Investigating Cellular Imaging Techniques for Cancer Cell Tracking

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Medical Biophysics

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Abstract

**Introduction:** Breast cancer remains a leading cause of mortality among women due to the propensity of breast tumours to spread and metastasize to distant sites, including the brain. Early detection of metastatic disease has been challenging, and typical methods of treatment often fail. A significant roadblock in advancing the detection and treatment of breast cancer brain metastases is the lack of representative experimental preclinical models and methods of studying its progression *in vivo.* **Methods:** First, we use iron-based cellular MRI to noninvasively track the progression of a brain metastatic breast cancer cell line in murine models with varying immune competencies. We then employ longitudinal BLI and MPI to visualize the tumour progression of a patient-derived xenograft (PDX) model of breast cancer brain metastasis. Finally, we used MPI to evaluate the magnetic performance of iron oxide particles following cell labeling with a brain metastatic breast cancer cell line to optimize cellular detection and cell tracking techniques. **Results:** Cellular MRI revealed significant differences in tumour progression throughout the brain and body between murine models. We then developed a novel method for labeling PDX cells with iron oxide particles and use MPI and BLI to provide measures of iron content and cellular viability. Finally, we demonstrate that cell labeling can change the magnetic performance of iron agents used for cell tracking with MPI. **Conclusion:** Comprehensive analysis of cancer cell arrest, clearance, and tumour progression with cellular imaging is important for understanding the metastatic cascade of a model of breast cancer brain metastasis in both cell lines and PDX models. Furthermore, we provide evidence that iron oxide particles are valuable tracers for MPI cell tracking, but their MPI performance may be altered following cell labeling.
Keywords
Breast cancer, metastasis, magnetic resonance imaging (MRI), bioluminescence imaging (BLI), magnetic particle imaging (MPI), superparamagnetic iron oxide particles (SPIOs), patient-derived xenografts (PDX)
Summary for Lay Audience

Introduction: The majority of cancer related deaths are due metastasis, which is the spread of cancer from the original tumour to distant locations in the body. For breast cancer, this is often the brain, lungs, liver, and bones. When breast cancer spreads to the brain, the prognosis is poor. Therefore, there is an urgent need to understand and develop new tools to study the progression of this disease. Methods: In this thesis, we study models of breast cancer that spread to brain using novel cellular imaging techniques. First, we use magnetic resonance imaging (MRI) to compare how cancer cells arrive in the mouse brain and grow and progress over time. We then used two complementary imaging technologies called bioluminescence imaging (BLI) and magnetic particle imaging (MPI) that allowed us to visualize how cells derived from patient brain metastases grows in a mouse model. Finally, we assessed different iron agents that are used for cell tracking and determined how they behave alone and once taken up by breast cancer cells. Results: Using MRI allowed us to measure and examine differences in cancer cell fate over time in different strains of mice. Using BLI and MPI was a complementary approach to study the development of a tumour formed from patient tumour cells, allowing us to measure both cell viability and iron content. Lastly, we demonstrated that some iron agents significantly change their behaviour and affect their utility for cell tracking once they have been taken up by breast cancer cells. Conclusion: Cellular imaging technologies, like MRI, BLI, and MPI, are critical in providing a comprehensive understanding of the progression of breast cancer and can be used to improve detection of cancer cells in the body.
Co-Authorship Statement

Chapter 1 contains material from 2 previously published review papers. The first review paper is Knier NN, Pellizzari S, Zhou J, Foster PJ, and Parsyan A (2022). “Preclinical Models of Brain Metastases in Breast Cancer”. Biomedicines 10(3), 667. NNK, AP, and PJF conceptualized this review and wrote the manuscript. SP and JZ also contributed to writing and editing the manuscript. SP contributed to figure creation. The second review paper is Sehl OC, Gevaert JJ, Melo KP, Knier NN, and Foster PJ (2020). “A perspective on cell tracking with magnetic particle imaging”. Tomography 6(4), 315-324. OCS, JJG, KPM, NNK and PJF all contributed to writing and editing the manuscript.

Chapter 2 is reprinted from Knier NN, Hamilton AM, and Foster PJ (2020). “Comparing the fate of brain metastatic breast cancer cells in different immune compromised mice with cellular magnetic resonance imaging”. Clinical & Experimental Metastasis 37(4), 465-475. NNK performed experiments and data collection, data analysis, and drafted the manuscript. AMH contributed to conducting animal experiments and histology. PJF designed experiments, assisted with imaging, and contributed to writing and editing the manuscript.

Chapter 3 is reprinted from Knier NN, Dubois VP, Chen Y, Ronald JA, and Foster PJ (2021). “A method for the efficient iron-labeling of patient-derived xenograft cells and cellular imaging validation”. Journal of Biological Methods 8(3). NNK contributed to experiment design, performed experiments and data collection, analyzed data, and drafted the manuscript. VPD assisted with performing animal experiments. YC performed histology. JAR contributed to editing and reviewing the manuscript. PJF contributed to experiment design, editing, and reviewing the manuscript.
Chapter 4 is in preparation for submission to Nanoscale. Knier NN, Gevaert JJ, Sehl OC, and Foster PJ (2023). “Evaluating superparamagnetic iron oxide particles for cell tracking with magnetic particle imaging (MPI)”. In preparation. NNK carried out all experiments, performed data analysis and interpretation, contributed to study design, and drafted and edited the manuscript. JJG and OCS contributed to the methodology and experimental design. PJF contributed to experimental design, data interpretation, and drafting and editing the manuscript.
I consider myself very lucky to have pursued my graduate studies in the Department of Medical Biophysics at Western University. Throughout the course of the past five years, I have had the privilege of collaborating with exceptional scientists, mentors who were truly outstanding, and remarkable peers who all have had a significant impact on both my personal growth and my professional achievements.

First, I would like to thank my supervisor, Dr. Paula Foster, for providing me with guidance and mentorship that shaped me into the scientist I am today. Thank you for taking a chance on me 5 years ago and continually inspiring me with your expertise and calm mentorship style. I will be forever grateful for the countless opportunities to share our findings around the world, build relationships with those in the field, and mentor students throughout my degree. Thank you for always supporting my interests in and out of the lab, including my love for chihuahuas and desire to teach. Your support and encouragement have been instrumental in my success, and I cannot thank you enough for all that you have done.

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appreciated and I am thankful for the time spent discussing my personal and professional goals over the years. Thank you to Dr. Savita Dhanvantari for leading the Molecular Imaging Graduate Program for the first 2 years of my studies and for providing a welcoming environment for interesting discussion.

I would like to thank previous Foster Lab members who were critical to my success and growth in the lab that I now consider my close friends. Dr. Katie Parkins, I cannot express how thankful I am to have met you. Your friendship means more than you know, and I could not have arrived at this stage without your unwavering support, patio dinners, and wise advice. Veronica Dubois, I am so glad we were able to start this graduate journey together and become good friends through the process. Thank you for all of the troubleshooting and support during the long experiment days. Kierstin Melo, I truly admire your calm approach to science and life and will always be grateful for your friendship and willingness to take the best photos with me during conference travel.

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A special thanks goes to my family, grandparents, aunts and uncles, and cousins. You all have always believed in me since the day I was born, no matter what, and been a source of strength on days when I didn’t believe in myself. I especially want to thank my parents for helping me move all over the place during the last 10 years of schooling, for providing meals and extra help when studying and when experiments were busy, and for letting me read the medical books we had in the house which inspired me to pursue this path.

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<td>$^{19}$F</td>
<td>Fluorine-19</td>
</tr>
<tr>
<td>231BR</td>
<td>MDA-MB-231BR</td>
</tr>
<tr>
<td>231BR-HER2+</td>
<td>MDA-MB-231BR-HER2+</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>arbitrary units</td>
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<tr>
<td>B$_0$</td>
<td>main magnetic field</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<td>BLI</td>
<td>bioluminescence imaging</td>
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<tr>
<td>bSSFP</td>
<td>balanced steady-state free precession</td>
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<tr>
<td>BW</td>
<td>bandwidth</td>
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<tr>
<td>CCD</td>
<td>charge coupled device</td>
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<tr>
<td>CK5</td>
<td>cytokeratin 5</td>
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<tr>
<td>CLUT</td>
<td>colour look-up table</td>
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<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CTC</td>
<td>circulating tumour cells</td>
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<td>CTE</td>
<td>concomitant tumour enhancement</td>
</tr>
<tr>
<td>CTR</td>
<td>concomitant tumour resistance</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>Abbreviation</td>
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<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>FA</td>
<td>flip angle</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FFR</td>
<td>field free region</td>
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<tr>
<td>FIESTA</td>
<td>Fast Imaging Employing Steady-State Acquisition</td>
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<tr>
<td>FLI</td>
<td>fluorescence imaging</td>
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<td>FLuc</td>
<td>Firefly luciferase</td>
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<td>FOV</td>
<td>field of view</td>
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<td>FWHM</td>
<td>full width at half maximum</td>
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<td>GE</td>
<td>gradient echo</td>
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<tr>
<td>GEMM</td>
<td>Genetically engineered mouse model</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
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<td>Gd</td>
<td>gadolinium</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
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LPS  lipopolysaccharide
MFP  mammary fat pad
MMTV-LTR  mouse mammary tumour virus-long terminal repeat
MPI  magnetic particle imaging
MPIO  micron-sized iron oxide particle
MRI  magnetic resonance imaging
Mn  manganese
mT  milli-Tesla
NMR  nuclear magnetic resonance
NOD/SCID  nonobese diabetic/severe combined immunodeficiency
NSG  NOD/SCID/IL1Rg<sup>−/−</sup>
PAI  photoacoustic imaging
PBS  phosphate buffered solution
PCR  polymerase chain reaction
PDX  patient-derived xenograft
PEG  polyethylene glycol
PEI  polyethylenimine
PET  positron emission tomography
PFC  perfluorocarbons
PMAO  poly(maleic anhydride-alt-1-octadecene)
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<tr>
<td>PPB</td>
<td>Perl’s Prussian Blue</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
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<td>PSF</td>
<td>point spread function</td>
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<tr>
<td>RF</td>
<td>radiofrequency</td>
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<tr>
<td>ROI</td>
<td>region-of-interest</td>
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<td>SCID</td>
<td>severe combined immunodeficiency disease</td>
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<tr>
<td>SE</td>
<td>spin echo</td>
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<td>SNR</td>
<td>signal to noise ratio</td>
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<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<td>superparamagnetic iron oxide particles</td>
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<td>transfection agents</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TR</td>
<td>repetition time</td>
</tr>
<tr>
<td>US</td>
<td>ultrasound</td>
</tr>
<tr>
<td>USPIO</td>
<td>ultra-small superparamagnetic iron oxide particles</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>vascular endothelial growth factor A</td>
</tr>
<tr>
<td>WAP</td>
<td>whey acidic protein</td>
</tr>
<tr>
<td>WBRT</td>
<td>whole brain radiotherapy</td>
</tr>
</tbody>
</table>
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Chapter 1

1 Introduction

This thesis employs magnetic resonance imaging (MRI), bioluminescence imaging (BLI), and magnetic particle imaging (MPI) to characterize animal models of brain metastatic breast cancer, to visualize and quantify the metastatic disease burden, and to develop iron-based cell tracking methods for detection of this disease. This introductory chapter discusses breast cancer metastasis, preclinical models of breast cancer, imaging technologies, and use of iron particles for cancer cell tracking to provide background and motivation for the studies presented in this thesis.

This chapter contains sections previously published. Section 1.3 and Section 1.4.2 contain excerpts from: Knier NN, Pellizzari S, Zhou J, Foster PJ, Parsyan A. “Preclinical models of brain metastases in breast cancer”. Biomedicines. 10(3), 667. March 2022. Section 1.4.4 contains excerpts from Sehl OC, Gevaert JJ, Melo KP, Knier NN, Foster PJ. “A perspective on cell tracking with magnetic particle imaging”. Tomography. 6(4):315-324. December 2020. Sections are reproduced with permission (Appendix B).

1.1 Motivation and Overview

Breast cancer remains a leading cause of mortality among women worldwide, with brain metastases resulting in extremely poor prognosis [1]. A major roadblock in advancing the treatment of breast cancer brain metastases is the scarcity of representative experimental preclinical models and methods studying its progression in vivo [2]. There is a clear need to characterize brain metastatic models of breast cancer in vivo, which can be accomplished through use of imaging modalities and optimizing cell tracking techniques.
In this thesis, experimental imaging technology and noninvasive cell tracking techniques were developed for the detection and monitoring of the progression of brain metastatic breast cancer in mouse models. In Chapter 2, we explore the differences in cancer cell fate and tumour progression in different mouse models with iron-based cellular magnetic resonance imaging and demonstrate substantial differences in cancer development between mice with different immune competencies. In Chapter 3, we develop a novel method for efficiently labeling patient-derived xenograft (PDX) breast cancer cells with iron oxide particles and concurrently validate our labeling method and monitor the subsequent tumour growth with BLI and MPI. In Chapter 4, we evaluate different iron oxide particles for cancer cell tracking with MPI and further, evaluate changes in their magnetic performance following cell labeling. Finally, Chapter 5 summarizes the overall conclusions and significance of this thesis. Additionally, experimental limitations and for future work and directions are discussed.

1.2 Metastatic Breast Cancer
Breast cancer is one of the most common cancers seen in women, with a lifetime risk of developing breast cancer being 1 in 8. This disease accounts for approximately one-third of cancer diagnoses and is the second leading cause of cancer death among women [1]. Mortality associated with this disease is caused most frequently by metastasis, which is the spread of cancer from the primary tumour to other distant locations in the body. In breast cancer, these locations most often include the brain, bone, lungs, and lymph nodes [4]. Up to 30% of all breast cancer cases will result in eventual metastasis [5]. Early detection of metastatic disease has been challenging, and typical methods of treatment often fail.
Clinically, the heterogeneity amongst breast cancer is typically understood and classified by the varied expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). When tumours do not express any of these three markers, it is classified as “triple-negative” breast cancer, a subtype that presents a major unmet clinical need due to its aggressive behaviour, poor prognosis, and lack of actionable targets for treatment [6]. While distant metastasis can occur in all subtypes of breast cancer, the triple-negative subtype has the highest rate of metastasis and shortest overall survival [7]. This thesis is focused on studying and monitoring the progression of human breast cancer cell lines that have been classified as the “triple-negative” subtype and have the propensity to metastasize to the brain.

1.2.1 Metastatic Colonization

Both clinical and experimental observations indicate that metastasis is a very inefficient process. The number of circulating tumour cells (CTCs) shed from the primary tumour and sampled from the blood is much greater than the number of distant metastases that develop [8], and only half of patients develop metastases from the cancer cells that do survive after reaching distant organs [9]. This is consistent with experimental mouse models, as the vast majority of cell populations injected fail to form macro or micro metastases [10]. More specifically, studies in mice have shown that overt metastases may only arise from 0.02% of the injected cells into circulation [11].

The metastatic process, termed the ‘metastatic cascade’, encompasses several sequential steps which must be completed successfully to allow for a metastatic tumour to grow (Figure 1.1). Cells that are shed from the primary tumour must first intravasate into the bloodstream or nearby lymphatic vessels and survive in circulation. If cells survive, they
may extravasate into distant tissues and organs. Once cells have arrived in the new location, these metastatic cells must initiate and sustain proliferation as well as promote angiogenesis within the tissue for adequate blood supply. All these steps must be accomplished while cells simultaneously evade immune cell responses and apoptotic signals. For metastatic cells to successfully colonize in the brain, cells must arrest in the microcirculation, penetrate the blood-brain barrier (BBB), and extravasate into the parenchyma, leptomeninges, or more rarely, the choroid plexus [12].
Figure 1.1 Schematic of metastatic colonization.

Metastatic colonization is comprised of several steps required for cells to invade distant locations. Cancer cells must leave the primary tumour and intravasate into the bloodstream and survive in circulation. These circulating cells then disseminate to distant locations and extravasate into the surrounding tissues. Created with BioRender.com.
1.2.2 Mechanisms of Metastasis

Many traditional models and theories of metastasis have suggested that metastatic progression follows a unidirectional process, whereby cancer cells leave the primary tumour and unidirectionally spread through circulation to seed distant sites or lymph nodes. However, both biological and clinical observations have led to an emerging idea of multidirectional metastasis, where cells that leave the primary tumour can either return to the primary tumour, travel through the circulation as a CTC, seed new distant sites, or seed other established metastatic lesions. This theory, described in 2006 by Norton and Massagué [13], was later termed tumour “self-seeding” or “self-homing”, and is thought to be attributed to a leaky vasculature and permissive tumour microenvironment that can contribute to both CTC recruitment and growth [14,15]. This rapidly evolving area of study has been shown by in animal models of breast cancer [16,17], colorectal cancer [18], and melanomas [19]. Kim et al. demonstrated that using the MDA-MB-231 model of breast cancer, as well as a colon and melanoma cell line, primary tumour lesions were actively seeded by CTCs from secondary tumour lesions, from distant metastatic tumours, and following direct injection of cancer cells into the bloodstream [20]. Radiotherapy, a localized therapy used for killing primary breast tumours, has also been shown to attract circulating tumour cells as a result of granulocyte-macrophage colony stimulating factor (GM-CSF) produced by irradiated cells [21]. More recently, our group has shown that CTCs labeled with iron particles and administered into the vasculature can be detected returning to the primary tumour with MPI in a mouse model of breast cancer [22]. Understanding this self-seeding model has novel therapeutic implications for breast cancer, as steps in the seeding process could be interrupted or exploited to disrupt metastasis and
further growth. Indeed, our group and others have shown that CTCs can be engineered to express both imaging and therapeutic agents, thereby reducing metastatic burden and allowing for in vivo visualization [23–25].

Two additional mechanisms of metastasis that have been studied are known as concomitant tumour resistance (CTR) and concomitant tumour enhancement (CTE). CTR is the phenomenon whereby the primary tumour can restrict the growth or establishment of distant secondary tumours. This was first described by Ehrlich in 1906 [26], who proposed that nutrients that would be required for secondary tumour growth are consumed by the primary tumour. Later in 1967, Gershon et al. supported these observations by demonstrating that hamsters with primary lymphoblastic lymphoma tumours inhibited secondary tumour growth to a much greater extent than control animals [27]. The relevance and convincing observations of this effect have been observed in both breast cancer patients [28] and murine models of breast cancer. This was shown by our group, where a model of CTR was generated in mice by implanting a primary tumour and producing brain metastases from the MDA-MB-231 and 231BR cell lines, respectively [29]. In this study, the primary tumour inhibited the development of brain metastases as detected with MRI. Studies have also observed CTE, whereby the presence of a primary tumour can stimulate the growth of other tumours. This phenomenon was first described by Ando et al. in 1979 [30]. In this work, they demonstrated that after the transplantation of fibrosarcoma into mice, significantly more secondary tumours developed in the lungs compared to controls after inoculation of cells intravenously. Our group has demonstrated this effect in a murine model of breast cancer using multimodality cellular and molecular imaging, when Parkins
and colleagues visualized that the presence of a 4T1 primary tumour significantly enhanced the development of brain metastases in immune competent mice [31].

