative Medicine

Corresponding Author:

Hai-Ling Margaret Cheng, PhD, PEng
Institute of Biomaterials & Biomedical Engineering
164 College Street, RS 407
Toronto, ON, Canada M5S 3G9
Telephone: 1-416-978-4095
Email: hailing.cheng@utoronto.ca

Key Words: magnetic resonance imaging, cellular imaging, cell tracking, stem cell therapy, human embryonic stem cells, regenerative medicine, tissue engineering
2.1 Abstract

**Purpose:** To develop a gadolinium-free, scalable, T1 contrast agent for cell tracking to be used in regenerative medicine for cell therapies.

**Materials and Methods:** A scalable manganese porphyrin contrast agent, MnP-TPPS-NH$_2$, was used to label human embryonic stem cells. MRI was performed on a 3 Tesla clinical scanner; T1 relaxation times for different labeling concentrations and incubation times were measured. Quantification of manganese content was performed using inductively coupled plasma atomic emission spectroscopy, and cellular distribution of the agent was observed with fluorescence microscopy. Viability and differentiation into embryoid bodies was performed and showed no adverse effects on cell function.

**Results:** MnP-TPPS-NH$_2$ is an effective T1 contrast agent that is passively uptaken by human embryonic stem cells. A two-fold decrease in T1 relaxation times was observed after MnP-TPPS-NH$_2$ was added to cell incubation media for 24 hours. Viability and cell proliferation, were undisturbed by the labeling process. Fluorescence microscopy shows MnP-TPPS-NH$_2$ is found mainly in the cell-cell boundaries and within the nucleus.

**Conclusion:** MnP-TPPS-NH$_2$ is an effective gadolinium-free T1 contrast agent with no adverse effects on cell survival and function. Due to the ease of synthesis, this agent is readily scalable to the large cell number for stem cell therapies.
2.2 Introduction

Human embryonic stem cells (hESCs) have the unique potential of developing into all the different cell types in our body and represent a clinically relevant cell source in the field of regenerative medicine$^{14,51,52}$. Stem cells therapies remain predominantly in the research area and require much optimization before they can successfully move into clinical practice. There are currently two major issues with existing therapies. Firstly, soon after injection or implantation, the vast majority, approximately 90%, of the cells quickly die$^{53}$. Secondly, even if the cells survive, there is little integration with the host tissue$^{54}$. Optimization of this issue is difficult at present, because there currently exists no standard method of quantifying stem cell survival and integration in real-time and non-invasively in deep tissue. While non-invasive optical imaging can be used on shallow tissues, the only method currently available for observing stem cells in deep tissue is explantation, which requires the trial animal to be sacrificed.

Magnetic resonance imaging (MRI) can be used to image soft tissue with fine spatial resolution noninvasively and without ionizing radiation. With the aid of contrast agents, MRI is also capable of tracking cells throughout the body$^{37,55}$. Until now, iron-oxide nanoparticles which are T2 agents have been the preferred choice due to their great sensitivity$^{56}$. However, there are many other sources of dark contrast naturally present in the body. These include hemorrhage and blood clots, which are often found in areas of trauma likely to be targeted by cell therapy. Furthermore, it has been shown that iron remains in the target area after cells have died as it is absorbed by the macrophages
present in the inflamed damaged tissues\textsuperscript{57}. T1 or positive contrast agents, which provide an increase in signal intensity, are a better choice, since they provide more accurate spatial delineation and do not suffer from the aforementioned issues\textsuperscript{37}. The most commonly used positive contrast agents today are gadolinium (Gd) based\textsuperscript{58}. While widely used, in recent years concerns have been raised about gadolinium's toxicity as it is not naturally found in the body\textsuperscript{59}.

An alternative to Gd agents are manganese (Mn) based agents that can provide the same or better contrast and lower toxicity. Manganese is naturally present in the body and excess at low concentrations can be eliminated. Amongst manganese contrast agents, manganese-porphyrins are particularly promising. These agents have been shown to have a much higher relaxivity than the current gadolinium chelates used in the clinical practice\textsuperscript{41}. Furthermore, the porphyrin structure is highly thermodynamically stable and will not readily release the manganese ion\textsuperscript{31}.

To address the need for a non-invasive method to monitor and guide cellular therapy in humans, we have explored a magnetic resonance (MR) imaging-based cell-tracking method that utilizes a manganese porphyrin agent, MnP-TPPS-NH\textsubscript{2}, that has better sensitivity and safety than gadolinium-based compounds and is easy to scale up due to simplicity of synthesis. In this study, we investigate for the first time the efficacy of cell-permeable MnP-TPPS-NH\textsubscript{2} for labeling human embryonic stem cells. We have shown that this agent is not only easy to synthesize but also an effective contrast agent showing a T1 reduction of over two-fold compared to unlabeled cells. The agent shows no adverse effects on cell viability, colony formation, and suspended cell aggregation.
behavior. Furthermore, we have characterized, for the first time, the cellular distribution of the agent by using the fluorescently active precursor molecule, which is the same as MnP-TPPS-NH$_2$ only without the manganese.

### 2.3 Materials and methods

**Cell culture**

Human embryonic stem cells from the line ESI –017 (ESIBio, SKU: ES-700) were cultured in sterile conditions on tissue culture plates coated with Corning™ Matrigel™ Membrane Matrix (Fisher Scientific Catalog No.08-774-552). The cells were maintained in an incubator at 37° at 5% CO2 in mTeSR ™1 (STEMCELL Technologies Catalog # 85850). The cells were grown in colonies and passaged using enzyme free dissociation to prevent differentiation and allow the cells to remain in small colonies using Gentle Cell Dissociation Reagent (STEMCELL Technologies Catalog #07174) and mechanical cell scraper separation.

**Preparing cells for imaging**

Stock solution of manganese porphyrin at 10 mM dissolved in sterile distilled water under sterile conditions was created to label cells without significantly changing the volume of media. This stock solution was then heated to a low boil for 5 minutes further
ensure sterility. Contrast agent was added under sterile conditions in the fume by simply pipetting from the stock solution directly into the well that was to be labelled and swirling the plate gently until the solution was mixed.

At the end of the labeling period the cell media was removed and the cells were rinsed with room temperature DPBS (Thermofisher Catalog # 21600010) three times with gentle swirling of each rinse to ensure that most of the contrast agent not inside the cells was washed off. The stem cells were then disassociated using stem cell technologies gentle cell disassociation reagent and then fully removed with a cell scraper. Cells were then resuspended in PBS and centrifuged at 300g three times for five minutes each time to ensure all extracellular contrast agent was fully removed. The cells were then transferred into 115 × 5 mm Wintrobe sedimentation tubes (Kimble Chase, Vinelad, NJ) and transported on ice to the MRI.

Sample imaging

Immediately after pelleting, the tubes containing cell pellets were placed inside an ULTEM™ resin holder for imaging. The pellets were then quickly imaged on a clinical MR scanner (Achieva 3.0 T TX, Philips Medical Systems) using a 32-channel head coil. T1 mapping was performed using inversion recovery turbo spin echo: TR = 3000 ms, TE = 18.5 ms, 5 cm field-of-view, 3 mm slices, 0.5 x 0.5 mm in-plane resolution, and TI = [50, 100, 250, 500, 750, 1000, 1250, 1500, 2000 and 2500] ms. After image acquisition, the data was analyzed on a 3-mm deep cylindrical volume within each cell pellet. T1 values
were calculated pixel-by-pixel (~50 pixels per vial) using in-house software developed in Matlab (ver. 8.1).