### 1.2.3 Dormancy

Dormancy is a stage in the progression of cancer whereby residual disseminated disease is present but a patient with cancer remains asymptomatic, and was originally ideated following clinical observations of cancer recurrence after a prolonged period of undetectable disease [32].

There are two types of dormancy: cellular dormancy and tumour dormancy. Once an individual metastatic cancer cell arrives at a distant site, one of three things can happen: (1) it may die, (2) it may proliferate to form micrometastases, or (3) it may remain viable but non-proliferative (Figure 1.2) [33]. This third option describes the cellular dormancy model, where dormancy is defined as individual cells that exit the cell cycle and exist in a G0/G1 arrested state. If solitary cancer cells proliferate to form micrometastases, they can again experience one of three fates: (1) they may die, (2) they may continue to proliferate and form macrometastases, or (3) they may persist as dormant micrometastases, where this tumour dormancy is defined as a balance between proliferation and apoptosis within the cell population such that there is no net growth [34]. This balance is maintained by factors such as the vasculature and surrounding immune cells. The factors that trigger proliferation following dormancy at both the single cell and micrometastasis stage remain poorly understood, however, chronic inflammation, angiogenesis, and other microenvironmental cues may play a role in this phenomenon [35].
Figure 1.2 Fate of an individual metastatic cancer cell.
Individual cancer cells will experience one of three fates upon intravasating into the bloodstream: A) apoptosis, or cell death, B) proliferation to form metastases, or C) cellular dormancy, where the cell remains viable but is not proliferating. Created with BioRender.com.
Naumov et al. developed a significant model of dormancy using a breast cancer cell line to compare non-angiogenic and angiogenic cancer progression [36]. Non-angiogenic MDA-MB-436 cells were subcutaneously injected in severe combined immunodeficiency disease (SCID) mice, where the majority of tumours remained microscopic for a prolonged period of time, and very few became angiogenic. These angiogenic tumours were isolated and reimplanted, and developed tumours at a much faster rate than non-angiogenic cells, suggesting that prolonged dormancy may be due to reduced angiogenesis. Albrengues et al. demonstrated the use of lipopolysaccharide (LPS) in mice as a preclinical method of studying the effects of chronic inflammation in MCF-7 and D2.0R breast cancer cells injected intravenously after a period of dormancy and showed that local inflammation in the lung could directly drive the awakening of disseminated dormant cancer cells [37].

Dormant cells present a substantial therapeutic problem, as they may be responsible for cancer recurrence after what was thought to be a successful treatment [38–40]. There are several challenges complicating the study of solitary dormant cancer cells, including the limited number of metastatic dormancy models available, and the difficulties associated with monitoring dormant cancer cells in vivo. Although solitary cancer cells have been identified in numerous cell lines, there are no definitive markers that can identify dormant cancer cells in vivo and it can be difficult to observe them due to the damage of tissues caused by proliferating metastases. An important study in 2002 demonstrated that the D2.0R and D2A1 breast cancer cells labeled with fluorescent nanospheres could be imaged with intravital video microscopy [41]. For both cell lines, solitary dormant cancer cells could be identified, and those that retained the fluorescent label over time and lacked the proliferative marker Ki67 were classified as ‘dormant’, as proliferating cells lose the label
over time with cell division. Several groups including ours have used cellular MRI technology to show that the retention of iron oxide particles in nonproliferative cells can longitudinally tracked and monitored in vivo [42–45]. More information on this technique can be found in the subsection “Iron-Based Cellular MRI”.

1.2.4 Brain Metastasis

Considerable progress has been made towards understanding the biology of breast cancer, leading to the development of effective treatments. However, up to 30% of breast cancer metastases occur in the brain, with a risk of death within a year reaching 62% [46–48]. The most common sites of breast cancer brain metastases include the frontal lobe, cerebellum, and to a lesser extent, the brain stem [49]. A substantial decrease in the quality of life is observed in breast cancer patients with brain metastases due to the neurological sequelae of the disease [50]. Since breast cancer is a molecularly heterogeneous disease, some of its molecular variants exhibit higher rates of brain metastases, such as those that do not express ER and PR receptors but are positive for HER2. These HER2+ subtypes tend to metastasize to the brain at a higher (~50%) rate and lead to ~6 months median survival [51–53]. While the reason for this increased metastatic potential for HER2+ cancer is likely multifactorial, it has been posited that the driving factors of brain metastases in this subtype may be attributed to interactions between HER2 and other receptors, including epidermal growth factor receptor (EGFR) and HER3 [54]. Treating metastases has been particularly challenging due to the unique anatomical and functional features in the brain. Novel therapies are being developed to improve systemic control, however, poor drug penetration of the BBB can create a sanctuary for tumour cells in the brain during treatment and lead to an increased incidence of brain metastases. The poor outcomes and failures of treatment
are also a reflection of the differences in the biology of brain metastases compared to that of the early stages of breast cancer [55,56]. These differences remain poorly understood. Hence, understanding the biology and subsequently testing novel therapeutic modalities that account for unique anatomic features of brain metastases is of clinical importance [57]. Success in developing effective treatments and understanding metastatic progression is founded on the availability of preclinical experimental models that effectively recapitulate metastases in patients and development of sensitive experimental detection methods.

### 1.3 Preclinical Models of Breast Cancer Brain Metastasis

Preclinical experimental models for breast cancer brain metastases should ideally represent a milieu in which metastases develop, capture the heterogeneity of breast cancer in patients [58], and incorporate brain anatomical nuances, such as the blood-brain barrier. The latter can cause brain metastases to become inaccessible to drugs and therefore, is an essential consideration for therapeutic testing [59]. Although in vitro microfluidics and ex vivo coculture models have been described to incorporate the blood-brain barrier [60,61], stromal cell interactions, and infiltration patterns [62], in vivo animal models remain the benchmark for preclinical models.

#### 1.3.1 Immortalized Human Brain Metastatic Breast Cancer Cell Lines

Various xenogeneic models of breast cancer brain metastasis in immunocompromised mice have been described based on human breast cancer cell lines. Some of the initially described MDA-MB-361 and MDA-MB-468 cell lines [63] have been followed in mice to study treatment responses [64], the tumour microenvironment [65], and blood-brain barrier impairment in response to the development of metastases [66]. Those models, however,
had poor selectivity for the formation of metastases in the brain. Therefore, attempts to establish models that preferentially form metastases in the brain have been made through the selection of cell populations that have a propensity to form brain metastases. One of the earliest examples of this is a mucin (MUC1) secreting MA11 cell line derivative [67], that after intracardiac injections in nude mice preferentially formed brain metastases in 87% of animals.

The most common approaches to increase the efficiency of breast cancer brain metastases formation are based on clonal selection of cell populations from parental cell lines. Yoneda et al. established a brain-seeking clone from the parental triple negative breast cancer MDA-MB-231 cell line [68]. Cells were injected intracardially into nude mice and after 3–4 weeks, cells from brain metastases were cultured in vitro and re-inoculated into mice. This procedure was repeated six times until the brain-seeking MDA-MB-231BR (231BR) cell line was established, resulting in 100% frequency of metastases to the brain and no metastases to other organs. This cell line is used to study metastatic development in Chapter 2. Additional subclones of the 231BR cell line have been developed by performing three rounds of selection and intracarotid injections in mice, resulting in the BR1, BR2, and BR3 sublines [69]. These sublines varied from the original 231BR cells in that they expressed elevated levels of VEGF-A (vascular endothelial growth factor A), which has been shown to be critical in the development of brain metastases in breast cancer [70]. Indeed, they led to the shorter survival of mice and development of more brain metastases compared to the 231BR cells. The MDA-MB-231-BrM2 subline has been established using a similar approach of intracardiac injections and clonal selection through an additional round of in vitro and in vivo culturing and led to metastases in the cerebrum, cerebellum, brainstem,
and leptomeninges [71]. Using a similar methodology but a different TNBC cell line, CN34, a CN34-BrM2 clone was described, that after intracardiac or mammary fat pad injections, metastasized to the same locations in the mouse brain [71].

Given that HER2+ has a higher propensity to metastasize to the brain [51,52], various approaches have also been used to establish HER2+ models. An MDA-MB-231BR-HER2+ (231BR-HER2+) line was developed by transducing 231BR cells with enhanced green fluorescent protein (eGFP), and then transfecting with HER2 cDNA [72]. When compared to 231BR cells after intracardiac injection, 231BR-HER2+ developed more aggressively with an increased number of large metastatic tumours. Other HER2+ brain-seeking sublines based on JIMT-1, SUM190, and BT474 lines have been described. JIMT-1-BR3 was established [73] through successive intracardiac injections, resulting in brain metastases in 100% of mice. A similar approach was utilized to establish the SUM190-BR3 HER2+ line [74]. BT474.br was established through right carotid injections of BT474 cells to select for brain-seeking cells in vivo [75]. Additionally, a combination treatment of neratinib and the c-MET inhibitor cabozantinib was tested in a model where brain-seeking SKBrM3 cells expressing high levels of c-MET and the EGFR were selected for markers of invasiveness (vimentin and ZEB1) to establish a brain-seeking SKBrM3+ subline [76]. The latter was injected into the mammary fat pad of nude mice and tumour growth was monitored with BLI. High incidences of brain and other organ metastases were observed using this subline and their occurrence and proliferation were inhibited by a combination treatment.

To address the shortcomings associated with the absence of the immune components in xenogeneic models, syngeneic models of breast cancer brain metastasis have been
developed. These models have important utility given the development of immunotherapies and their introduction into clinical practice for the treatment of breast cancer [77]. A brain metastatic subline (4T1BM), a derivative of 4T1 cells, was established by orthotopic implantation but resulted in poor brain metastatic development [78]. However, after four rounds of selection in vitro and in vivo via inoculation into a BALB/c mouse mammary gland, a 4T1Br4 subline was developed that metastasized to the brain, with a higher incidence (20%) than the parental 4T1 cell line (7%) [79]. Similarly, a 4T1Br5 subline was developed through the intracardiac injection of the parental cell line and tumours were grown out in the brain for 4-6 weeks [59]. A 4T1 cell-based model using either intracranial or intracardiac injection of luciferase-transduced 4T1 cells into mice has also been described, which resulted in higher (compared to subcutaneous injection) rates (25%) of BCBM as assessed by BLI [80].
Table 1.1 Established cell lines used in studies of breast cancer brain metastasis.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Site of Origin</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA11</td>
<td>Bone marrow</td>
<td>ER-/PR-/HER2-</td>
</tr>
<tr>
<td>MDA-MB-231BR (-BR1, -BR2, -BR3, -BrM2)</td>
<td>Pleural effusion</td>
<td>ER-/PR-/HER2-</td>
</tr>
<tr>
<td>MDA-MB-231BR-HER2+</td>
<td>Pleural effusion</td>
<td>Above, transfected with HER2</td>
</tr>
<tr>
<td>CN34-BrM2</td>
<td>Pleural effusion</td>
<td>ER-/PR-/HER2-</td>
</tr>
<tr>
<td>JIMT-1-BR3</td>
<td>Pleural effusion</td>
<td>ER-/PR-/HER2+</td>
</tr>
<tr>
<td>SUM190-BR3</td>
<td>Primary tumour</td>
<td>ER-/PR-/HER2+</td>
</tr>
<tr>
<td>BT474.br</td>
<td>Primary tumour</td>
<td>ER+/PR+/HER2+</td>
</tr>
<tr>
<td>SKBrM3+</td>
<td>Plural effusion</td>
<td>ER-/PR-/HER2+</td>
</tr>
<tr>
<td>4T1BM</td>
<td>Murine breast cancer</td>
<td>ER-/PR-/HER2-</td>
</tr>
<tr>
<td>4T1Br4, Br5</td>
<td>Murine breast cancer</td>
<td>ER-/PR-/HER2-</td>
</tr>
</tbody>
</table>
1.3.2 Patient-Derived Models of Brain Metastatic Breast Cancer

To better capture disease heterogeneity and patient treatment responses, patient tissue-derived models, such as PDX models, have been developed. PDX models involve the excision of tissue fragments or cells from a patient tumour and engrafting directly into immunodeficient animals to form tumours. These systems serve as a basis for the next-generation of preclinical translational research and personalized medicine, as they have been shown to recapitulate the tumour microenvironment and represent the biology and tumour heterogeneity seen clinically [81–83]. Therefore, there has been growing interest in applying these models to studies of breast cancer brain metastasis.
Figure 1.3 Development of patient-derived xenograft models.
Tumours are harvested during a biopsy or surgery and directly implanted into immunodeficient mice. Tumours are then expanded from mouse to mouse and can then be used for a variety of applications such as characterization, therapeutic testing, or biobanking. Created with BioRender.com
Generation of metastases by PDX models is relatively challenging. Orthotopic PDX in the mammary fat pad of NOD/SCID/IL1rg−/− (NSG) mice have been described where 1/7 models developed brain metastases that represented <2% of overall metastases [84]. The low rates of brain metastasis reported in this study could be partially attributed to the use of an orthotopic model and to the histological analysis of brain metastasis that could fail to detect small tumour deposits.

A triple-negative PDX cell line from patient brain metastases, F2-7, has been developed by Contreras-Zárate and colleagues [85], where tumour cells were dissociated to a single cell suspension and labeled with luciferase *in vitro*. Subsequently, labeled cells were injected into the mammary fat pad of NSG mice and BLI was used to monitor the presence of metastases. This group also developed the triple-negative BM-E22-1 model, where tumour tissue was propagated through implantation into the mammary fat pad of NSG mice. After two generations, tumours were dissociated to single cells and injected intracardially. MRI-detectable brain macrometastases were observed in 50% of mice and later, histological analysis showed micrometastases in 100% of mice after 8–12 weeks post-injection [85]. The WHIM2 and WHIM5 models were established from a triple negative primary tumour and brain metastases, respectively, from the same patient, and were implanted into the mammary fat pads of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice that had been humanized through fibroblast injections [86]. In a later study, xenografts from WHIM2 were cultured *in vitro* for further expansion and subsequent transplantation by intracardiac injections to generate brain metastases [87]. In this model, 100% of mice developed brain metastases, however, animals also developed metastases in the liver (50%), lung (33%), ovaries (83%), and
adrenal glands (25%). This study highlighted the importance of studying cancer therapeutics, such as carboplatin and cyclophosphamide, at different metastatic sites, as drug efficacy was shown to vary depending on metastatic location.

Alternative methods to establish patient-based animal models of breast cancer brain metastasis have been described, including those using direct implantation into the animal brain. In order to provide a more direct pathway to disseminating cells within the brain and to extend survival of the experimental model by minimizing metastatic growth elsewhere in the body, novel protocols of intracarotid injection [88] and intracranial injections [89–91] have also been developed. It is important to note that intracranial injections and implantations of cancer cells are associated with several major limitations, such as injection-induced BBB disruption and generation of only one single large tumour lesion.

1.3.3 Mouse Models for Cancer Studies

Mouse models are most commonly used to study the progression of breast cancer experimentally, with several variations existing in immune competencies and genetic background. Syngeneic mouse models, also known as allograft tumour systems, describe mice that retain intact immune systems and are used for studies of tumour tissues from the same genetic background. A common example of this for studies of breast cancer brain metastasis would be studying the murine derived 4T1 or 4T1-Br5 cell lines in BALB/c mice, which have an immune-competent system. This approach is often used to model the progression of breast cancer brain metastasis and responses to anti-cancer therapies in the presence of a functional immune system [31,59,92]. The main drawback to this approach is the lack of clinical translation and representation of human tumour heterogeneity as a result of using murine derived models and immune systems as a platform.
Immune-deficient or compromised mice are also common in breast cancer research in order to facilitate xenograft transplantation models, which involve the use of human-derived cancer cells or tumours. In these systems, immune compromised mice are used to prevent rejection of human cells by the host, with various strains being develop with differing immune deficiencies. In breast cancer research, commonly used strains are the nude, NOD/SCID, and NSG mice [93]. Nude mice have a genetic mutation that results in an absent or deteriorated thymus, causing a T-cell deficiency [94]. NOD/SCID mice have both the SCID mutation and NOD background, resulting in impaired T and B cell development and impaired NK cell function [95]. NSG mice are currently one of the most immunodeficient strains, lacking T cells, B cells, NK cells, as well as most innate immune cell functions [96], and allow for the successful implantation of human tumour fragments and PDX cell lines. These mouse models are ideal for monitoring growth and metastasis and provide a platform for evaluating anti-cancer therapies but may not be ideal for studies of immunotherapies due to their lack of immune function. Further experimental considerations for commonly used mouse strains are described in Table 1.1. In this thesis, both NSG and nude mice are used to study the development and progression of the MDA-MB-231BR cell line (Chapter 2), where it was demonstrated that cancer develops differently in mice with varying immune competencies. In Chapter 3, we demonstrate the development of a primary tumour formed from a PDX breast cancer cell line in the NSG mouse.
Table 1.2 Experimental considerations for commonly used murine models in preclinical breast cancer research.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Immune System</th>
<th>Considerations</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Competent immune system</td>
<td>Suitable for immunological studies, but low mammary tumour incidence</td>
</tr>
<tr>
<td>Nude</td>
<td>T cell depleted, innate immunity intact</td>
<td>Lack of hair makes subcutaneous tumour development observable, high radiation tolerance, engrafts cell lines</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>T and B cell depleted, reduced NK cell activity, low levels of innate immunity</td>
<td>Engrafts many tissues efficiently, sensitive to radiation</td>
</tr>
<tr>
<td>NSG</td>
<td>T and B cell depleted, impaired NK cell and macrophage function and impaired innate immunity</td>
<td>High success for tumour engraftment, long lifespan for longitudinal study, poor tolerance for radiation</td>
</tr>
</tbody>
</table>
Genetically engineered mouse models (GEMMs) are mice that through genetic editing techniques have specific genetic alterations, whereby a mouse may express or lack specific genes. GEMMS of breast cancer have contributed considerably to cancer research, allowing for further understanding of cancer genes, genetic pathways, and therapeutic strategies. Three commonly used promoters in GEMMs of breast cancer are the mouse mammary tumour virus-long terminal repeat (MMTV-LTR) [97], C(3)1 [98], and the whey acidic protein (WAP) promoter [99], which all drive and increase the expression of oncogenes. For studies of metastasis, GEMMS can be ideal for investigating and modeling all stages of the metastatic cascade, as cancer is studied following spontaneous tumour initiation. Because of this, two significant limitations of GEMMs are their low incidence of metastatic spread and the long latency period before the development of metastases.