**Quantifying intracellular magnesium content**

To prepare cells for manganese quantification 0.2 mL aliquots of the previously imaged cell pellets were digested by the addition of one millilitre of 70% ultrapure analytical grade HCl and sonication at 40 C for 30 minutes. The solution was then diluted to a final volume of 6 mL with ultrapure water. The final solution was run through a 0.22 micrometer filter to remove any large protein compounds leaving a solution containing the MnP-TPPS-NH2 that was inside the cells. These samples were then run on a inductively coupled plasma atomic emission spectrometer ICP-AES (Optima 7300 DV ICP AES). ICP-AES passes this solution through a plasma flame exciting the manganese, which then emits a signal at 293 nm which is converted into ppb of Mn in the solution. This value was then divided by the number of cells to give an approximation of the amount of Mn in one cell.

**Measuring cell viability**

Human embryonic stem cells were grown in 6-well plates until the colonies reached 60% confluency. Wells were labelled with MnP-TPPS-NH2 for 24 hours at 0 mM, 0.1mM, 0.2mM, 0.3mM, 0.4mM, and 0.5mM. Once the labeling period was complete, the contrast agent containing media was aspirated, and the cells were rinsed thrice with room
temperature PBS to eliminate any residual extracellular contrast agent. Since hESCs grow in colonies, complete dissociation into single cells was necessary to perform the trypan blue assay\textsuperscript{60}. The cells were then removed from the wells and suspended in 3mL of PBS in 15-mL tubes for counting. 1mL aliquots from each sample was then automatically mixed with trypan blue and counted on a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter) with default image gating. Fifty separate images were taken and counted for viability. This trial was repeated three separate times to ensure statistical relevance.

Differentiation Potential via embryoid body differentiation

Four wells of a six-well plate with roughly 60% confluent HESC colonies were labelled with MnP-TPPS-NH2 for 24 hours with two wells each at 0.2 mM and 0.5 Mm concentrations. Once the labeling period was complete, the contrast agent containing media was aspirated and the cells were rinsed thrice with room temperature PBS to eliminate any residual extracellular contrast agent. The cells were the removed and plated into 12-well untreated, uncoated plates for suspension culture. Each original well was split into 6 of the 12-well plate wells with 2mL of complete mTeSR media and incubated on a shaker spinning at 60 rpm and left for 5 days to assist aggregation.

Media was subsequently changed every four to five days with care not to disturb the EBs until day 14 for imaging. To prepare for imaging, the embryoid bodies were gently
rinsed three times with PBS, fix with 4% paraformaldehyde for 10 minutes at room temperature, and labelled with DAPI nuclear stain (Thermofisher Catalog # D1306).

**Fluorescence imaging of Apo-porphyrin (TPPS-NH₂)**

To determine intracellular localization of the porphyrins, hESCs colonies grown on glass cover slips to 60% confluency were labelled with mTeSR media containing 0.5 mM of TPPS-NH₂ for 24 h. When labelling was complete the cells were rinsed 3 times with room temperature PBS and fixed with 4% paraformaldehyde at room temperature for 10 minutes. The cover slips were then mounted and imaged on a Leica DMi8 Inverted Microscope using the excitation filter of a DAPI filter cube (350/50 nm) and a DSRED emission filter (605/75 nm). A control sample of unlabeled hESCs was also prepared as above minus the TPPS-NH₂.

**2.4 Results**

Figures 2.1 and 2.2 show that under even short labelling times and low concentrations, MnP-TPPS-NH₂ is an effective contrast agent showing significant decrease in T1 relaxation times. To test the significance of the increase in contrast the Kruskal-Wallis test (non-parametric one way ANOVA) was used since the pixel values did not pass the Kolmogorov- Smirnov normalcy test. It was found that the contrast of all labeled cells were significantly different from the unlabeled control and there is significant difference of contrast between the two labeling times. The relaxation times measured for
cells that were incubated for 2 hours with MnP-TPPS-NH2 show relatively the same decrease in relaxivity. This implies that the rate of absorption of the contrast agent is too slow and the rate limiting step with such a short labelling time. To get the benefit of changing concentrations a 24-hour labeling interval is necessary. Alternatively, it shows that there is no need to use high concentrations for short labelling times, and the lowest concentration 0.1 mM is going to perform as well as the highest, 0.5 mM. Figure 2.4 corroborates the T1 relaxivity results by quantifying the intercellular Mn content post labelling.
Figure 2.1: (A) T1-weighted inversion-recovery image, repetition time (TR) = 3000 ms, inversion time (TI) = 1250 ms showing the signal from a 3 mm slice of pelleted unlabelled human embryonic stem cells, three concentrations of MnP-TPPS-NH2, and two labeling intervals of 2 and 24 hours. (B) Quantitative T1 relaxation map for each corresponding cell pellet.
Figure 2.2: Mean T1 relaxation times for cell pellets containing unlabeled and MnPNH$_2$ labelled human embryonic stem cells. Error bars represent standard deviation of the individual pixel T1 values in a cell pellet (approximately 50 pixels per pellet).

* Denotes significantly different via Kruskal-Wallis test (P<0.01) between control and the different incubation times

** Denotes significantly different via Kruskal-Wallis test (P<0.01) for ingroup labeling concentration
Figure 2.3, shows that the success of the labeling method can be qualitatively observed by the colour of the cell pellets. A gradient of colour from unlabelled white cell pellets to progressively darker green can be seen correlating exactly with manganese porphyrin concentration. The staining can even be seen at a cellular level.

Figure 2.3: Cell pellets in borosilicate glass tubes that were imaged with MRI showing the dark green dyeing effect that MnP-TPPS-NH2 has on the cells. A darker green corresponds with increased porphyrin uptake and increased MRI contrast.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mn Concentration / Pellet (ppb)</th>
<th>Mn Concentration / Cell (mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Below Detection Threshold</td>
<td>Below Detection Threshold</td>
</tr>
<tr>
<td></td>
<td>2 Hour Label</td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>8.82</td>
<td>1.05E-11</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>13.5</td>
<td>1.61E-11</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>19.14</td>
<td>2.28E-11</td>
</tr>
<tr>
<td></td>
<td>24 Hour Label</td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>12.36</td>
<td>1.47E-11</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>17.98</td>
<td>2.14E-11</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>46.9</td>
<td>5.59E-11</td>
</tr>
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</table>

**Figure 2.4:** Inductively Coupled Plasma Atomic Emission Spectroscopy results from the digested cell pellets shown in Fig 2.3 showing the total amount of Mn in the pellet and the amount of Mn per cell.

Figures 2.5 to 2.7 show that the agent has no adverse effects on the cell colony morphology, cell survival, and proliferation. In Figure 2.5, microscopy images of representative colonies of the ~50 colonies observed for labeled and unlabeled cells are shown. These images show no difference in colony size, shape, and distribution post MnP-TPPS-NH₂ labeling other than a slight darkening due to the dark coloration of the agent. Viability results show that the longest contrast agent labelling time had no effect on cell viability regardless of concentration (Figure 2.6). To further show that there were no adverse effects on the behavior of the cells, they were differentiated into
embryoid bodies showing proper function aggregation as described here \(^6\). Six wells of embryoid bodies were prepared for every labelled sample, and every well developed embryoid bodies. The embryoid bodies were of the same size and appearance as the one generated from unlabelled cells (Figure 2.7). This also shows that the cells retain their ability to divide and proliferate after labelling.

**Figure 2.5:** Representative phase contrast microscope (**A-B**) and bright-field (**C-D**) images showing representative hESC colonies before and after labeling with MnP-TPPS-NH2. Cell morphology and colony shape is unchanged in the ~50 colonies observed. The labeled cells appear slightly more opaque due to the strong absorption of the contrast agent.
Figure 2.6: Graph showing the average hESC viability from trypan blue assay measured for the different concentrations of manganese porphyrin contrast agent after 24 hours incubation. Error bars represent the standard deviation (N=3).
**Figure 2.7**: (A) Brightfield image at 10 x magnification of a 21 day-old embryoid body made from hESCs (B) a different embryoid body 21 days after cells were labeled at 0.5 mM MnP-TPPS-NH2 for 24 hours (C) corresponding DAPI nuclear stain fluorescence image (B). Shown in each image is one single embryoid body formed from thousands of individual embryonic stem cells, the individual cells can be seen in the fluorescence image.