1.3.4 Methods for Generating Breast Cancer Metastases

Spontaneous models of metastasis allow the spread of cancer from the primary tumour to distant secondary locations. In breast cancer models, this occurs most often following the injection of cells or tissues into the mouse mammary fat pad, or what is otherwise known as an orthotopic implantation. The main advantage of this model in cancer research studies is that this allows for the full metastatic cascade to be modeled and recapitulated in the mouse, increasing its clinical relevance [100]. Additionally, orthotopic models provide a site where cancer cells can interact with the tissue of origin, impacting the tumour microenvironment, initial invasion, and eventual extravasation into circulation [101]. The main drawback of this method is that formation of brain metastases may only occur after long periods of latency, if at all, and spontaneous metastases in breast cancer are typically only seen in allograft models, such as the 4T1 cell line. In this thesis (Chapter 3), we inject
patient-derived breast cancer cells into the mouse mammary fat pad to develop an orthotopic tumour that develops in the mouse breast tissue environment.

There also are several experimental models of metastasis that allow for the arrest, growth, and clearance of cells in particular locations of interest to be examined following the injection of cells into circulation (Figure 1.3). For breast cancer, the most common experimental is intravenous injection of cells, which generally leads to the formation of lung metastases, as cancer cells are rapidly trapped in the lung microvasculature. Another method is intracardiac injection, where cells are delivered into left ventricle of the mouse heart and is often performed with ultrasound guidance. This method of injection leads to the arrest of ~3.5-9.5% [102] of cells into the brain microcirculation, which may lead to the development of metastases in this location as well as the bone or liver. Intracarotid and intracranial injections are also often used in the formation of brain metastases, however, these methods are much more invasive and require advanced surgical techniques. In contrast to spontaneous models, the drawback to these experimental models is that they do not mimic the initial steps of the metastatic cascade, however, they are useful for studying metastatic progression and treatment response at various metastatic sites. In this thesis (Chapter 2), we use intracardiac injection with ultrasound guidance to study the arrest, proliferation, and clearance of a brain trophic breast cancer cell line (231BR) in the brain of NSG and nude mice.
Figure 1.4 Common methods of generating metastases for preclinical studies of breast cancer brain metastasis.

Breast cancer cells can be injected into either the mammary fat pad for orthotopic implantation, injected into the tail vein for intravenous administration, or injected into the left ventricle of the mouse heart with ultrasound guidance to administer cells into arterial circulation. Created with BioRender.com
1.4 Preclinical Imaging of Breast Cancer

Preclinical imaging methods are critical for developing a better understanding of the progression of breast cancer metastasis and for the dynamic monitoring of therapeutic responses. In experimental models, several imaging modalities have been widely used for the detection of breast cancer, including magnetic resonance imaging (MRI), iron-based cellular magnetic resonance imaging, magnetic particle imaging (MPI), bioluminescence imaging (BLI), fluorescence imaging (FLI), photoacoustic imaging (PAI), ultrasound (US), positron emission tomography (PET), computed tomography (CT), and single photon emission computed tomography (SPECT). Due to each modality having their own strengths and limitations, multiple imaging modalities are often used in a complementary way to acquire multi-layered information.

In this thesis, we use magnetic resonance imaging, iron-based cellular magnetic resonance imaging, bioluminescence imaging, and magnetic particle imaging for cell tracking of breast cancer brain metastasis which will be discussed further in this section.

1.4.1 Anatomical MRI

MRI is a non-invasive and non-ionizing imaging technology that provides superior soft tissue contrast, high spatial resolution, and allows for detailed anatomical reference in three dimensions. Therefore, MRI is well suited for the diagnosis and longitudinal monitoring of breast cancer and treatment response, with anatomical MRI often used to identify tumour masses and provide information about tumour sizes and volumes. Conventional MRI is based on the properties of hydrogen atoms ($^{1}$H or protons), which, as part of tissues that contain water or lipids, makes up approximately 75-80% of the human body [103]. In the
absence of an external magnetic field, these positively charged particles spin in a random orientation. To generate signal with MRI, a large magnetic field \((B_0)\) which is constantly present causes a small proportion of the protons to align with \(B_0\), resulting in a net magnetization vector that precesses around \(B_0\) (Figure 1.5A). A radiofrequency (RF) excitation pulse is then temporarily applied which changes the orientation of the net magnetization vector away from its alignment with \(B_0\). When the RF excitation pulse is turned off, these protons then dephase and relax via transverse relaxation and longitudinal relaxation, returning to their alignment with \(B_0\) (Figure 1.5B). This generates energy that produces detectable signal that is translated into an image. Because protons in different tissues relax at different rates (i.e., have different \(T_1\) and \(T_2\) values), this can be exploited to compare healthy tissues to pathological tissues. This is extremely valuable in cancer research, as malignant tissue generates different contrast, and therefore, MRI can be used to determine information about the spread and composition of tumours within the body. Additional discussion of basic MRI physics is described by McRobbie et al. in the textbook “MRI from Picture to Proton” [103].
Figure 1.5 Mechanisms of MRI.
A) The precession of protons in response to an external magnetic field (B_0). B) Protons are excited upon the application of an RF pulse, with both longitudinal and transverse relaxation of the protons occurring following its removal. Created with BioRender.com
In general, MR images have contrast that are weighted by proton density (PD) and two characteristic times called spin-lattice relaxation time (T₁) and spin-spin relaxation time (T₂). Proton density is related to the number of hydrogen atoms present in a particular volume, and therefore, fluids like blood and cerebral spinal fluid have higher proton density than other tissues like bone. T₁ weighted contrast is dependent on how long the protons take to return to equilibrium and realign with the \( B_0 \) after the application of an RF pulse. Contrast for T₂-weighted imaging illustrates the rate of dephasing after the RF pulse. Generally, images have different contrast which will depend on either PD, T₁ or T₂. T₁ and T₂ are dependent on different types of tissues, with fluids having long T₁s, and fat-based tissues having short T₁s. Fluids also have longer T₂s, while fat-based tissues have short T₂s. In PD images, tissues with high PD will result in higher signal intensities, creating bright pixels in the image. In T₁-weighted images, tissues with short T₁s are associated with high signal, however, in T₂-weighted images, tissues with long T₂ produce higher signal intensities. Contrast agents can further change the signal intensity by shortening either the T₁ or T₂ times of surrounding tissues. Commonly used contrast agents to shorten T₁ are paramagnetic agents like gadolinium (Gd) and manganese (Mn) to produce bright regions in a T₁-weighted image [103]. Superparamagnetic agents like iron oxide particles can shorten T₂, which appears as dark regions of signal loss, and are used throughout this thesis.

There are two principal types of pulse sequences that control contrast in MR images: spin echo (SE) and gradient echo (GE) sequences. SE sequences use two RF pulses, which creates an echo that measures signal intensity. This type of pulse sequence can produce very high-quality images that can be T₁-, T₂- or PD-weighted but can take a relatively long time to acquire. GE sequences use a signal RF pulse followed by a gradient pulse, and can
also produce T₁-, T₂- or PD weighted images. GE sequences are typically much faster to acquire; however, they are influenced by inhomogeneity of the main magnetic field. The combined T₂ and magnetic field inhomogeneity is known as T₂*, as pure T₂ contrast is not possible in GE sequences [103].

In the Foster lab, we use the balanced steady-state free precession (bSSFP) MR pulse sequence to simultaneously image the development of tumours in the brain and track cancer cells labeled with iron oxide particles. This sequence is also known as Balanced Fast Field Echo (balanced FFE), True Fast Imaging with Stead-State Precession (True FISP), or Fast Imaging Employing Steady-State Acquisition (FIESTA) based on the MRI vendor. This pulse sequence is a type of GE sequence that produces T₂/T₁-weighted image contrast due to the magnetization “steady state”. It is also one of the most signal to noise ratio (SNR) efficient (high signal per unit time) sequences [104]. Using this sequence, tumours appear as regions of hyperintensity (bright pixels), and iron-labeled cells appear as regions of signal void.

Anatomical MRI has been widely used to study tissues in the brain, as grey and white matter both have distinct T₁ and T₂ from each other and cerebral spinal fluid, providing excellent anatomical imaging. Since cancerous lesions in the brain have differing T₂ values from grey and white matter in the brain, they are also detected with MRI. Breast cancer brain metastases [43,105,106] and other brain tumours [107,108] have been studied extensively with MRI due the superior soft tissue contrast and ability to detect small tumours without ionizing radiation. Various contrasts generated with MRI can be used to specifically highlight the tumour boundary associated edema, necrosis, and hemorrhage, and three-dimensional imaging allows for tumour volumes to be measured and tracked over
time. Post-Gd images can provide excellent sensitivity to detect pathological processes that break down the BBB, such as leaky tumour neovasculature, tumours, and inflammation. In turn, this allows for Gd penetration of the BBB, resulting in signal enhancement in images post-administration. This strategy has been used in many preclinical cancer models to evaluate BBB permeability associated with brain tumours, and effects of radiotherapy and chemotherapy [42,109].

1.4.2 Iron-Based Cellular MRI

Cellular MRI is a tool that allows for the in vivo and dynamic tracking of cells though the use of various contrast agents, most commonly iron oxide particles. This technique has been used to image cells non-invasively and provide an understanding of cellular and therapeutic administration [110–112], visualize cellular uptake and migration [113–115], as well as observe the longitudinal fate of cells [43,45,116,117].

Iron oxide nanoparticles are the most used contrast agents for MRI cell tracking and this technology has been thoroughly developed and reviewed [118–122]. The strong magnetic susceptibility of these iron particles leads to local magnetic field inhomogeneities and shorter T₂ and T₂* relaxation times of nearby nuclei. In turn, this causes a region of signal loss in MR images which is much larger than the size of the cells, a so-called “blooming” effect [123]. In MR images, iron can appear as large areas of hypointensity or discrete, punctate regions of signal voids. This leads to very high cellular sensitivity and even single iron-labeled cells can be detected under optimal conditions [43].

Many different varieties of iron oxide particles exist and for MRI they have been grouped into three categories based on their hydrodynamic diameter; micron-sized iron oxide
particles (MPIO) that are the largest with diameters \( \geq 1 \mu m \), superparamagnetic iron oxide particles (SPIO) which have diameters ranging from \( \sim 50-150 \) nm, and ultra-small superparamagnetic iron oxide particles (USPIO) with diameters of \( \leq 50 \) nm [118]. These agents can label cells through two main techniques that have been developed with an aim to maximize cellular uptake of iron without impacting cellular function. The first technique involves pre-labeling cells in culture and can often be achieved through simple co-incubation [43,44,123,124]. Other strategies used to pre-label cells include incubation with transfection agents, electroporation, or magnetofection [125,126]. The second labeling strategy is achieved through systemic injection of iron for \textit{in situ} cellular labeling, which is primarily used for imaging of endogenous macrophages and other immune cells due to their phagocytic activity [117,127]. In Chapter 2 and 3, we pre-label breast cancer cells and patient-derived breast cancer cells through simple co-incubation using an MPIO. In Chapter 4, we pre-label breast cancer cells through simple co-incubation and incubation with transfection agents with a variety of different iron particles. Following administration of iron labeled cells or systemic injection and cellular uptake, iron particles are diluted in the progeny of proliferative cells and therefore labeled cells become undetectable after repeated cell divisions [45]. In contrast to proliferative, non-proliferative cancer cells do not dilute the SPIO and can be identified by MRI as persistent signal voids [42,45].

Iron-based cellular MRI has been used to study different models of disease and track a variety of different cell types, including cancer cells, stem cells, and immune cells [43–45,110,124,128–130]. Importantly, previous work has shown that labeling of cells with iron has very few negative effects on cellular function and viability. For cancer cells, it has
been shown that iron labeling also has no significant effect on cellular proliferation, apoptosis, or metastatic development [43].

Iron-based cellular MRI has been used to study breast cancer brain metastases to study the arrest, proliferation, clearance, and retention of disseminated cancer cells in the brain. The main advantage of this technique for tracking the fate of cancer cells in vivo is that it has high cellular sensitivity. In 2006, Heyn and colleagues demonstrated that solitary MDA-MB-231BR cells could be visualized in the mouse brain for the first time with in vivo cellular MRI [43]. This was achieved by pre-labeling the brain trophic cell line with iron, injecting labeled cells into the left ventricle of the mouse heart, and imaging with MRI. Discrete signal voids which represent iron-label cells were co-registered with confocal microscopy images to validate their findings and confirm the ability to visualize individual cells. Additionally in this study, the retention of iron in non-proliferative cells was exploited to simultaneously track the fate of both proliferative and non-proliferative cell populations in the brain. This subpopulation of non-proliferative cancer cells is thought to represent “quiescent” or “dormant” cancer cells and may proliferate to form metastases in the future. Clinical relevance of this finding is emphasized by studies of whole brain radiotherapy (WBRT) in MDA-MB-231BR-HER2 models [42], where WBRT prevented almost all tumor growth in the brain; however, MRI illustrated persisting signal voids due to non-proliferative, iron-retaining cancer cells over time.

While iron-based cell tracking has proved advantageous for cell tracking, this technique does have its limitations. For proliferative cells, such as with cancer cell tracking, dilution of iron particles during repeated cell division results in a loss of detectability by MRI [45,131]. However, this could be exploited in studies of cancer dormancy, as non-
proliferative or dormant cancer cells may retain iron over time and be detected. Additionally, iron-based MRI lacks specificity due to regions in the body that are naturally hypointense, such as the lungs. This could result in signal voids caused by iron-labeled cells to be indistinguishable in images and undetectable. Several studies also demonstrate the limitation of bystander cell uptake of iron-labeled cells following transplantation and cell death [128,132–135]. In turn, this may lead to low specificity following uptake of iron particles by phagocytic immune cells. Another limitation to consider is quantification of the presence of iron-labeled cells with MRI. Current approaches are challenging and are semi-quantitative in nature. These methods include counting individual signal voids, calculating the volume of signal loss, determining the fractional signal loss, measuring T2/T2* relaxation rate, and calculating the percentage of black pixels throughout the brain [115,136–139], the latter of which was used in Chapter 2.

In this thesis, Chapter 2 demonstrates how iron-based cellular MRI technology can be used to visualize the fate of brain metastatic breast cancer cells in nude and NSG mice, where we saw substantial differences in tumour development and metastatic distribution.

1.4.3 Bioluminescence Imaging

BLI is a valuable preclinical imaging technique that allows for the detection and tracking of viable cells longitudinally. Since it was first described in 1995 [140], many groups have used this imaging technology for the tracking of a variety of cell types in vitro and in animal models. BLI has often been used as a complementary modality for cell tracking of cancer cells, as this form of optical imaging provides a highly sensitive and low-cost method for the detection of live cell populations, providing insight on biodistribution and proliferative potential of this disease [141]. In order to track cells, cells must be engineered to stably
express a luciferase transgene. Many bioluminescent systems have been identified in nature and described; however, the most commonly used reporter is *Firefly* luciferase (FLuc), which reacts with the corresponding substrate, D-luciferin. This enzymatic reaction requires adenosine triphosphate (ATP) as a cofactor and so the BLI signal produced is directly proportional to the number of viable cells at a particular location. To acquire images using BLI, mice are first administered the substrate by injection to produce signal from the engineered cells through the enzymatic reaction. This reaction produces light through oxidation of the substrate, and photons are collected by a cooled charge coupled device (CCD) camera (Figure 1.6).
Figure 1.6 Schematic of bioluminescence imaging protocol.

Prior to imaging, cells are engineered to express a luciferase transgene, which can then be implanted into small animal models. BLI requires the administration of an appropriate substrate which reacts with luciferase. The enzymatic reaction for *Firefly* luciferase (Fluc) is shown here. In the presence of O$_2$ and ATP, Fluc catalyzes the oxidation of D-luciferin into oxyluciferin, and light is emitted as a byproduct. The emitted light is detected by a cooled charged-coupled device (CCD) camera. Created with BioRender.com.
Images are then immediately obtained for approximately 30 minutes until peak signal has been reached. The signal generated can then be overlayed onto a photographic image, CT, or X-ray for anatomical reference. Images can then be analyzed to determine the relative number of viable cells in a given location, which is pertinent for preclinical studies of cancer metastasis and assessing therapeutic responses. A main advantage for using BLI for this application is the fact that BLI provides a high throughput method for assessing the growth, dissemination, and viability of cancer cells over time. This has been applied to preclinical models of breast cancer metastasis, where primary tumours or spontaneous metastases can be monitored longitudinally. For example, Jenkins and colleagues demonstrated that BLI could be used to noninvasively compare the tumour growth rate and metastatic potential of two luciferase expressing cell lines derived from the triple-negative MDA-MB-231 cell line [142]. Following intracardiac injection of the luciferase expressing subclones, multiple metastases were detected through longitudinal imaging with BLI out to 5 weeks, with no tumours appearing palpable or visible superficially. BLI is also used in conjunction with other modalities like MRI to provide complementary information on cell viability. Parkins and colleagues demonstrated that Firefly luciferase BLI could be used with cellular MRI as a multimodal imaging technique to follow the fate of breast cancer cells delivered to the brain via intracardiac injection over time [44]. While the cellular MRI provided excellent anatomical information and quantification of tumour volumes in the brain, BLI provided a more holistic view of the cancer progression, allowing for a direct measure of cell viability in this model. In this thesis, we use a luciferase expressing patient-derived xenograft model of breast cancer brain metastasis to track the growth of a mammary fat pad tumour in vivo with longitudinal BLI.
1.4.4 Magnetic Particle Imaging

MPI is an emerging imaging modality that was first presented in 2005 by Weizenecker and Gleich [143]. Later, Connolly and Goodwill developed alternative MPI scanners with the same principals but different reconstruction approaches at the University of California, Berkeley [144]. In collaboration with Phillips, Bruker Biospin released the first preclinical scanner to market in 2013 [145]. This was followed by the founding of Magnetic Insight, Inc. who was the second company to release a preclinical scanner in 2016. MPI directly detects the nonlinear magnetization of SPIOs in response to an alternating applied magnetic field [146,147] and is built around a gradient magnet system. Two opposing electromagnets form strong gradient magnetic fields (in the order of T), and a field free region (FFR) is created where these gradient magnetic fields nearly cancel out. The gradient field (also known as the selection field) saturates the magnetization of all SPIOs except for those SPIOs present at the FFR. The FFR is shifted over an imaging volume, by changing the current through the electromagnets, to produce an image. When the FFR traverses a location containing SPIOs, the SPIO’s magnetization changes nonlinearly in response to the drive field. This induces a voltage that is detected via a receiver coil, and the resulting signal can be assigned to the instantaneous FFR location to reconstruct the final image and achieve spatial localization [148]. This is illustrated in Figure 1.7. The voltages induced are linearly proportional to the number of SPIOs at the FFR location, enabling quantification of SPIOs. MPI cell tracking has the potential to address many of the limitations presented by iron-based cell tracking [149]. First, as the MPI signal is generated only when the magnetic moments of the SPIOs rotate in response to the applied fields, there is no signal from tissue. This imbues MPI with a positive “hot-spot” contrast that
provides spatial localization without ambiguity. Second, MPI has high sensitivity, as the signal derives from the direct detection of the electronic magnetization of SPIO, which is 108 times larger than the nuclear magnetization of protons seen in MRI [150]. Third, the MPI signal is linearly quantitative with SPIO concentration, and therefore, the number of SPIO-labeled cells can be calculated [151]. Currently, the highest cellular sensitivity to date was achieved by Song et al., who developed MPI-tailored particles that allowed for the detection of 250 cells in vivo [152]. The shortcomings of MPI include a relatively low spatial resolution, compared to MRI, and the requirement that anatomical images must be acquired separately with a different imaging modality, commonly MRI or CT.
Figure 1.7 Schematic of MPI Physics.

A) The gradient magnetic field creates a field-free-region (FFR) where SPIOs are unsaturated and can rotate freely to generate a signal in response to an alternating applied magnetic field. Particles in the field gradient are saturated and do not generate signal. SPIOs can be characterized by MPI relaxometry, which measures the net magnetization and MPI relaxation rate of SPIO. B) The output is the derivative of the Langevin function, called the point-spread function (PSF). Created with BioRender.com
For MPI, spatial resolution is defined as the ability to distinguish two different signals with the same intensity in space. This is driven primarily by the gradient strength and the physical and magnetic properties of the SPIO. If the gradient strength is increased, the FFR is narrower, and thus, greater spatial resolution can be achieved as generated SPIO signal is assigned to a narrower space. This comes at the cost of lower sensitivity due to less iron being contained within the FFR. Likewise, a decrease in the gradient strength results in a larger FFR, leading to lower resolution and higher sensitivity. With respect to the magnetic properties of the SPIO, resolution strongly depends on SPIO relaxation. In response to the applied drive field, the dynamics of SPIO magnetization changes are influenced both by Néel relaxation and Brownian relaxation [153,154]. Néel relaxation refers to the internal flip in magnetization of the particle (on the order of nanoseconds), whereas Brownian relaxation refers to the physical rotation of the particle (on the order of microseconds). While both relaxation mechanisms jointly contribute to particle relaxation, in general, SPIOs with smaller core sizes are dominated by Néel relaxation, while larger core sizes are guided mainly by Brownian relaxation, as larger particles may experience increase drag, slowing their magnetization response [155]. While the Langevin model of superparamagnetism predicts a cubic improvement of spatial resolution with core diameter, a study by Tay et al. determined experimentally that resolution increases with increasing core sizes up to around 25 nm, after which size the effects of Brownian relaxation influence SPIO relaxation more prominently [156].

Particle sensitivity in MPI refers to the lowest mass of SPIO per imaging unit. For MPI cell tracking, the aim is to optimize cellular sensitivity, which refers to the lowest number of SPIO-labeled cells detected per imaging unit. The sensitivity of MPI depends on both
particle and scanner specific factors. Particle factors include the strength of the particle magnetization (stronger magnetization improves MPI signal) and the rate of particle relaxation at the FFR (faster change in magnetization leads to higher MPI signal). With cell tracking, the efficiency of the iron oxide particle cell labeling is also important, as more iron per cell leads to higher sensitivity. There are also scanner-specific factors that affect sensitivity. This includes increasing the amplitude of the excitation field, as a higher excitation field amplitude will excite SPIOs more rapidly, resulting in a faster change in magnetization and inducing a larger voltage in the receiver coil. Decreasing the gradient strength can also improve sensitivity by creating a larger FFR, which will result in more iron within the FFR. Signal averaging is another approach which may increase MPI sensitivity, although this has not been studied in depth.

While it is well established that MPI sensitivity and resolution are closely related to the type of SPIO, the ideal SPIOs for MPI are still under investigation. In early MPI cell tracking, commercially available SPIOs used for MRI were evaluated, including ferucarbotran and ferumoxtyol. Ferucarbotran improved MPI characteristics [110,157,158], and it has been used in MPI studies of mice to detect mesenchymal stem cells [110,157,159–161], neural stem cells [161], neural progenitor cells [162], pancreatic islets [163], T-cells [164], and macrophages [151,158,165]. Although widely used, ferucarbotran is no longer considered optimal for MPI because it has a bimodal size distribution, predominantly containing small cores ~5 nm in diameter (70%) with a small fraction (30%) of multicore aggregates with an effective size of 24 nm [166]. The individual cores are too small to magnetize significantly and so the MPI signal predominately originates from the clustered multicore structures. Approaches to improve
MPI sensitivity include increasing the fraction of these larger aggregates [166] or by fractionation of ferucarbotran [167]. Lastly, the synthesis of homogeneously distributed single-core SPIOs with optimized core diameters is being investigated [168]. Optimizing SPIOs expressly for MPI is emerging as a powerful area of research and will be critical for improving sensitivity and spatial resolution [156].

MPI relaxometry is commonly used as a first step to characterize SPIOs. MPI relaxometry measures the net magnetization and MPI relaxation rate of SPIOs by turning off the selection field and applying a negative magnetic field and then a positive field, and back. SPIOs in a sample are driven from a negative magnetic saturation to positive, and vice versa. The output is called the point spread function (PSF). The signal intensity, or height, of the PSF reflects the sensitivity of the SPIO. The full-width half maximum (FWHM) relates to the spatial resolution of the SPIO [169]. A narrower tracer response indicates superior spatial resolution, and a greater signal intensity per mass of iron indicates superior sensitivity (Figure 1.8). Relaxometry has significant value for testing SPIOs before use with MPI as certain SPIOs may be more suitable than others depending on the particle characteristics and the application they will be used for. In this thesis, we evaluate iron oxide particles for MPI cell tracking using MPI relaxometry to assess their sensitivity, resolution, and magnetic performance after cell labeling (Chapter 4).
Figure 1.8 Comparison of particle performance using the RELAX module.  
A) Peak signal intensity from each particle can be compared to evaluate particle sensitivity. B) Normalized curve to maximum signal value allows for the comparison of the FWHM to evaluate particle resolution. Created with BioRender.com.
Applications of MPI in cancer research have focused on cancer cell labeling with SPIOs, visualizing tumours by injection of tracers *in vivo*, and visualization of inflammatory cells within the tumour microenvironment. Early utilization of these techniques have been applied to studies of breast cancer brain metastasis, including a study by Melo and colleagues, where MDA-MB-231BR and 4T1BR5 cells were labeled *in vitro* with iron oxide particles and imaged in the mouse brain using complementary MRI and MPI [124]. In this study, they showed that MPIO-labeled cells could be detected in the mouse brain following intracardiac injection more readily than cells labeled with VivoTrax, however, imaging was only performed at one timepoint. This method was also implemented by Parkins et al., who labeled breast cancer CTCs with iron oxide particles and sensitively detected tumour self-homing in a mouse model with MPI [22]. Other studies of breast cancer metastasis have also been investigated. Wang and colleagues detected metastatic 4T1 cancer cells in lymph nodes with MPI and fluorescence molecular imaging by developing a novel breast tumour targeting SPIO tracer [170]. Spontaneous metastases were also observed using multimodality MPI and BLI following the implantation and development of primary tumours from 4T1BR5 cells expressing Akaluc labeled with Synomag-D [171]. To investigate and target cells in the tumour microenvironment, Makela et al. was the first to evaluate two SPIOs for visualization of tumour-associated macrophages in a 4T1 breast cancer model with MPI [158]. Later, Makela et al. combined MPI, BLI and CT to track MPIO-labeled 4T1BGL cells that developed as a primary tumour and subsequent metastases, as well as free iron in the liver and lymph nodes [172].
1.5 Purpose of Thesis

This thesis uses iron-based cellular MRI, BLI, and MPI to monitor the development of breast cancer brain metastasis in mouse models over time and investigate the influence of cellular uptake on iron-based agents. The objectives of this work were to compare the progression of metastases in mouse models with varying immune competencies and to develop a platform to study the growth of patient-derived brain metastases. The final objective was to investigate SPIOs for cancer cell tracking and how cellular uptake of SPIOs by breast cancer cells influences MPI sensitivity and resolution.

1.5.1 Hypothesis

1. There will be an increase in the overall tumour growth in the brain and potential body metastases resulting from the MDA-MB-231BR cell line in the severely immune-compromised NSG mice compared to nude mice. Nude mice will have preferential tumour growth in the brain, with no metastatic growth elsewhere as detected with cellular MRI.

2. Heterogeneous breast cancer cells derived from patient brain metastases can be efficiently labeled with iron oxide particles and tracked over time with BLI and MPI following mammary fat pad injection to provide complementary measures of cell viability and iron content.

3. Labeling breast cancer cells with iron oxide particles with varying characteristics will result in a reduction of the magnetic performance of particles for MPI cell tracking as assessed with MPI relaxometry.
In Chapter 2, iron-based cellular MRI was used to noninvasively track the fate of breast cancer cells from their initial arrest in the brain to the formation of tumours in the nude and NSG mouse models. This work explores the differences in tumour development from the same cell line between animal models with varying immune competencies. This chapter was published in Clinical & Experimental Metastasis (Knier NN et al., “Comparing the fate of brain metastatic breast cancer cells in different immune compromised mice with cellular magnetic resonance imaging” Clinical & Experimental Metastasis 2020 37(4), 465-475).

In Chapter 3, we developed a novel method to label cells derived from a patient brain metastasis and used BLI and MPI to track the growth of these cells as a primary tumour over time. In this work, we demonstrated efficient iron labeling of the patient-derived cells and complementary measures of proliferation and iron content of the tumour was assessed with BLI or MPI, respectively. This work was published in the Journal of Biological Methods (Knier NN et al., “A method for the efficient iron-labeling of patient-derived xenograft cells and cellular imaging validation” Journal of Biological Methods 2021 8(3), e154).

In Chapter 4, MPI relaxometry and imaging was used to evaluate the impact on the magnetic performance of SPIOs following cell labeling to understand SPIO features that produce higher sensitivity and resolution for in vivo cell tracking. We demonstrated that certain SPIO experience a performance reduction following labeling of breast cancer cells, which may impact their utility for cell tracking applications. This chapter is currently in preparation for submission to Nanoscale.
1.6 References


Chapter 2

Comparing the fate of brain metastatic breast cancer cells in different immune compromised mice with cellular magnetic resonance imaging

Purpose: Metastasis is the leading cause of mortality in breast cancer patients, with brain metastases becoming increasingly prevalent. Studying this disease is challenging due to the limited experimental models and methods available. Here, we used iron-based cellular MRI to track the fate of a mammary carcinoma cell line (MDA-MB-231-BR) in vivo to characterize the growth of brain metastases in the nude and severely immune-compromised NOD/SCID/IL2rg−/− (NSG) mouse. Methods: Nude and NSG mice received injections of iron-labeled MDA-MB-231-BR cells. Images were acquired with a 3T MR system and assessed for signal voids and metastases. The percentage of signal voids and the number and volume of metastases were quantified. Ex vivo imaging of the liver, histology, and immunofluorescence labeling was performed. Results: Brain metastases grew more rapidly in NSG mice. At day 21 post cell injection, the average number of brain tumours in NSG mice was approximately four times greater than in nude mice. The persistence of iron-labeled cells, visualized as signal voids by MRI, was also examined. The percentage of voids decreased significantly over time for both nude and NSG mice. Body images revealed that the NSG mice also had metastases in the liver, lungs, and lymph nodes while tumours were only detected in the brains of nude mice. Conclusion: This work demonstrates the advantages of using the highly immune-compromised NSG mouse to study breast cancer metastasis, treatments aimed at inhibiting metastasis and outgrowth of breast cancer metastases in multiple organs, and the role that imaging can play toward
credentialing these models that cannot be done with other in vitro or histopathologic methods alone.

2.1 Introduction

Breast cancer is a leading cause of death in women mainly due to the propensity of breast tumours to metastasize to regional and distant sites, such as the lymph node, lung, liver, bone and brain [1]. The incidence of brain metastases is increasing due to the introduction of more sensitive diagnostic methods and improved systemic therapies leading to improvements in extra-cranial control and survival [2,3,4]. Breast cancer is a disease with a number of subtypes and patients with metastatic ‘triple-negative’ breast cancer tend to develop brain metastasis at a high rate [5,6]. For the HER2 amplified subtype, the frequency of brain metastasis has been reported to be as high as 50% [7].

Once a metastatic cancer cell arrives in the brain one of three things can happen: (1) it may die, (2) it may proliferate to form micrometastases, or (3) it may remain viable but dormant (‘non-proliferative’) [8,9]. If the solitary cells proliferate to form micrometastases, they may again experience one of three fates: (1) they may die, (2) they may continue to proliferate and form macrometastases, or (3) they may persist as “dormant” micrometastases, where dormancy is defined as a balance between proliferation and apoptosis within the cell population such that there is no net growth [9,10]. The factors that tip the balance between dormancy and proliferation are poorly understood. Both dormant single cells and dormant micrometastases are believed to be sources of cells that contribute to tumour recurrence [11]. Dormant cancer cells also present a substantial therapeutic problem; since they are quiescent, they are non-responsive to current therapies which target proliferating cells [8].
Currently, only a handful of models specific to breast cancer brain metastasis have been described and even fewer allow for *in vivo* investigation of cancer cell dormancy. Both murine and human cancer cell lines have been developed to mimic as many steps in the metastatic cascade as possible. A well characterized murine breast cancer cell line is the 4T1-BR5 cell line, a highly tumourigenic and invasive cell line that has undergone multiple rounds of selection to preferentially grow in the mouse brain [12]. There are several advantages to using a murine breast cancer cell line to study brain metastasis, as growth and maintenance is easy and relatively inexpensive, and it can be grown in immune-competent mice, which is of particular interest for studying this disease in a way that recapitulates the tumour microenvironment [13].

A small number of human breast cancer brain metastatic cell lines have also been developed, including the commonly used MDA-MB-231BR (231BR), MDA-MB-231BR-HER2, MA11, JIMT1-BR3, and SUM190-BR3 cell lines. The MDA-MB-231BR cell line has been particularly well characterized for studying the progression of brain metastases in nude mice. Nude mice are the most commonly used immune-deficient strain for models which use human cell lines. They have a genetic mutation that causes a deteriorated or absent thymus, resulting in a lack of T cells [14]. The 231BR cell line grows selectively in the brain of nude mice, without metastatic growth seen in other distant organs [15]. Human breast cancer cell lines are one of the most widely used models to study the metastatic growth of cancer *in vitro* and *in vivo*, as they have been used to provide extensive insight into the characteristics of human cells and can be used for high throughput screening of various drugs [16]. There are, however, significant limitations to using human and murine breast cancer cell lines, as the quick progression *in vivo* can limit opportunities for adequate
therapeutic testing, and the growth of these cells in vitro prior to establishment in a mouse can cause changes in the genetic composition due to clonal selection [17]. Many groups have shown that these genetic changes result in these models failing to maintain tumour heterogeneity, which is now recognized as a critical element for developing personalized treatments [18,19,20]. Studying breast cancer with immunocompetent mice also has limitations, as tumour latency and growth can be variable and slow [21]. There is also increased difficulty in establishing human tumours successfully in these mice. Immune-compromised mice lack a comparable tumour microenvironment to clinical tumours and fail to provide a realistic result of interactions with the natural immune response, particularly in the case of studying anticancer therapeutics [22].

In more recent years, researchers have moved towards studying breast cancer and its subtypes with patient-derived xenografts (PDX), which allow for the growth of human primary breast cancer tissue that has been recently resected from consenting patients into immune-compromised rodents [23]. NOD/SCID/IL1Rg−/− (NSG) mice are the preferred strain of mice to engraft a PDX, as they are highly permissive to growing breast cancer metastasis, and resembles the metastatic pattern seen in human patients. NSG mice are the most immunodeficient mouse strains to date, lacking T cells, B cells, NK cells, and have defective macrophages and dendritic cells [24,25,26]. Even fewer PDX models have been developed specific to breast cancer brain metastasis, including the novel F2-7 and E22-1 PDX cell lines [27], as well as others who have established low passage PDX models of breast cancer brain metastasis and those that implant fresh tumour tissue directly into the rodent brain [28].
The D2.0R and related D2A1 mouse mammary tumour cells have been well characterized as model systems for tumour cell dormancy. In mice, D2.0R/R cells invade distant metastatic sites, remain as single quiescent cells for prolonged periods of time, and occasionally proliferate into metastatic tumours. D2A1/R cells have a much shorter dormancy period before forming metastases in the lung, liver, and other organs [29]. A human breast cancer cell line, MCF-7 has also been studied to examine the mechanisms of dormancy, however it is poorly metastatic [30].

For all of these brain metastasis models—cancer cell growth, metastasis and dormancy are typically studied using methods such as histology, flow cytometry, immunohistochemistry, and fluorescent microscopy [31]. While these techniques provide useful information about molecular and cellular markers and morphology, they are limited to studying this disease after endpoint has been reached. There is a clear need to characterize brain metastatic models of breast cancer in vivo, which can be accomplished through use of imaging modalities and cell tracking techniques.

Cellular magnetic resonance imaging (MRI) combines the ability to obtain high resolution MRI data with the use of iron oxide nanoparticles for labeling specific cells, thereby enhancing their detectability [32, 33]. The presence of the iron in cells causes a distortion in the magnetic field and leads to abnormal signal hypo-intensities in iron-sensitive images (T2*-weighted images are most often used). Areas containing iron-labeled cells therefore appear as regions of low signal intensity on MR images, creating negative contrast [34]. We have previously shown that it is possible to use cellular MRI to track iron-labeled 231BR cancer cells in the nude mouse brain [35,36,37]. Proliferative cancer cells lose the iron label over time; as the cells divide, the particles are apportioned to daughter cells and
eventually some cells contain too little iron to be detectable by MRI. As brain metastases form, changes to the tissue result in the tumour appearing brighter than the surrounding brain in MRI. Non-proliferative cancer cells retain the iron particles and can be detected over long periods of time [37].

In this study, we used these cellular MRI techniques to characterize and compare the growth of 231BR brain metastases and the persistence of iron-retaining cancer cells in nude and NSG mice. Our goal was to evaluate the NSG mouse as a model for breast cancer brain metastasis and dormancy.

2.2 Methods

2.2.1 Cell Culture and MPIO Labeling Procedure

Brain trophic human breast cancer cells (MDA-MB-231BR) expressing green fluorescent protein (GFP) were maintained with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (complete DMEM) at 37 °C and 5% CO₂. 2 × 10⁶ of these cells were seeded and allowed to adhere for 24 h. To iron label these cells, cells were supplemented with 25 μg Fe/mL of micron-sized iron oxide particles (MPIO) (0.9 μm diameter, ~63% magnetite, labeled with Flash Red; Bangs Laboratory, Fishers, IN, USA) in 10 mL of complete DMEM in a T75 cm² flask. Following this, cells were washed once with phosphate buffered solution (PBS) within the flask and trypsinized with 0.25% Trypsin–EDTA. After, cells were harvested and washed three additional times with PBS in the flask to thoroughly remove unincorporated MPIO prior to cell injections. Cell viability was assessed and calculated using the Trypan blue exclusion assay. To visualize MPIO labeling, labeled cells were affixed to a glass slide with a ThermoFisher Cytospin 4
cytocentrifuge and fixed with a Methanol/Acetic acid solution. Slides were then stained with a Perl’s Prussian Blue (PPB) solution and counterstained with Nuclear Fast Red. Slides were dehydrated with increasing concentrations of ethanol, cleared with xylene, and coverslipped with a xylene-based mounting medium. These PPB-stained slides were examined to assess the localization of MPIO within the cell and to determine the labeling efficiency using a Zeiss AXIO Imager A1 Microscope (Zeiss Canada, Toronto, ON, Canada). Iron oxide nanoparticles appear dark blue, and cells appear light pink in colour.

2.2.2 Animal Model and Workflow
All animals were cared for in accordance with the standards of the Canadian Council on Animal Care, under an approved protocol of the University of Western Ontario’s Council on Animal Care and housed in a pathogen-free barrier facility. Female nude mice (nu/nu Foxn1, aged 6–8 weeks, from Charles River Laboratories, Wilmington, MA) and female NSG mice (NOD.Cg-PrkdcscidIl2rgm1Wjl/SzJ, 6–8 weeks, Humanized Mouse and Xenotransplantation Facility, Robarts Research Institute, University of Western Ontario, London, ON) were first anesthetized with isoflurane (2% in 100% oxygen). Nude (n = 11) and NSG mice (n = 10) were then injected with a suspension of $1.5 \times 10^5$ MPIO-labeled MDA-MB-231BR/GFP + cells in 100μL of sterile saline and 15% Vevo MicroMarker microbubble solution (FUJIFILM, VisualSonics Inc., Toronto, ON, Canada). Cell suspension was loaded into a 100μL Hamilton syringe with a 30G needle. Cells were administered by slow intracardiac injection into the beating left ventricle of the heart with ultrasound imaging guidance on a Vevo 2100 ultrasound system (FUJIFILM, VisualSonics Inc., Toronto, ON, Canada). Cancer cell arrest was evaluated with MRI on the day of the injection (Day 0) and used to determine whether mice had a successful intracardiac
injection. Absence of signal voids in the brain indicated an unsuccessful injection, resulting in exclusion from the remainder of the study. Nude mice with successful injections had MRI performed at day 21 and day 32 post-injection. NSG mice with successful injections had MRI performed at day 21 post-injection (Figure 2.1). Additionally, 2 nude mouse and 2 NSG mice received intracardiac injections of $1.5 \times 10^5$ MPIO labeled 231BR cells to assess cancer cell arrest and metastases in the liver. One of each mouse type was sacrificed on Day 0, the other NSG mouse was euthanized on day 21 and the other nude mouse on day 32. These mice were exsanguinated, and the livers were removed for ex vivo imaging.
Figure 2.1 Experimental design for the NSG and nude mouse model.

Created with BioRender.com.
2.2.3 Magnetic Resonance Imaging

All brain and body MRI examinations were acquired on a 3.0T GE MR750 clinical MR scanner (General Electric, Mississauga, ON, Canada) using a custom-built gradient coil (inner diameter = 17.5 cm, gradient strength = 500 mT/m, and peak slew rate = 3000 T/m/s). Brain and ex vivo liver images used a custom-built solenoidal mouse brain radiofrequency (RF) coil. Body images were acquired using a 4.3 × 4.3 cm dual tuned \(^{1}\)H/\(^{19}\)F surface coil (Clinical MR Solutions, WI, USA), originally built for imaging small ROIs in humans. Mice were anesthetized with 2% isoflurane in 100% oxygen administered through a nose cone. In vivo brain and body images were acquired using a 3D balanced steady-state free precession (bSSFP) sequence [Fast Imaging Employing Steady State Acquisition (FIESTA) on a GE system] that has been optimized for simultaneous detection of signal voids produced by iron-loaded cells and hyperintense metastases. This permitted the assessment of both cell arrest and retention as well as the number and volume of metastases throughout the mouse brain. Brain images for nude mice were acquired on day 0, day 21, and day 32. Brain imaging for NSG mice occurred on day 0 and day 21. Scanning parameters were as follows: resolution = 100 × 100 × 200 μm, repetition time (TR) = 7 ms, echo time (TE) = 3.5 ms, bandwidth (BW) = 20.83 kHz, flip angle (FA) = 35°, signal averages = 2, phase cycles = 8, matrix = 150 × 150, scan time = approximately 33 min per mouse. Body images were acquired on Day 20. Body imaging parameters were as follows: resolution = 200 × 200 × 200 μm, repetition time (TR) = 4.7 ms, echo time (TE) = 2.3, bandwidth (BW) = ± 31.25 kHz, flip angle (FA) = 35°, signal averages = 2, phase cycles = 8, matrix = 250 × 250, scan
time = approximately 20 min. Day 0 *ex vivo* liver imaging parameters were acquired using a spoiled gradient echo sequence that also allows for the detection of signal voids throughout the liver. Day 32 images were acquired using the 3D bSSFP sequence to visualize tumour burden. *Ex vivo* liver samples were immersed in tubes containing Fluorinert™ FC-40 Electronic Liquid (3M, St. Paul’s, MN, USA), a fluorocarbon liquid which produces a black background in proton images. Images were taken of *ex vivo* livers acquired on day 0 for both strains, day 21 for the NSG mouse, and day 32 for the nude mouse. The scanning parameters for *ex vivo* liver images at Day 0 were as follows: resolution = 100 × 100 × 200 um, repetition time (TR) = 43 ms, echo time (TE) = 4.844 ms, bandwidth (BW) = 31.25 kHz, flip angle (FA) = 60°, signal averages = 1, phase cycles = 8, matrix = 250 × 250, scan time = approximately 23 min.

### 2.2.4 Image Analysis

Images were analyzed using open-source OsiriX image software (Pixmeo, SARL, Bernex, Switzerland), version 10.0.4 and Horos image software, version 3.3.5. Brain images were evaluated for successful cell delivery by assessment of signal voids on day 0. To quantify cancer cell arrest throughout the brain, the number of black pixels within the total brain volume was determined from day 0 MRI images. The brain was manually segmented as a region of interest and then a threshold value was determined based on the mean pixel intensity value of a representative signal void within the brain region ± 2 standard deviations. The total number of black pixels under this threshold value within the entire brain volume signal was obtained from a pixel intensity histogram. To quantify brain metastases at day 21 and day 32, tumours were counted in all image slices by a single observer. Each tumour was manually segmented, and the tumour volumes were
reconstructed using the OsiriX and Horos volume algorithm. To calculate brain tumour burden at day 21 and day 32, an ROI was drawn around the outline of the brain in each slice, and a 3D reconstruction using the OsiriX and Horos volume algorithm provided a quantification of the total brain volume. The total volume of all segmented tumours was then determined by adding each individual tumour volume measurement and calculating the percentage of the total brain volume occupied by tumours. Body images were qualitatively assessed for the presence of metastases in the liver, lung, and lymph nodes. All quantitative values were presented as the mean ± standard error. Statistical analysis was performed using Welch’s $t$ tests on GraphPad Prism version 8 software (GraphPad, San Diego, CA).

2.2.5 Histology and Immunohistochemistry

At each strain’s respective endpoints, mice were euthanized by isofluorane overdose and perfused with 4% paraformaldehyde. Brains were removed and placed into paraformaldehyde for another 24 h. Fixed brains were processed, paraffin embedded and then cut into 6 or 8 μm sections. Sliced sections were deparaffinized and stained with either hematoxylin and eosin (H&E) or immunofluorescently labeled for Ki67.

2.2.6 Hematoxylin and Eosin (H&E) Staining

Sections were washed briefly in distilled water, stained in Harris hematoxylin solution for 5 min and differentiated in 1% acid alcohol for 30 s. After washing in 0.2% ammonia for 5 min, sections were counterstained in eosin-phloxine solution for 30 s; dehydrated through 95% alcohol, 2 changes of absolute alcohol, and 5 min each. Sections were then dehydrated and cleared through 95% ethyl alcohol, absolute alcohol and xylene, and mounted with resinous medium.
2.2.7 Immunofluorescent Labeling

Ki-67 immunostaining was performed using a rat anti-Ki-67 antibody (1:400 dilution; Catalog #14-5698-82, Invitrogen). All sections were permeabilized with 0.2% Triton X-100 in PBS for 15 min and non-specific protein binding was then blocked by incubation in a commercial blocking agent (ab156024, Abcam) for 1 h at room temperature. Sections were then incubated with the Ki67 primary antibody in commercial antibody dilutant (ab64211, Abcam) at room temperature for 1 h. Negative controls (without addition of primary antibody) were performed on adjacent sections. Unbound primary antibody was washed away through three 5-min exchanges of 1xPBS. Sections were then incubated with an anti-rat Alexa Fluor-488 secondary antibody (1:300 dilution; Catalog #A-11006, Invitrogen) for 1 h. Unbound secondary antibody was washed away through three 5-min exchanges of 1xPBS. Finally, nuclei were counterstained with Hoechst 33,258 for 5 min and rinsed sections cover slipped for microscopic examination.

2.3 Results

2.3.1 In Vitro Studies

MDA-MB-231BR cells were efficiently labeled with MPIO, as demonstrated by the Perl’s Prussian blue staining of cells shown in Figure 2.2a; cancer cells appear pink, and the intracellular iron is blue. Labeling efficiency of 96.33 ± 1.20% was achieved with 97.33 ± 0.88% cell viability.
Figure 2.2 Iron labeling and injection of 231BR cells.

a) Perl’s Prussian Blue stain showing intracellular iron (blue, PPB) detected within MDA-MB-231BR cells (pink, Nuclear fast red) *in vitro*. b) Representative Day 0 image showing visualization of successful iron-labeled cancer cell delivery by intracardiac injection as regions of signal void (orange arrows).
2.3.2 In Vivo Studies

In our experience, intracardiac injection of $1.5 \times 10^5$ 231BR cells in nude mice results in brain tumour burden, significant weight loss and neurological impairments which leads to a requirement for euthanasia at 28–34 days post injection. In this study the nude mice ($n = 10$) reached experimental endpoint on day 32. In contrast, NSG mice ($n = 10$) could only be studied until day 21, at which time they had reached a weight loss greater than 15% of their body weight, resulting in extreme cachexia and anorexia. Additionally, these mice showed severe signs of neurological impairment, resulting in the inability to perform normal functions and exhibiting paralysis and circling. Post-mortem examination also revealed significant liver tumour burden, which also contributed to the early, unexpected endpoint.

On day 0, bSSFP brain images confirmed the successful intracardiac injection of MPIO-labeled cancer cells in all mice; iron-labeled cancer cells appeared as distinct regions of signal void throughout the brain due to their initial arrest in brain vasculature (Figure 2.2b). Figure 2.3a shows representative day 0 images for each mouse strain. When quantifying the percentage of the brain consisting of black signal voids, approximately $4.69\% \pm 1.71$ and $6.26\% \pm 1.86$ of the brains of NSG and nude mice contained arrested cancer cells, respectively. At each strain’s endpoint, the number of black pixels was again determined, with percentages significantly reduced to approximately $1.89\% \pm 0.57$ in NSG mice (day 21) and $2.45\% \pm 0.48$ in nude mice (day 32). While the number of arrested cancer cells significantly differs from day 0 to endpoint for both NSG ($p = 0.03$) and nude mice ($p = 0.02$), there was no significant differences between strains at each time point (Figure 2.3b).
Figure 2.3 Cancer cell arrest and clearance.

a) Balanced steady-state free precession images showing initial cancer cell arrest comparing NSG and nude mice at day 0 and clearance and retention at endpoint. The orange arrows indicate regions of signal void. b) The percentage of black pixels, representing regions of signal void was not significantly different between strains, however there was a significant decrease within strains from day 0 to endpoint, suggesting similar cancer cell arrest and clearance between nude (n = 5) and NSG (n = 6) mice.
Brain metastases grew more rapidly in NSG mice. At day 21 post cell injection, the mean number of brain tumours in NSG mice was approximately 4 times greater than in nude mice. Brain metastases were detectable in bSSFP brain images as regions of signal hyperintensity in both strains of mice (Figure 2.4a). On day 21, the mean number of tumours per mouse brain was 63.70 ± 5.37 for NSG and 15.33 ± 4.29 for nude mice (Figure 2.4b). While the nude mice had fewer tumours, they were significantly larger. The mean volume of tumours in nude mice was 0.06 mm$^3$ ± 0.01, compared to 0.04 mm$^3$ ± 0.003 for NSG mice (Figure 2.4c). At day 21, the mean tumour burden in the brain was significantly higher for NSG mice (2.39 mm$^3$ ± 0.18) compared to nude mice (0.82 mm$^3$ ± 0.35) (Figure 2.4d).
Figure 2.4 Day 21 visualization and quantification of brain metastases.

a) Balanced steady-state free precession images showing brain metastases in both strains of mice as regions of signal hyperintensity (orange arrows) at day 21. b) There were significantly more brain metastases on average detected with MRI in NSG mice (n = 10) than nude mice (n = 6) at day 21. c) The mean volume of tumours present in the brain was significantly greater in nude mice (n = 6) than NSGs (n = 10). d) The brain tumour burden of NSG mice (n = 10) was significantly higher than in nude mice (n = 6) at day 21. Data is presented as mean ± SEM. * - P < 0.05.
Nude mice (n = 10) survived longer than NSG mice and were followed out their endpoint of day 32 (Figure 2.5a). The mean number and volume of brain metastases in nude mice increased between days 21 and 32. On day 32 the mean number of brain metastases in nude mice was 39.60 ± 10.99. When comparing the mean number of metastases for NSG and nude mice at their respective endpoints there was no longer a significant difference (p = 0.07) (Figure 2.5b).

On day 32 the mean volume of brain metastases in nude mice was 0.32 ± 0.23 mm³, compared to 0.04 ± 0.03 mm³ on day 21. This was significantly higher (p ≤ 0.0001) than the mean volume of brain metastases at the NSG endpoint (Figure 2.5c). Accordingly, nude mice had significantly more tumour burden than NSG mice (Figure 2.5d), which was a reversal from the previous timepoint at day 21.
Figure 2.5 Endpoint visualization and quantification of brain metastases.

a) Magnetic resonance images of both NSG and nude mice at each strain’s respective endpoints (Day 21 for NSG, Day 32 for nude) showing regions of signal hyperintensity where brain metastases have developed (orange arrows). b) There was no significant difference between the number of brain metastases detected with magnetic resonance imaging between NSG (n = 10) and nude mice (n = 10) at endpoint. c) The tumours in the nude mice (n = 10) were significantly larger in volume than those in the NSG mice (n = 10). d) The total tumour burden within the brain at endpoint in both nude (n = 10) and NSG (n = 10) mice was not significantly different. Data is presented as mean ± SEM. * - P < 0.05.
Because NSG mice were perishing relatively early after the cancer cell injection, we performed exploratory MRI on the mouse body \((n = 1)\) to determine if metastases were developing in other locations. Notably, there was significant tumour burden detected by MRI within the liver, lungs (Figure 2.6a), and lymph nodes (Figure 2.6b). Nude mice had no MRI-detectable tumours within the body outside the brain (Figure 2.6c).

### 2.3.3 Ex Vivo Liver MRI and Histology

When NSG mice were euthanized and examined post-mortem liver tumours were very clearly visible. Because of this observation, MRI was performed on ex vivo livers removed the same day MPIO-labeled cells were injected from one nude and one NSG mouse to determine if cancer cells arrested in the liver in both strains. Discrete signal voids were visible in the day 0 images of ex vivo livers from both NSG and nude mice, suggesting the arrest of iron-labeled 231BR cells throughout the liver (Figure 2.6d, e). Next, MRI was performed on ex vivo livers removed on day 21 (NSG) or day 32 (nude) from one nude and one NSG mouse to determine if metastases formed within the liver. Ex vivo liver images obtained from the NSG mouse showed numerous regions of abnormal high signal intensity associated with liver metastases, confirming the in vivo image results (Figure 2.6f). A photo of an NSG liver clearly shows the liver metastases on the surface (Figure 2.6h). Since MRI acquisitions were 3D, images were visualized in all three dimensions and metastases were detected in all lobes throughout the entire organ. No regions of abnormal signal hyperintensity were observed in ex vivo images of the nude mouse liver (Figure 2.6g). H&E stained sections of the liver tissue from NSG mice confirmed the presence of metastases (Figure 2.6i) and ki67 staining showed that they were highly proliferative (Figure 2.6j).
Figure 2.6 Body and liver imaging with correlative histology and immunofluorescence.

a) Body imaging of an NSG mouse (n = 1) allowed for detection of significant tumour burden in the liver, lungs. b) Tumours were detected in the lymph nodes of an NSG mouse (n = 1) with magnetic resonance imaging. c) No tumours were detected with magnetic resonance imaging in the nude mouse body (n = 1). d) Ex vivo liver MRI showing initial arrest of cancer cells at Day 0 for NSG mouse. e) Ex vivo liver MRI at Day 32 showing regions of hyperintensity where liver metastases have developed in the NSG mouse. f) Ex vivo liver MRI from a nude mouse showing signal voids, representing arrested cancer cells at Day 0. g) Day 32 ex vivo liver of a nude mouse with no magnetic resonance detectable metastases. h) Photo of a representative ex vivo liver from a NSG mouse showing tumours on the exterior of the organ. i) H&E-stained section showing liver metastases that had developed in the NSG mouse.
(outlined in yellow). j) Ki67 staining showing a highly proliferative tumour within the NSG liver.
2.4 Discussion

This work demonstrates for the first time the use of in vivo longitudinal MRI based cell tracking to compare murine models of brain metastatic breast cancer. We show that there are significant differences in tumour progression for 231BR cells in NSG versus nude mice. While the initial arrest, clearance, and retention of iron-labeled cells was similar, brain metastases developed more quickly in NSG mice and NSG mice developed substantial body tumour burden, particularly in the liver.

The 231BR cell line was developed by Yoneda et al. [15] and is a brain-colonizing subline of the metastatic triple-negative MDA-MB-231 human breast cancer cell line, which was isolated by six repeated cycles of intracardiac injection and harvesting from brain metastases grown in nude mice. In our lab, the intracardiac injections are performed using ultrasound (US) guidance. Of note, was our observation by US of a thicker heart muscle surrounding the left ventricle of the NSG mouse. This anatomical observation made intracardiac injections more difficult, as the ventricular space appeared smaller with US.

The observation of signal voids in brain images acquired on day 0 was used to identify successful delivery of cells to the brain. In this study, all mice imaged had successful injections and were included in the study. Delivery of cells to a specific organ is related to the cardiac output that is delivered to that organ. The cardiac output to the brain of a mouse is ~9.5% [38]. Here, we injected 150,000 cells into the left ventricle of the heart, and therefore we can expect ~14,250 cells to be successfully delivered to the brain. Only a percentage of these cells will arrest; previous studies, however, have shown that less than 1% of cells are retained in the microcirculation of the brain after 2 h post-injection [38].
previous studies, we have used PPB staining and fluorescence microscopy to demonstrate that these signal voids correspond to the presence of iron-labeled cells in the brain [35, 36].

The size of the signal void created by iron-labeled cells is much larger than the actual cell size, due to what is known as the blooming effect. The blooming effect is a susceptibility artifact that occurs as a result of the iron oxide nanoparticle, which causes a local magnetic field inhomogeneity [34]. We have previously shown that we can detect single iron-labeled cells arrested in the mouse brain using cellular MRI [35, 39, 40]. Our image showed that the number of signal voids in the brain was similar for nude and NSG mice on day 0 and at endpoint. While we acknowledge that there is potential for immune cell uptake of the iron oxide nanoparticles that may be released by dead cancer cells, we believe that the majority of the signal voids that remain present are live cancer cells retaining iron. For example, in Parkins et al. [39] we investigated this mouse model using luciferase-positive 231BR cells and measured a strong correlation on day 0 between the number of signal voids detected in day 0 brain MR images and the brain signal measured in bioluminescence images, which is only detected from viable cells, providing evidence that signal voids represent live iron-labeled cells. In Hamilton et al. [41] we used fluorescence activated cell sorting (FACS) to successfully isolate live 231BR cells that were GFP-positive and labeled with red fluorescent (Flash Red) MPIO from the brains of mice. The GFP + Flash Red + cells were collected and expanded in vitro. The majority (~ 90%) of cells adhered to tissue culture plastic and successfully expanded, displaying the same cell morphology as the original cultured 231BR cells. This provides additional support for our claim that signal voids in MRI represent viable iron-positive cancer cells.
Our previous work has shown that the number of signal voids detected in MRI of the brain decreases over the course of the experiment, from day 0 to day 8, which is expected as the large majority of cancer cells that arrest in the brain do not survive and are cleared with time [39]. The signal voids which do persist in the brain over time are thought to represent iron-retaining, non-proliferative or dormant cancer cells. These cells have been shown to contribute to tumour recurrence [41]. It appears that both nude and NSG mice are capable of clearing dead cells from the brain.

3D MRI of the entire mouse brain at high resolution allowed us to view brain metastases in all 3 orientations and to digitally re-slice images to carefully interrogate image data. We counted and measured the volume of all MRI-detectable brain metastases. Brain metastases developed more quickly in NSG mice. At day 21 post cell injection, the mean number of brain tumours in NSG mice was approximately 4 times greater than in nude mice. The NK cells and the remaining innate immune cells in nude mice likely contributed to the reduced tumour growth at this timepoint. NSG mice had to be euthanized at day 21/22. Exploratory mouse body MRI on NSG mice injected with 231BR cells revealed additional tumour burden due to metastases in the liver, lungs, and lymph nodes. Metastases did not develop outside of the brain in nude mice, though cancer cells still arrest elsewhere, as demonstrated in day 0 ex vivo imaging of the liver. The 231BR cell line was developed to selectively grow distant metastases in only the brains of nude mice, and so the proliferation of metastases of this cell line in the liver demonstrates the loss of selectivity to the brain in the NSG mouse.

Our findings are in agreement with the results of other groups that show that more immune compromised mouse models are more permissive for tumour growth and metastasis
Puchalapalli et al. [45] have previously reported an increase in the metastatic burden (in liver, lungs, brain and bones) in NSG compared to nude mice that were injected with the parental 231 breast cancer cell line in an intracardiac experimental metastasis model. In this study, metastases in each organ were enumerated from *ex vivo* fluorescence microscopy images. While our findings agree with this study, our work demonstrates the advantages of using *in vivo* and longitudinal 3D MRI for the accounting of metastases throughout the whole brain, as well as volumetric analyses of individual tumours detected over time. We have used MRI previously to evaluate the growth of brain metastases in nude mice with various other human breast cancer cell lines including SUM190-BR3, JIM-T1-BR3 and MDA-MB-231-BR-HER2, as well as a human melanoma cell line called A2058 [40, 46]. In all of these prior studies, brain metastases developed more slowly, and significantly fewer brain metastases developed, compared to the growth of 231BR cells in NSG mice reported here.

Nude mice were imaged at a third timepoint. On day 32, the number of brain metastases in nude mice was similar to the number of brain metastases in NSG mice on day 21. The mean volume of the brain metastases in the nude mice on day 32 was more than 3 times greater than those in NSG mice at day 21, having had more time to develop. Overall, the brain tumour burden at necessary endpoint was significantly greater in nude mice.

### 2.5 Conclusion

In summary, high resolution cellular MRI allowed us to characterize the 231BR cell line in both the NSG and nude mouse models. We found marked differences in tumour incidence, volumes, and body tumour burden between strains. Our *in vivo* comprehensive analysis of cancer cell arrest, clearance, and tumour progression is important for
understanding the metastatic cascade of a model of breast cancer brain metastasis that can be challenging to obtain with *in vitro* or *ex vivo* methods alone. Our data demonstrates that NSG mice may provide a better model for studying human breast cancer metastasis compared to the more commonly used nude mouse model.
2.6 References


Chapter 3

A method for the efficient iron-labeling of patient-derived xenograft cells and cellular imaging validation

**Purpose:** There is momentum towards implementing patient-derived xenograft models (PDX) in cancer research to reflect the histopathology, tumour behavior, and metastatic properties observed in the original tumour. To study PDX cells preclinically, we used both bioluminescence imaging (BLI) to evaluate cell viability and magnetic particle imaging (MPI), an emerging imaging technology to allow for detection and quantification of iron nanoparticles. The goal of this study was to develop the first successful iron labeling method of breast cancer cells derived from patient brain metastases and validate this method with imaging during tumour development. **Methods:** The overall workflow of this labeling method is as follows: adherent and non-adherent luciferase expressing human breast cancer PDX cells (F2-7) are dissociated and concurrently labeled after incubation with micron-sized iron oxide particles (MPIO; 25 μg Fe/ml), with labeling validated by cellular imaging with MPI and BLI. In this study, NOD/SCID/ILIIrγ−/− (n = 5) mice received injections of $1 \times 10^6$ iron-labeled F2-7 cells into the fourth mammary fat pad (MFP). BLI was performed longitudinally to day 49 and MPI was performed up to day 28. **Results:** *In vivo* BLI revealed that signal increased over time with tumour development. MPI revealed decreasing signal in the tumours over time. **Conclusion:** Here, we demonstrate the first application of MPI to monitor the growth of a PDX MFP tumour and the first successful labeling of PDX cells with iron oxide particles. Imaging of PDX cells provides a powerful system to better develop personalized therapies targeting breast cancer brain metastasis.
3.1 Introduction

Breast cancer is one of the most common cancers seen in women, currently affecting 1 in 8 women in North America [1]. Mortality associated with this disease is caused most frequently by metastasis, which is the spread of cancer from the primary tumour to other distant locations in the body. In breast cancer, these locations often include the brain, bone, lungs, and lymph nodes [2]. For brain metastases in particular, prognosis is poor, with mean 1-year survival rates of only 20% [3], and 2-year survival rates of < 2% [4]. Additionally, the incidence of brain metastasis is increasing, as neuroimaging techniques improve and treatments that allow for longer patient survival permits more time for cells to metastasize to the central nervous system [5]. Insight into the mechanisms and pathophysiology of breast cancer brain metastasis have long relied on the use of immortalized cell lines that have been injected intracardially into the left ventricle of the mouse heart in order to selectively grow tumours within the brain. While these cell lines have been well characterized, they do not represent the tumour heterogeneity, metastatic behaviours, and histopathology seen clinically, and are unsuitable for evaluating therapies due to their fast progression in vivo [6].

Patient-derived xenografts, or PDXs, have begun to supplant traditional cell lines due to their retention of clinical biomarkers and heterogeneity from the original tumour [7]. In recent years, PDXs have been developed to grow in the mammary fat pad of NOD/SCID/IL2rg−/− (NSG) mice, with a long-term objective to advance personalized medicine. This strategy has shown exciting progress for the development of novel PDXs from brain metastases in breast cancer patients. In 2017, Contreras-Zárate et al. developed BM-PDXs to study the biology of brain metastasis and to serve as tools for testing novel
therapeutic approaches [8]. These novel PDXs retained intratumoural heterogeneity and metastatic potential, providing a clinically relevant model to study mechanisms of brain metastatic colonization and slower progression to allow for therapeutic testing. Currently, most PDX models are typically studied using methods such as histology, immunohistochemistry, and fluorescent microscopy, limiting the ability to study these models before an experimental endpoint has been reached. Tools that enable the longitudinal study of the fate of BM-PDXs would provide valuable information to characterize to the mechanisms and metastatic patterns in vivo.

Cellular imaging and cell tracking can be used to study cancer cell populations and metastatic processes in vivo. Bioluminescence imaging (BLI) with the reporter firefly luciferase (Fluc) has been widely utilized for tracking preclinical models of cancer. BLI is a valuable imaging modality as it allows for the longitudinal study of cell viability. Fluc BLI requires adenosine triphosphate (ATP) as a co-factor, and thus, the signal measured with BLI is directly proportional to the number of viable cells in a region of interest [9]. This modality is exceptionally useful when measuring treatment and therapeutic response, as cell viability may change but tumour volumes can remain unaltered.

Our group has previously shown that BLI can be complemented with iron-based cellular MRI technology to provide longitudinal measures of cancer cell viability in preclinical models [10]. Iron-based cellular MRI requires cells to be loaded with superparamagnetic iron oxide particles (SPIOs) and has shown to provide single cell sensitivity [11]. However, a limitation of this modality is that SPIOs create regions signal loss where iron-labeled cells are present in images, and so, determining the quantitation of signal loss is challenging and it is not possible to determine cell number [12]. Magnetic particle imaging (MPI) is an
emerging imaging modality that directly detects SPIOs [13], resulting in a positive signal that appears as a “hot spot” in images. The signal strength detected is linearly proportional to the number of SPIOs, allowing for quantitation [14]. Presently, MPI has been used as a cell tracking modality for immortalized cancer cell lines [15], stem cells [16-18], pancreatic islets [19], T-cells [20] and macrophages [21,22]. However, no studies exist studying the growth of patient-derived xenografts labeled efficiently with an SPIO in vivo.

Efficient iron labeling of a patient-derived xenograft cell line presents a challenge due to the mixed and heterogeneous population of cells. In this work, we report the first iron-labeling method for a patient-derived xenograft cell line and validate its utility for cell tracking with MPI and BLI.

3.2 Methods

3.2.1 Cell Line Origin and Cell Culture

A firefly luciferase and enhanced green fluorescent protein (eGFP) expressing patient-derived xenograft cell line (F2-7) was obtained from the Cittely Lab (UC Denver) and was originally developed from a triple-negative brain metastatic patient-derived xenograft [8]. F2-7 cells were maintained in T75 cm² flasks coated with collagen-I (1 mg/ml) for 2 h to encourage attachment. 12 ml of Dulbecco’s modified Eagle’s medium (DMEM)-F12 supplemented with 10% of fetal bovine serum (FBS), 1 μg/ml hydrocortisone, 100 ng/ml of cholera toxin, and 1 nM of insulin was added to flasks after collagen coating. Cells grew under 37°C and 5% CO₂. Since cells grow as both non-adherent mammospheres and adherent single cells, both populations require maintenance with each passage.
3.2.2 MPIO Labeling Procedure

On day 1, DMEM-F12, PBS, and trypsin were heated at 37°C for 30 min in a water bath. Conditioned media and non-adherent cells were collected into 15 ml conical tubes from confluent flasks of F2-7 cells. Conical tubes were centrifuged with a ThermoFisher Cytospin 4 for 5 min at 900 rpm, and the supernatant was removed and stored for future use as conditioned media. 1 ml of 0.25% Trypsin-EDTA was added to the cell pellet within the conical tube and was placed in the water bath for 2 min to trypsinize cells. In parallel, 1 ml of 0.25% Trypsin-EDTA was added to the flask with remaining adherent cells and incubated for approximately 5 min. Once adherent cells and mammospheres were dissociated, cell populations were mixed and resuspended in 5 ml of fresh media. After, the mixed population of cells were centrifuged again at 900 rpm for 5 min. The supernatant with trypsin was removed, and the cell pellet was resuspended in 2–5 ml. Cells were assessed for viability with Trypan blue exclusion assay and counted to achieve the correct volume of media to plate 2 × 10^6 cells. Cells were plated in uncoated T75 cm^2 flasks to discourage attachment with 50% fresh media and 50% conditioned media. To iron label these cells, 3 methods of iron labeling were performed to determine the most efficient labeling technique. In the first trial, cells were supplemented with 25 μg Fe/ml of micron-sized iron oxide particles (MPIO) (0.9 μm diameter, ~63% magnetite, labeled with Flash Red; Bangs Laboratory, Fishers, IN, USA), and swirled in the flask to mix. The second trial used the 50 ug Fe/ml of the same MPIO, and the third used 25 ug Fe/ml with a magnetic plate under the flask. Following this, all flasks were incubated for 24 h.

After 24 h, cells from the flasks were harvested and dissociated in accordance with the protocol in Day 1. Adherent cells were harvested and washed with 10 ml PBS in the flask
to remove unincorporated MPIO. Both cell populations were combined following
dissociation and centrifuged at 900 rpm for 5 min. This process was repeated thrice to
thoroughly remove unincorporated MPIO prior to cell injections. Cell viability was
assessed and calculated using the Trypan blue exclusion assay. A simplified visual of this
protocol is shown in Figure 3.1.
Figure 3.1 A simplified illustration of the workflow to efficiently label patient-derived xenograft cells with iron oxide particles.

Created with BioRender.com.
3.2.3 Assessing Iron Labeling

To visualize MPIO labeling, labeled cells were affixed to a glass slide with a ThermoFisher Cytospin 4 cytocentrifuge and fixed with a Methanol/Acetic acid solution. Slides were then stained with a Perl’s Prussian Blue (PPB) solution and counterstained with Nuclear Fast Red. Slides were dehydrated with increasing concentrations of ethanol, cleared with xylene, and coverslipped with a xylene-based mounting medium. These PPB-stained slides were examined to assess the localization of MPIO within the cell to determine the labeling efficiency using a Zeiss AXIO Imager A1 Microscope (Zeiss Canada, Toronto, ON, Canada). Iron oxide particles appear dark blue, and cells appear light pink in colour.

3.2.4 Animal Model and Workflow

All animals were cared for in accordance with the standards of the Canadian Council on Animal Care, under an approved protocol of the University of Western Ontario’s Council on Animal Care and housed in a pathogen-free barrier facility. Female NSG mice (n = 5) (6–8 weeks, Humanized Mouse and Xenotransplantation Facility, Robarts Research Institute, University of Western Ontario, London, ON) were first anesthetized with isoflurane (2% in 100% oxygen). NSG mice were then injected into the fourth mammary fat pad (MFP) with a suspension of $1 \times 10^6$ MPIO-labeled F2-7Luc/eGFP+ cells in 50 μL of sterile saline and 50 μL of Matrigel. Mice were imaged with BLI (n = 2) out to day 49 and MPI (n = 3) out to day 28. At endpoint, mice were sacrificed by isoflurane overdose. Tumours were excised and placed in paraformaldehyde. Ex vivo MFP tumour volumes were estimated using the following formula $= 0.52 \times \text{width}^2 \times \text{length}$ to approximate the volume of an ellipsoid (mm³). Fixed MFP tumours were then processed, paraffin embedded
and then cut into 5 μm sections with 5 sections placed on each slide. Immunohistochemistry was performed on contiguous slides for epidermal growth factor receptor (EGFR) and cytokeratin 5 (CK5) and the subsequent slide was stained with hematoxylin and eosin (H&E).

3.2.5 In Vitro BLI Procedure
To evaluate the relationship between cell number and BLI signal, cells were seeded in 24-well plates in 0.5 ml of growth medium at concentrations of $2 \times 10^5$, $6 \times 10^5$, $1 \times 10^6$ cells per well. Cells were allowed to adhere for 24 h and then 5 μl of D-luciferin (30 mg/ml) was added to the cell media 2 min prior to imaging using a hybrid optical/X-ray scanner (IVIS Lumina XRMS In Vivo Imaging System, PerkinElmer). Region-of-interest (ROI) analysis was performed for each well using Living Image Software (IVIS Imaging Systems, PerkinElmer) and data is expressed as the average radiance (photons/sec/cm²/steradian).

3.2.6 In Vivo BLI Procedure
BLI was performed on NSG mice ($n = 2$) on days 4, 28, 35, 42, and 49 using a hybrid optical/X-ray scanner (IVIS Lumina XRMS In Vivo Imaging System, PerkinElmer). Mice were anesthetized with isoflurane (2% in 100% oxygen) using a nose cone attached to an activated carbon charcoal filter. Mice received a 100 μL intraperitoneal injection of D-luciferin (30 mg/ml), and luminescent images were captured for approximately 35 min to obtain the peak signal at each imaging session.

3.2.7 MPI Acquisition
Full body MPI images of mammary fat pad tumor bearing NSG mice ($n = 3$) were acquired at days 0 (30 min post-injection), 7, 14, and 28. Images were collected on a Momentum™
scanner (Magnetic Insight Inc., Alameda, CA, USA) using the 3D high sensitivity isotropic (multichannel) scan mode. In this mode, images were acquired using a 3 T/m gradient, 21 projections and a FOV of $12 \times 6 \times 6$ cm, for a total scan time ~18 mins per mouse. Mice were anesthetized with 2% isoflurane in 100% oxygen for the entirety of the scan. 3D high sensitivity isotropic images of ex vivo tumors ($n = 2$) removed on Day 40 were acquired using the same parameters and a FOV of $4 \times 6 \times 6$ cm, for a total scan time of ~12 min.

### 3.2.8 MPI Calibration and Signal Quantification

To generate a calibration curve for converting MPI signal to iron content, samples were made with 2 μL aliquots of MPIO and 2 μL PBS and were imaged using the 3D high sensitivity isotropic parameters. In this mode, images were acquired using a 3 T/m gradient, 21 projections, and a FOV of $12 \times 6 \times 6$ cm. The following samples were scanned separately: 0.09 μg, 0.175 μg, 0.35 μg, 0.70 μg, 1.40 μg, 2.80 μg. Images were analyzed using the open-source Horos imaging software, version 3.3.5 (Annapolis, MD USA). To quantify the MPI signal in each image set, signal intensities were set to full dynamic range in order to represent the full range of signal in each specific ROI, such as the calibration samples and mammary fat pad tumours. Areas of interest were then segmented manually, and 3D volumes were reconstructed and calculated using the Horos volume algorithm. The total MPI signal was calculated using the equation mean signal $\times$ volume (mm$^3$).

### 3.2.9 Hematoxylin and Eosin Staining

Sections were washed briefly in distilled water, stained in Harris hematoxylin solution for 5 min and differentiated in 1% acid alcohol for 30 s. After washing in 0.2% ammonia for 5 min, sections were counterstained in eosin-phloxine solution for 30 s; dehydrated through 95% alcohol, 2 changes of absolute alcohol, and 5 min each. Sections were then dehydrated
and cleared through 95% ethyl alcohol, absolute alcohol and xylene, and mounted with resinous medium.

### 3.2.10 Immunohistochemistry

EGFR and CK5 immunostaining were performed using a rabbit anti-EGFR antibody (1:200 dilution; Cat. # ab32077, Abcam) and a rabbit anti-cytokeratin 5 antibody (1:200 dilution; Cat. # ab64081, Abcam). All sections were rinsed in PBS, incubated for 10 min in PBS containing 3% hydrogen peroxide, and immersed for 30 min in blocking reagent (Dako Protein Block, S0909, Dako). Sections were then incubated with primary antibody at 4°C for 24 h followed by biotinylated horse anti-rabbit IgG secondary antibody (1:300 dilution; BA1100, Vector Laboratories) for 30 min at room temperature and rinsed in PBS for 3 changes for 5 min each. Tissue sections were processed using the (ABC) solution for 30 min (Vector Laboratories, Burlington, ON), and visualized with 3,3’-diaminobenzidine (0.5 mg/ml; Sigma-Aldrich Canada, Oakville, ON). Negative control staining (without addition of primary antibody) was performed on adjacent sections. Counterstaining of EGFR and cytokeratin 5 stained sections was performed using hematoxylin. Slides were then dehydrated through 70–100% alcohol, cleared with xylene, and cover slipped for microscopic examination.

### 3.2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 Software (GraphPad, San Diego, CA, SA). Pearson’s rank correlation was used to determine the relationship between total MPI signal and iron content. *In vivo* data was expressed as mean ± SD and analyzed with a one-way ANOVA. Differences were considered statistically significant at $P < 0.05$. 
3.3 Results

3.3.1 Cell Labeling

For each trial of labeling, F2-7 cells had varying labeling efficiencies demonstrated by a PPB stain shown in Figure 2, with cancer cells appearing pink, and intracellular iron in blue. For trial 1 with 25 μg Fe/ml of MPIO, F2-7 cells were efficiently labeled with labeling efficiency of 81.80 ± 10.14% (Figure 3.2A) and viability of over 90%. This labeling efficiency was deemed successful and the 25 ug Fe/ml of MPIO labeling procedure was used for the remainder of the study. The second trial, using the 50 ug Fe/ml of the same MPIO, resulted in a labeling efficiency of 27.51 ± 1.19% (Figure 3.2B). Finally, the third trial which used 25 ug Fe/ml with a magnetic plate resulted in a labeling efficiency of 5.55 ± 1.65% (Figure 3.2C).
Figure 3.2 Perl's Prussian Blue (PPB) stains of F2-7 cells labeled with MPIO.

A) PPB stain showing F2-7 cells labeled with 25 μg Fe/ml of MPIO, with labeling efficiency of 81.80 ± 10.14%. B) PPB stain showing F2-7 cells labeled with 50 μg Fe/ml of MPIO, with a labeling efficiency of 27.51 ± 1.19%. C) PPB stain showing F2-7 cells labeled with 25 ug Fe/ml and a magnetic plate, resulting in a labeling efficiency of 5.5 ± 1.65%.
3.3.2 Bioluminescence Imaging

F2-7/eGFP-luc cells were seeded at concentrations of $2 \times 10^5$, $6 \times 10^5$, $1 \times 10^6$ cells per well and *in vitro* BLI was performed (Figure 3.3A). A significant positive correlation was found between the number of F2-7/eGFP-luc cells and BLI signal ($R^2 = 0.9664$). Specifically, as seeded cell number increased, BLI signal also increased (Figure 3.3B). In Figure 3.3C, the BLI signal from a representative tumour is shown to increase over time with tumour development. No BLI signal was detected in any other region of the mouse body.
Figure 3.3 *In vitro* and *in vivo* bioluminescent imaging of F2-7luc/eGFP+ cells.

A) F2-7luc/eGFP+ cells seeded at various concentrations. B) A strong linear correlation is seen between cell number and BLI signal; $R^2 = 0.9664$. C) BLI signal from a representative tumour is shown to increase over time with tumour development from day 4 to day 49.
3.3.3  Magnetic Particle Imaging

A calibration line was generated to determine iron content for a given MPI signal based on the 3D, high sensitivity, isotropic parameters used to image MPIO. An example of the images of samples measured to generate calibration curves are shown in Figure 3.4A for MPIO and the calibration line generated from this data is shown in Figure 3.4B. Based on this data, we determined here was a strong linear relationship between iron content and MPI signal (arbitrary units, A.U.) for MPIO ($R^2 = 0.9836$, $P < 0.001$). The equation of the line was: MPI Signal = 69.559*(Iron Content) for MPIO. Using this relationship, iron content could be determined for a given MPI signal.
Figure 3.4 MPI calibration line generation and signal quantification.

A) Images of MPIO samples measured to generate calibration curves. B) A strong linear relationship is seen between iron content and MPI signal. The asterisks in the equation represents a multiplication sign. $R^2 = 0.9836$.  

$$\text{MPI Signal} = 69.559 \times (\text{Iron Content})$$
Figure 3.5A shows representative MPI of a tumour bearing mouse on day 0, 7, 14, and 28 post-injection of $1 \times 10^6$ MPIO-labeled F2-7 cells. MPI signal is clearly visualized in the lower, right MFP tumour, with signal decreasing over time (orange box). The mean iron content in the MFP tumours decreased significantly between day 0 ($M = 4.06 \pm 2.09 \text{ ug}$) and day 28 ($M = 0.41 \pm 0.25 \text{ ug}$) ($P = 0.0095$) (Figure 3.5B). Note that some MPI signal can also be detected elsewhere in the body. Additionally, *ex vivo* MPI was performed on tumours removed on day 40 (Figure 3.5C). A representative *ex vivo* tumour is shown in Figure 3.5D. The tumours measured 152.88 mm$^3$ and 178.36 mm$^3$, and the iron content was 0.05 μg and 0.75 μg, respectively.
Figure 3.5 In vivo and ex vivo MPI of mammary fat pad tumours.

A) Representative MPI of a tumour bearing mouse on day 0, 7, 14, and 28 post-injection of 1x10⁶ MPIO-labeled F2-7 cells, with the tumour shown in the orange box.

B) Quantification of mean iron content over time in MFP tumours decreasing significantly between day 0 (M = 4.06 ± 2.09 μg) and day 28 (M = 0.41 ± 0.25 μg) (P = 0.0095). The double asterisks represent statistical significance, indicating (P = 0.0095).

C) Representative MPI of an ex vivo tumour removed on day 40. D) A representative ex vivo tumour of F2-7 cells.
3.3.4 Histology and Immunohistochemistry

Tumours that formed from both the MPIO labeled and the unlabeled F2-7 cells retained expression of both EGFR and CK5, H&E stained sections confirmed the presence of tumour growth in the MFP (Figure 3.6).
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Figure 3.6 Expression of cytokeratin 5 (CK5) and estrogen growth factor receptor (EGFR) in MPIO-labeled and unlabeled F2-7 MFP tumours and corresponding hematoxylin and eosin (H&E) staining.
3.4 Discussion

The increasing incidence of breast cancer brain metastases and its poor prognosis has highlighted the critical need for clinically relevant models to develop new therapeutic strategies and to understand the mechanisms underlying its progression. PDX models have been used increasingly to understand the role of tumour heterogeneity in the development of novel drugs and metastatic progression, and while other groups have made exciting progress in this area, most methods employed have been performed ex vivo, through histology, or with immunohistochemistry. Alone, these do not allow for the longitudinal study of cancer progression in vivo, and therefore, tools such as cellular imaging would be extremely valuable in further investigation and characterization of these models. Additionally, only a handful of studies exist that validate the engraftment and growth patterns of PDX models with imaging. At this time, only four studies have used MRI to image PDX models of breast cancer brain metastasis. In 2016, Ni et al. monitored therapeutic response to combination therapies by both MRI and BLI of an orthoptic PDX brain metastatic model [23]. In 2017, two novel brain metastatic PDX cell lines, including F2-7, as well as BM-E22-1, were imaged to determine successful engraftment and detect metastases within the mouse brain [8]. Sharma et al. also confirmed the tumour establishment of two PDX cell lines (PDX2147 and PDX1435) with MRI after 30 days [24]. In 2020, Oshi et al. developed new implantation techniques for higher engraftment and growth rates of a PDX model and monitored tumour development with MRI [25]. Including the mentioned study by Ni et al., only three studies have used BLI to monitor cell viability in PDX models of breast cancer brain metastasis. Turner et al. used BLI to monitor the response of two basal-like, triple-negative PDX cell lines (WHIM2 and
WHIM30) to treatment [26]. Liu et al. described establishment of orthotopic mouse models of BCBM-PDXs and monitored their engraftment with BLI [27]. To address this, we developed a novel method to label luciferase expressing PDX cells with iron oxide particles to allow for the \textit{in vivo}, longitudinal cell tracking with two imaging modalities for the first time.

Our group has previously demonstrated that BLI is a valuable tool for cell tracking preclinical models of breast cancer brain metastasis, as it is able to provide longitudinal measures of tumour growth and indicates cell viability [10]. MPI is an emerging, highly sensitive imaging modality that can be used for cell tracking and offers the benefits of directly detecting iron oxide nanoparticles to allow for the quantification of iron in a region of interest. These modalities are complementary, as BLI allows for real-time monitoring of viable cells longitudinally. However, experiences tissue attenuation of emitted light. MPI addresses this by providing a quantitative method to directly measure iron content within cells longitudinally but does not provide measures of viability or proliferation. Additionally, while MPI has been used to detect cell lines of breast cancer [15,28], to date, no studies exist demonstrating the labeling of PDX breast cancer cells with iron oxide nanoparticles to allow for MPI cell tracking.

In this work, we visualized the iron labeling of the luciferase expressing F2-7-eGFP with PPB stains and demonstrated that 25 μg Fe/ml was the most successful labeling trial. The addition of the magnetic plate for labeling was likely ineffective due to the fact that the majority of these cells are in suspension during labeling, which differs from most studies which have labeled adherent cells. Previous work by our group has shown that this concentration of MPIO effectively labels immortalized cancer cells for the purposes of cell
tracking [29]. However, the methods of labeling described in this work varies from the traditional labeling protocol of adherent cells due to the differences in homogeneity and complex cell culture required to grow PDX cells. Consequently, a limitation of this method is that labeling efficiency may vary due to the mixed cell populations within established PDX cell lines. Future work will investigate labeling trials with different iron particles and whether this changes the visualization of these cells in vivo with MPI or MRI.

In this model, F2-7 mammary fat pad tumours were monitored out to day 42 with BLI. BLI signal increased over time, demonstrating that the PDX cells had successfully engrafted and were proliferating over time. In contrast, the MPI signal from the MFP decreased over time during the 28-day period. This may be related to the clearance of iron released from dead labeled cancer cells. We observed MPI signal elsewhere in the body, which may be signal from the gut and attributed to iron in the mouse feed, or signal from the liver, which may result from clearance of iron from the tumour. Wang et al. have shown that when iron-labeled islets were transplanted under the kidney capsule MPI signal is detected in the kidney on day 1 post-transplant but on day 14 MPI signal also appears in the liver, where the released iron particles accumulate [19]. These observations require more study. Future work will investigate this signal located in elsewhere in the body through ex vivo imaging and iron quantification with MPI.
3.5 Conclusion

Our group and others have used either BLI or MPI to detect iron-labeled cancer cells in vivo. However, only one study has used both modalities [29], although they performed imaging at a single timepoint in a single mouse. We have previously shown that labeling cancer cells with iron oxide nanoparticles does not significantly affect cell viability, proliferation, apoptosis, or metastatic efficiency, demonstrating that this labeling agent is an effective technique to track cells in vivo [11]. With the increased use of PDX models as a platform to study cancer metastasis and develop novel drugs and therapeutics, this method provides a reliable and efficient method to determine the fate of these cells in vivo and their response to therapeutic treatments.
3.6 References


Chapter 4

4 Evaluating superparamagnetic iron oxide particles for cell tracking with magnetic particle imaging (MPI)

Purpose: Magnetic particle imaging (MPI) is an emerging modality that detects superparamagnetic iron oxide particles (SPIOs), which can be used to label and track cells. Sensitivity and resolution of MPI depend heavily on the SPIO properties. The SPIO core size, coating, and interactions with the local environment are known to impact the MPI signal and resolution. Therefore, it is important to evaluate SPIO performance with free SPIO in solution and with SPIO labeled cells. Here, we evaluate the MPI performance of several SPIOs alone and after cell labeling, with a goal of further understanding what particles are best suited for cell tracking applications.

Methods: MPI relaxometry and 2D images were acquired for SPIOs alone (free iron), following labeling of MDA-MB-231BR cells, and after mixing with transfection agents (TAs). Perl’s Prussian Blue (PPB) stains were performed to assess labeling efficiency. Average peak MPI signal (sensitivity) and full width at half maximum (FWHM) values (resolution) were determined using known iron content and compared between groups.

Results: MPI tailored SPIOs, specifically Synomag-D (50 nm and 70 nm) and PMAO2, lose their initial advantage of high sensitivity as free SPIOs after cell labeling. When comparing free Synomag-D, Synomag-D mixed with TAs, and cells labeled with Synomag-D and TAs, we show that TAs reduce the MPI signal and that this is magnified once in cells. MPIOs did not show a loss of MPI signal following cell labeling. Reduction in signal for Synomag-D-PEG particles was less than that of the plain Synomag-D particles. There was a reduction in resolution for all particles other than MPIO1 and Molday-G after cell labeling.

Conclusion: This work demonstrates that the magnetic behaviour of SPIOs can be strongly influenced by the local environment.
and cell labeling process. Evaluating SPIOs will provide further understanding of \textit{in vivo} magnetic performance and ideal SPIOs for cell tracking applications.

4.1 Introduction

In Chapter 4, SPIOs were evaluated for cell tracking by magnetic particle imaging (MPI). As described in Chapter 1, cell tracking with MRI has some limitations. Notably, the inability to quantify iron content and cell number. MPI has the potential to provide quantitative information allowing estimates of cell number, and a long-term goal in our lab is to use MPI to quantify metastatic cancer cells in the brain in mice \textit{in vivo} (described in Chapter 5). A previous MSc student in our lab, Kierstin Melo, explored the use of MPI for detecting and quantifying cancer cells \cite{1} labeled with the micron-sized iron oxide particles (MPIO) used in Chapters 1 and 2. Although these iron-loaded cells could be detected in the brain, the resolution was poor, and sensitivity was limited. Therefore, our next objective was to investigate different SPIOs for cancer cell tracking with the overall goal of determining the optimal SPIOs for \textit{in vivo}, longitudinal studies of brain metastatic breast cancer.

Cell labeling with superparamagnetic iron oxide (SPIO) particles has been widely used for tracking cells \textit{in vivo} using magnetic resonance imaging (MRI). Various cell types including mesenchymal stem cells \cite{2–4}, progenitor cells \cite{5}, T lymphocytes \cite{6}, dendritic cells \cite{7}, cancer cells \cite{8}, and pancreatic islets \cite{9,10}, have been labeled with SPIOs and tracked with MRI. The iron label has minimal impact on cell function or phenotype at a wide range of iron loading levels. This technique is highly sensitive, permitting the imaging of single cells \textit{in vivo} \cite{11,12} under ideal conditions. However, SPIOs produce negative contrast in MRI, or signal loss, that is indirectly detected through its relaxation effects on
protons, making it impossible to reliably quantify the local tissue concentration of SPIO particles. MRI cell tracking with SPIO also has low specificity due to other low-signal regions in images, i.e. SPIO labeled cells in the lung or in some regions of hemorrhage cannot be detected [13].

Fluorine-19 (\(^{19}\)F)-based cell tracking uses perfluorocarbons (PFC) to label cells. PFC-labeled cells can be imaged by \(^{19}\)F MRI with high specificity since there is very little endogenous fluorine in biological tissues. \(^{19}\)F MRI is quantitative since the signal intensity is linearly related to the number of \(^{19}\)F atoms. A sample of PFC labeled cells can be analyzed by nuclear magnetic resonance (NMR) spectroscopy to determine the average \(^{19}\)F per cell and this can be used to estimate the number of cells which contribute to the signal in the \(^{19}\)F image [14]. The ability to determine cell number \textit{in vivo} from \(^{19}\)F images is the most compelling reason for using \(^{19}\)F-based MRI over SPIO-based MRI for cell tracking. However, \(^{19}\)F MRI has low sensitivity, requiring thousands of PFC-labeled cells/voxel [15].

Magnetic particle imaging (MPI) is a newly emerging tracer-based imaging modality that directly measures the response of SPIOs to an alternating magnetic field (kHz) - the excitation field [16,17]. A selection field is used to localize the signal. This is established by two opposing electromagnets which form a strong gradient magnetic field (T/m) with a field free region (FFR) in the centre. Only SPIOs in the FFR can respond to the excitation field. The position of the FFR is shifted over the field of view (FOV) to detect the response of SPIOs to the excitation field. The non-instantaneous response of the ensemble SPIO magnetization to the applied magnetic field in MPI is referred to as relaxation [18]. This response varies for different SPIO. MPI has great potential for overcoming the challenges
of MRI based cell tracking. It has high specificity because it only detects SPIOs and there is no background signal. It has high sensitivity to nanogram quantities of SPIOs, which translates to hundreds of cells, and further improvements in sensitivity are expected as tailored SPIOs are developed [19,20]. Importantly, the MPI signal is linearly related to iron mass allowing for quantitation of the amount of iron from images, and with knowledge of the amount of iron/cell after SPIO labeling, the cell number can be calculated [21], like $^{19}$F MRI.

In general, the SPIOs used in MPI are like those used for MRI. They are typically composed of a spherical iron crystal core, often of maghemite and/or magnetite, stabilized by coatings such as dextran, carboxy-dextran, or polyethylene glycol (PEG) [22]. Particle characteristics such as core size and shape, hydrodynamic diameter, and surface functionalization have all been shown to affect their magnetic properties [23–25]. SPIO can be single or multi-core. Single core particles, as the name implies, have just one magnetic core per iron particle. Multi-core SPIO have several closely aggregated iron cores per cluster. Most SPIO are multi-core. For MPI, sensitivity and resolution are strongly dependent on the choice of SPIO [18,23].