Using the precursor porphyrin molecule which contains no manganese, the distribution of the porphyrin within cells is visible under fluorescent microscopy. This non-metallated porphyrin has a peak absorption around 415nm and emission around 650nm\(^62\). There is fluorescent signal found throughout the cells but a stronger signal is observed as clumps around the cell periphery and within the nucleus (Figure 2.8) We have also shown the cellular distribution of our agent by incubating human stem cells with the non-metallic precursor porphyrin. This was used in conjunction with fluorescent microscopy to determine after 24 hours of labelling where the contrast agent
is localized at the cellular level. Images show peak brightness at the edges of the cells, indicating that the contrast agent is grouped together potentially in vacuoles in the cytoplasm of the cell. The nucleus also appears brighter than the cytoplasm, indicating a higher concentration of contrast agent in the nucleus. This shows that the agent is internalized in the cell and not simply bound to the edges. There are also large bright regions that showed that during incubation some of the contrast agent might have bound and crystallized on top of the cells. This should be eliminated prior to injection with sufficient washing. The cells cannot be washed as thoroughly without dissociating as would be done prior to injection in vivo.
Figure 2.8: Fluorescence images of the apo-porphyrin showing the subcellular distribution of contrast agent after 24 hours labelling. (A) 63x magnification of unlabeled cells showing no fluorescent signal, (B) 63x magnification of cells labeled with 0.5 mM of apo-porphyrin for 24 hours (C) Further Digital zoom at 63x magnification. Note in the red circles many bright accumulations of cells around the periphery and inter-connective space of the colony. Green circles highlight increased signal from increased porphyrin concentration inside the nucleus.
2.5 Discussion

The two main driving motivations of this work was to create a contrast agent that is both easy to manufacture and easy to use. We have shown that our contrast agent is easy to manufacture as a direct structural precursor can be purchased directly, and from this a simple one-step chemical reaction alone is sufficient to produce the final functional contrast agent. This is done by purchasing a precursor porphyrin and performing the metalation step shown here. Through our labelling process, we have shown that the agent is easy to use, as it requires simply dissolution in a stock solution and addition to the incubation media. The ease of use of this method is very important to ensure the maximum likelihood of implementation of this agent by labs that are not familiar with MR contrast agent chemistry and MRI physics.

The other major consideration necessary, if this agent were to ever be used even pre-clinically, is proof that it has no adverse effects on the cells that are to be labelled. We have shown that on a cellular level this agent is safe to use: this was accomplished using trypan blue viability assay, proliferation and embryoid body differentiation. Similar work from our lab has also shown that the same agent has no effect on the viability of three different cancer cell lines. These studies have used the trypan blue exclusion test which tests for membrane integrity. This does not rule out cell death due to apoptosis and an investigation using the TUNEL assay would strengthen these viability results. Further characterization is required, to make sure there are no genetic or long term effects on cell function. A chromosomal karyotype, or DNA sequencing study is
necessary to see if the agent is having adverse genetic effects since it is present in the nucleus\textsuperscript{67}. Differentiation potential of the hESCs needs to be tested via in-vitro differentiation assays or through an in-vivo teratoma formation study\textsuperscript{68}.

There has also lately been much evidence and controversy over the use of the common gadolinium-based T1 agents. Many studies have shown that gadolinium, if released from contrast agents, settles and deposits in the brain and kidney causing fibrosis\textsuperscript{59}. We have already shown in previous works that manganese-based agents show significantly higher reductions in T1 relaxation times to the current clinical gadolinium based agents\textsuperscript{41-49}. This is important from an imaging perspective, as higher contrast equals enhanced ability to visualize smaller population of cells. Even more important is the medical standpoint, as an increase in sensitivity would allow the same amount of contrast change at lower concentrations of the contrast agent. It is always critical to minimize the amount of agent a patient may be exposed to in the clinical setting.

The intracellular distribution of the agent was observed for a labeling time of 24h with a concentration of 0.5mM. This was done to ensure the maximum visible fluorescent signal. This agent was not designed as a standard fluorophore and has a very large stoke shift. This makes visualization at lower concentration difficult on a standard fluoresce microscope. Optimization of the agent on a confocal microscope with a tunable spectral filter would allow investigation at lower contrast agent concentrations and labeling times. Investigating the agent real time in living cells would give better insight into how this agent and others like it are absorbed and distributed by the cells. This could also be used on other cell types to see if the distribution varies or is consistent. A
previous study on the apo-porphjyrin confirms the nuclear penetration of the agent. Since the agent is present in the nucleus, chromosomal assay or DNA sequencing should be performed to show it is not adversely interacting with the cell's DNA.

As with all exogenous contrast agents used for cell labelling, there is the issue of signal dilution as the cells divide. This dilution is further exacerbated by contrast agent leakage out of the cells. Since this agent is not cell trappable we expected it to leak out at similar rates to other non-trackable agents such as Gd-HPDO3A. Over the course of several days, maybe up to a week, all signal will be lost. However, this signal dilution effect is not of great concern for many regenerative medicine applications. Many of the parameters of interest -- such as cell survival, cell integration, and in tissues under motion such as the heart, whether the cells remain in the target area -- are parameters that can be measured within the first few hours or days after cell injection. Once the cells have integrated into the host tissue or have at least survived 24 hours, the major risks of cell death lessen significantly and, at this point, it would be more beneficial if the agent were to leak out and dissipate allowing the cells to continue normal function and allowing the agent to clear the body.

Since we have no observable effects on cell viability and aggregation behavior at our highest labeling concentration and longest incubation, it is reasonable to believe that either of these can be further increased. However, more extensive and detailed assays will need to be performed to ensure no off-target effects are present in labeled cells before we can conclusively recommend effective doses for labeling. Furthermore, should
there be a need for increased signal and if it is safe to do so, the amount of contrast agent loaded in the cell can be improved using electroporation or other common methods\textsuperscript{70,71}.

### 2.6 Conclusions

In conclusion, we have shown that it is possible to get a significant reduction in T1 relaxation time in human embryonic stem cells times using a manganese porphyrin agent that is both simple to synthesize and easily added to any in-vitro cell culture. We have also shown that this agent does indeed pass through the cell membrane and accumulates in small clumps as well as within in the cell nucleus. This agent and labeling method should be applicable to all cell types in regenerative medicine. A simple imaging target such as the spinal cord or muscle would be a prime first in-vivo target to allow in-vivo optimization before a moving target such as a myocardial infarct.
3.0 Supplementary experiments

3.1 Other cell types labeled with MnP-TTPS-NH₂

Initial characterization of the porphyrin was performed on primary neonatal mouse atrial fibroblast cells. This was done as an initial feasibility test to see if any signal was to be had on MRI. The results were very positive, and we saw for a labeling concentration of 0.2 mM a T1 of 300 ms, which was almost exactly half the T1 of control unlabelled fibroblast cells. The fibroblast cells themselves had a very short T1 time; for this reason, they are not a good target for T1 contrast agents as the contrast to noise ratio will be very low. Again, as before, the difference in colour, due to the dyeing effect of the porphyrin can be seen on the cell pellets. Once we saw that the agent was effective as a contrast agent and had no cytotoxic effects, we moved to our final target cell type of human embryonic stem cells. These results do show that this labelling method and contrast agent can be readily applicable to other cell types with no modification.
**Figure 3.1:** Three pellets of neonatal mouse arteriolar fibroblast cells and their associated T1 relaxation times. Unlabeled cells appear white, while MnP-TPPS-NH₂ labeled cells are dyed green.