Spatial resolution in MPI is defined as the ability to distinguish between two signals with the same intensity. MPI resolution is driven primarily by SPIO relaxation. In response to the MPI excitation field, Neel relaxation refers to the reversal of the SPIO magnetic moment, whereas Brownian relaxation refers to the physical rotation of the SPIO [26]. The dominant relaxation mechanism depends primarily on the SPIO core size [18,23]. Theoretical modeling based upon the Langevin theory of SPIOs predicts that resolution improves with increasing core size. However, Tay et al. [18] found that resolution increases
with core size up to approximately 25 nm when the effects of SPIO rotational times become significant owing to increasing Brownian relaxation. SPIOs above this size range experience increased drag, slowing their magnetization response and limiting resolution [21,27]. Notably, these studies have focused on SPIO dispersed in aqueous media which are able to move freely in response to the applied magnetic field.

For MPI, particle sensitivity refers to the lowest mass of SPIO detected per imaging unit. For MPI cell tracking, the aim is to optimize cellular sensitivity, which refers to the lowest number of SPIO-labeled cells detected per imaging unit. MPI sensitivity also relies heavily on the strength of the SPIO magnetization (stronger magnetization improves MPI signal) [28,29] and the rate of SPIO relaxation, where a faster rate of change in magnetization leads to higher maximum MPI signal. For cell tracking, the efficiency of the particle cell labeling influences MPI sensitivity; more iron per cell leads to higher sensitivity. The optimal core size is thought to be between 20-30 nm [30].

Many SPIO are commercially available which can produce MPI signal. Ferucarbotran (available as VivoTrax™, Magnetic Insight Inc.), was originally developed for MRI and has been widely used for MPI. It is currently considered a “gold standard” because of its ease of commercial availability, even though its properties are not ideal for MPI resolution and sensitivity. Ferucarbotran has a carboxyextran coat and has been reported to have a bimodal core size distribution with approximately 30% of the cores being 25-30 nm and 70% of the cores being ~5 nm [31]. Synomag-D (micromod, Germany) is a SPIO tailored for MPI [32] which has 30 nm nanoflower-shaped iron oxide cores coated and approximately four times higher MPI signal than VivoTrax in solution. Other custom-made tailored tracers have also showed much greater MPI signal and better spatial resolution.
than existing commercial tracers [30]. Extensive research in the area of the synthesis of MPI tailored SPIOs is ongoing, still, it remains a challenge to optimize MPI tracers for maximum resolution and sensitivity.

The magnetic response generated by SPIOs is also strongly influenced by the local environment. Previous studies have demonstrated that the magnetic behaviour of SPIOs changes in biological media [33], after SPIO immobilization [34], after cell labeling [35] and during cellular binding [36] and internalization. This tends to lead to lower the MPI signal and resolution most likely due to aggregation of the SPIO particles and changes in viscosity which impact SPIO relaxation. Arami et al. have shown that for SPIO with core sizes smaller than 20 nm the MPI signal strength, but not resolution, is affected by immobilization [34]. Other differences between SPIO used for MPI cell tracking, such as the hydrodynamic size, surface coating, and need for transfection agents to facilitate efficient cell labeling may also influence changes in their magnetic performance for MPI [24,37]. In this study, we evaluated SPIOs, free in solution and after cell labeling with the goal of understanding SPIO features which produce the highest sensitivity and resolution for in vivo cell tracking.

4.2 Methods

4.2.1 Superparamagnetic Iron Oxide Particles

Several SPIO particles were examined using MPI relaxometry. Particles were examined either as free SPIO, SPIO combined with transfection agents (TAs), or following internalization within cells. Table 4.1 lists all particles that were assessed. Synomag®-D is a commercially available MPI tailored particle. It is a multicore particle with a nanoflower
substructure, the agglomerated iron core size is \(~30\) nm. The cores are coated with dextran to improve stability and provide biocompatibility. We tested two plain Synomag-D particles with hydrodynamic diameters of either 50 nm or 70 nm. These were purchased from micromod Partikeltechnologie GmbH (Rostock, Germany). We also tested one additional particle from micromod called Perimag®. Perimag has also been used as a tracer for MPI [38,39]. It is a cluster-typed, multicore unmodified dextran coated particle with a core diameter of 19 nm and hydrodynamic diameter of 130 nm.

Two custom MPI particles synthesized in the lab of our collaborator, Dr. Carlos Rinaldi-Ramos, were tested. These are called PMAO1 and PMAO2, with particle synthesis previously reported [40]. These particles have a single 22 nm iron core and are coated with Poly(maleic anhydride-alt-1-octadecene) (PMAO). The hydrodynamic diameter is 100 nm. PMAO1 and 2 are two samples from the same batch of synthesized particles, coated separately.

MPIO used in previous MRI cell tracking experiments in both this thesis [41,42] and for previous studies in the Foster lab [1,8,11,43] were also tested for MPI. These MPIO particles, purchased from Bangs Laboratories (Fishers, IN, USA) are synthesized using a process that results in the distribution of iron oxide crystals throughout a polymer matrix. We tested two versions of these, the “classical” version which has some exposed iron oxide on the surface and a mean hydrodynamic diameter of 0.9 \(\mu\)m (MPIO1) and a range of 0.5 to 2 \(\mu\)m, the “encapsulated” version is free of iron oxide on the surface and has a diameter of 1.63 \(\mu\)m (MPIO2) and a range of 0.5 to 2 \(\mu\)m. We also tested one additional MPIO from Bangs Laboratories called ProMag1. ProMag1 are similar to the encapsulated MPIO2 but
are highly uniform spheres with a hydrodynamic diameter of 1 \( \mu \text{m} \) (MPIO3). All MPIO particles are functionalized with a hydrophilic carboxyl group (COOH).

Two SPIOs previously used for MRI cell tracking were tested. VivoTrax\(^\text{TM}\), a ferucarbotran, which is the same as Resovist, used for MRI, was purchased from Magnetic Insight Inc. (Alameda, CA, USA). VivoTrax is made up of \( \sim30\% \) of the iron cores being 25-30 nm cores and \( \sim70\% \) of the iron cores being 5 nm. This particle is coated with carboxydextran and has a hydrodynamic diameter of 62 nm. Molday ION EverGreen\(^\text{TM}\) (Molday-G) was purchased from BioPal (Worcester, MA, USA). This particle is an amine-functionalized single core SPIO with an iron core size of \( \sim8 \) nm and a dextran coat, the hydrodynamic diameter is 35 nm.
Table 4.1 Characteristics of SPIOs used in study.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Iron Content (µg/µL)</th>
<th>Mean Core Diameter</th>
<th>Mean Hydrodynamic Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synomag-D (50 nm)</td>
<td>10</td>
<td>~30 nm</td>
<td>50 nm</td>
</tr>
<tr>
<td>Synomag-D (70 nm)</td>
<td>10</td>
<td>~30 nm</td>
<td>70 nm</td>
</tr>
<tr>
<td>Perimag</td>
<td>8.5</td>
<td>19 nm</td>
<td>130 nm</td>
</tr>
<tr>
<td>PMAO1</td>
<td>0.74</td>
<td>22 nm</td>
<td>100 nm</td>
</tr>
<tr>
<td>PMAO2</td>
<td>1.17</td>
<td>22 nm</td>
<td>100 nm</td>
</tr>
<tr>
<td>MPIO1</td>
<td>4.48</td>
<td>5-10 nm*</td>
<td>0.9 µm</td>
</tr>
<tr>
<td>MPIO2</td>
<td>3.075</td>
<td>5-10 nm*</td>
<td>1.63 µm</td>
</tr>
<tr>
<td>MPIO3</td>
<td>4.79</td>
<td>5 – 10 nm*</td>
<td>1.0 µm</td>
</tr>
<tr>
<td>VivoTrax</td>
<td>5.5</td>
<td>25-30 nm and 5 nm</td>
<td>62 nm</td>
</tr>
<tr>
<td>Molday ION EverGreen</td>
<td>2</td>
<td>~8 nm</td>
<td>35 nm</td>
</tr>
</tbody>
</table>

*5-10 nm core is manufacturer reported (Bangs Laboratories).
4.2.2 Cell Culture

Human brain metastatic breast cancer cells (MDA-MB-231BR) were cultured with Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific, Waltham, Massachusetts) containing 10% fetal bovine serum (FBS) and antimycotic/antibiotic. Cells were maintained at 37°C and 5% CO$_2$ in T75 cm$^2$ flasks and passaged every 2-3 days at passage numbers between 15-35. This cell line was tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, Maine) and was found to be negative.

4.2.3 Cell Labeling

To label cells with iron particles, $2 \times 10^6$ of 231BR cells were seeded in T75 cm$^2$ flasks and allowed to adhere overnight for 24 hours. For labeling with TAs, labeling was performed using protamine sulfate and heparin to form SPIO-TA complexes which are known to enhance cellular uptake [44–46]. To label with TAs, 60 µL of protamine sulfate (stock 10 µg/µL) was added to 2.5 mL of serum-free DMEM, and 20 µL of heparin (stock 1 U/µL) with 90 µL of each particle was added to an additional tube with 2.5 mL of DMEM. Both mixtures were individually vortexed, and then combined and added to culture. Following 4 hours of incubation at 37 °C, an additional 5 mL of complete DMEM was added to culture for a total volume of 10 mL in T75 cm$^2$ flasks. To label particles through simple co-incubation, cells were supplemented with either 54 µL of MPIO1, 100 µL of MPIO2, 54 µL of MPIO3, 90 µL of Perimag, 90 µL of PMAO2, or 250 µL of Molday-G in a total volume of 10 µL of complete DMEM in T75 cm$^2$ flasks. It was determined these particles did not require further labeling with TAs based on evaluation of successful labeling through
Perl’s Prussian Blue (PPB) staining (described below) and from prior work in our lab. All labeling protocols were based on methods developed in previous studies [37,41,47,48].

All T75 cm² flasks with cells were incubated at 37°C and 5% CO₂ overnight with SPIO, for a total of 24 hours. Cells were then washed three times with phosphate buffered solution (PBS) within the flask, trypsinized with 0.25% Trypsin–EDTA, and then washed an additional three times with PBS prior to centrifuging the cell pellets to thoroughly remove unincorporated iron. Cells were then counted, and viability was assessed using a Trypan blue exclusion assay.

4.2.4 Evaluation of Cell Labeling

To visualize and evaluate labeling for each particle, 2.0 x 10⁵ labeled 231BR cells were affixed to a glass slide with a ThermoFisher Cytospin 4 cytocentrifuge and fixed with a 3:1 Methanol/Acetic acid solution. Slides were then stained with a PPB solution and counterstained with Nuclear Fast Red. Samples were then dehydrated with increasing concentrations of ethanol, cleared with xylene, and coverslipped with a xylene-based mounting medium. These PPB-stained slides were examined to determine the labeling efficiency using an ECHO Revolve microscope (ECHO, San Diego, CA, USA), where iron particles should appear blue within the light pink cells. Labeling efficiency was calculated by counting the number of positively stained cells in three random 40x microscopy images using Fiji (ImageJ version 2.1.0/1.53c).

4.2.5 MPI Relaxometry

MPI was conducted on a MOMENTUM™ system (Magnetic Insight Inc.). All samples were individually scanned using the RELAX™ module provided on the Momentum™
scanner. The RELAX™ module allows for the characterization of the magnetic performance of SPIOs by turning off the selection field and measuring the net magnetization by varying an applied magnetic field from -100 mT to 100 mT. Rather than producing an image, the output of the RELAX™ module provides a point spread function (PSF) for each sample measurement. MPI Relaxometry was performed for all particles as (1) free SPIO, (2) SPIO with TAs, and (3) intracellular SPIO.

To evaluate free SPIO, triplicate samples of volumes between 1-40 µL of each agent were prepared in PCR tubes. The volumes for each agent were selected so the amount of SPIO in each sample would produce signal that would not magnetically oversaturate the system. To evaluate SPIO mixed with TAs, triplicate samples of SPIO were prepared with protamine sulfate, heparin, and serum-free media as described previously [47] using a volume of SPIO that would be below magnetic saturation. To evaluate the magnetic performance of intracellular SPIO, triplicate samples of 5 x 10^5 labeled cells in a 40 µL volume of PBS were prepared.

4.2.6 MPI Relaxometry Analysis

To evaluate sensitivity and resolution, all PSFs produced by the RELAX™ module were analyzed using GraphPad Prism version 9.5.0 software (GraphPad, San Diego, CA) to assess the signal, represented by the peak height, and the resolution, represented by the full-width half maximum (FWHM) value of each sample. Relaxometry curves for free SPIO and SPIO-labeled cells were evaluated. To assess sensitivity, MPI signal (A.U) was normalized to the amount of iron (µg/µL) in each sample for each agent based on iron content values provided by each manufacturer (for free SPIO) or iron content determined using a calibration line (for SPIO-labeled cells) and multiplied by 1000 to report signal as
A.U/mg Fe. To assess resolution, the curves were normalized to the maximum signal value for each sample. FWHM values in milli-Tesla (mT) were converted to millimeters by dividing each value by the 3.0 Tesla per meter (T/m) gradient strength.

### 4.2.7 Imaging

An individual enzyme-linked immunosorbent assay (ELISA) well was secured to the MPI bed to allow the precise placement of polymerase chain reaction (PCR) tubes containing each sample for imaging. 2D images were acquired of the same triplicate samples of (1) free SPIO, (2) SPIO with TAs, and (3) intracellular SPIO with a 3.0 T/m selection field gradient and drive field strengths of 22 mT and 26 mT in the X and Z axes, respectively, resulting in a scan time of approximately 2.5 minutes for a 12 x 6 cm FOV. These scans were performed to measure iron content per cell sample.

### 4.2.8 Image Analysis

All MPI images were imported and viewed with a custom MPI colour look-up table (CLUT) using the open-source Horos™ image analysis software (version 3.3.6, Annapolis, MD USA). For MPI signal calibration, 7 samples of each agent in a dilution series in PBS were prepared and then imaged with MPI using the same 2D imaging parameters. A linear relationship was then found between iron mass and MPI signal. The equation of the line was used to determine the iron content from each cell sample for each particle. A 2D image of the empty bed and sample holder was performed at the start of each imaging session. A region of interest (ROI) encompassing the entire background image was used to determine the standard deviation of the background noise. A threshold value of 5 times the standard deviation of the background noise was applied for subsequent image analysis to ensure only signal above this limit was measured. We have studied various image analysis
methods for MPI and found the threshold method to produce accurate quantification with low user variability [49]. The total MPI signal for 2D images was calculated by multiplying the area by the mean signal. All MPI images were quantified using this method. To determine the iron content per cell sample, the samples containing $5.0 \times 10^5$ iron-labeled cells were imaged in 2D and iron mass was determined using the calibration line. To determine the pg of iron per cell, the iron mass determined by the calibration line was divided by the number of cells in each cell pellet ($5.0 \times 10^5$).

### 4.2.9 Statistical Analysis

All statistical analysis were performed using GraphPad Prism version 9.5.0 software. The relationship between the measured MPI signal and the known iron content was determined by performing a simple linear regression, which determined the calibration equation. Differences between (1) free SPIO, (2) SPIO with TAs, and (3) intracellular SPIO were analyzed with ordinary one-way ANOVA tests and Welch’s unpaired t-tests to compare relaxometry results between groups. A nominal p-value < 0.05 was considered statistically significant. The data are expressed as mean ± standard deviation (SD).

### 4.3 Results

#### 4.3.1 MPI Relaxometry of Iron Particles

Figure 4.1A shows the average MPI signal (A.U.) values (n=3) from MPI relaxometry for each particle tested as free SPIO. For the MPI-tailored particles (shown in purple), Synomag-D (50 nm) had the highest MPI signal measurement (175.63 A.U.). PMAO2 (155.93 A.U.), Synomag-D 70 nm (155.18 A.U.), PMAO1 (115.46 A.U.), and Perimag (110.24 A.U.) also had high MPI signal (over 100 A.U). Of the MPIO particles (blue),
MPIO3 demonstrated the highest signal (64.57 A.U.), followed by MPIO1 (28.30 A.U.) and MPIO2 (27.01 A.U.). VivoTrax and Molday-G (orange) had relatively low signal, measuring 51.74 A.U. and 14.49 A.U., respectively.

The average FWHM (mT) values for each particle (n=3) are shown in Figure 4.1B. Perimag had the smallest FWHM for all particles (7.24 mT, 2.41 mm), representing superior resolution. Additional MPI-tailored particles, which included Synomag-D (50 nm) (7.27 mT, 2.42 mm), Synomag-D (70 nm) (7.44 mT, 2.48 mm), PMAO2 (8.41 mT, 2.80 mm) and PMAO1 (10.7 mT, 3.57 mm) also had good resolution. The MPIO1 (20.28 mT, 6.76 mm), MPIO2 (35.02 mT, 11.67 mm) and MPIO3 (23.22 mT, 7.74 mm) had larger FWHM values, indicating poorer resolution. Interestingly, VivoTrax had a small FWHM value (9.36 mT, 3.12 mm) while Molday-G had a FWHM value of 69.76 mT or 23.26 mm.
Figure 4.1 MPI relaxometry data comparing free SPIOs.
A) Average peak MPI signal for each particle tested as free SPIO for MPI-tailored particles (purple), MPIOs (blue), and MRI-tailored particles (orange). B) Average FWHM (mT) values for each particle tested as free SPIO.
At this point in the study, PMAO1 was eliminated because PMAO2 showed a higher MPI signal and better resolution compared to PMAO1. The average MPI signal and PSFs for the rest of the particles which were subsequently used for cell labeling trials are shown in Figure 4.2A and 4.2B. Numerical values for each particle are shown in Figure 4.2C. Average FWHM values and normalized PSFs are shown in Figure 4.3A and 4.3B, with numerical values shown in 4.3C.
Figure 4.2 MPI relaxometry data for average peak MPI signal for free SPIOs selected for cell labeling trials.

A) Average MPI signal for each particle tested as free SPIO for MPI-tailored particles (purple), MPIOs (blue), and MRI-tailored particles (orange). B) PSFs showing peak MPI signal from relaxometry for free SPIO grouped by MPI-tailored particles, MPIOs, and MRI-tailored particles. C) Numerical values for each sample.
Figure 4.3 MPI relaxometry data for average FWHM values for free SPIOs selected for cell labeling trials.

A) Average FWHM values for each particle tested as free SPIO for MPI-tailored particles (purple), MPIOs (blue), and MRI-tailored particles (orange). B) PSFs showing resolution from relaxometry for free SPIO grouped by MPI-tailored particles, MPIOs, and MRI-tailored particles. C) Numerical values for each sample.
4.3.2 Evaluating Cell Labeling

Cells could be labeled with the MPIOs, PMAO2, and Molday-G through simple co-incubation, without the use of TAs. The use of TAs (protamine sulfate and heparin) was required for successful labeling of 231BR cells with