### 3.1 Similar T1 agents

The only commercially available T1-contrast cell-labelling agent, to our knowledge, is a gadolinium oxide nanoparticles called Gado-CellTrack. This agent was also tested in our lab and showed very high contrast at very low labelling concentrations. However, this agent proved to be toxic in human embryonic stem cells, which was visible qualitatively under microscope as well as quantitatively by a trypan blue cell viability assay. Once we saw the detrimental effect in our cell experiments, we discontinued further investigations with this agent. It did show a very strong T1 contrast enhancement at

<table>
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<th>Sample</th>
<th>T1 (ms)</th>
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</tr>
<tr>
<td>0.1 mM</td>
<td>497</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>330</td>
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very low concentrations. Gado-celltrack has been used in our lab previously to successfully label human aortic endothelial and smooth muscle cells with no detrimental effect on viability\textsuperscript{72}. The reason why it proved highly toxic to human embryonic stem cells but not endothelial and smooth muscle cells is unclear and warrants further investigation.

3.2 Reporter genes for generating contrast

All exogenous contrast agents have an inherent limitation of signal dilution caused by cell division. The only method to circumvent this issue is to genetically modify cells to express their own contrast. The two most common methods are overexpression of either ferritin or the divalent metal transporter protein, which are proteins in which upregulation would increase cellular levels of iron and manganese respectively\textsuperscript{73,74}. To create a stable cell line expressing either of these two genes is significantly more complicated than our exogenous labelling method and would at best have significantly lower signal\textsuperscript{75,76}. Additionally, with these transfections, incubation with metal ions at a higher concentration than would be found in the body is usually required. Ultimately, for many applications, genetic modification to express contrast is impractical for the current amount of signal gain, and may never be seen in clinical use due to the ethical concerns around gene editing.
4.0 – Future work and perspectives

Our contrast agent, MnP-TPPS-NH₂, has been demonstrated to be effective with no effect on the viability and proliferation potential of human embryonic stem cells, mouse cardiac fibroblasts, as well as some cancer cell lines. However, further characterization is required, to ensure there are no genetic or long-term effects on cell function such as differentiation potential. The labeling procedure and methods do not need to be changed amongst cell types making investigating into other cell types for cell therapy very easy and appealing. The first step to further characterizing this agent is to demonstrate its use in vivo. A study in which different numbers of cells are injected subcutaneous in vivo to determine the minimum number of detectable cells would be the first step. Following this, this agent could be added to any cell therapy model to see how long signal remains after cell injection. This data would be correlated with histology to investigate how our agent behaves as an accurate and predictive measure of cell viability.

Once in-vivo characterization is complete, and assuming the results are positive, this agent can now be added to a variety of regenerative medicine models including: myocardial infarct, spinal cord injury, diabetes, and more.

It will also be beneficial for the field of MRI contrast agents if further investigation are carried out on methods of internalization and cellular distribution of MnP-TPPS-NH₂ in cells. A study showing how the intracellular distribution of the agent changes over time could give insight into how to make the agents more effective and potentially cell-trappable for long term imaging.
Our lab is also developing methods incorporating MnP-TPPS-NH$_2$ for the labeling of scaffolds and other extracellular support systems as a measure of degradation and non-invasive imaging.

5.0 – Summary and conclusions

The principle goal of this work was to create a diagnostic method for the real time visualization of cell viability of an implanted population of cells. Furthermore, the work focused on developing a method that was not only effective but easy to implement and adapt to different cell types and protocols to make it more accessible to stem cell researchers unfamiliar with MRI techniques. The human embryonic stem cells investigated were chosen as a clinically relevant target molecule and MRI scanning was done on a 3T clinical scanner to show the impact of our contrast agent MnP-TPPS-NH$_2$ on the current state of stem cell therapies. We also chose to use a manganese-based contrast agent as we strongly believe that gadolinium agents and iron-oxides are falling out of popularity in clinical work and the eventual goal is for this agent to be used in a clinical setting.®
References


48. Davidson, L., Cederblad, A., Lonnerdal, B. & Sandstrom, B. Manganese


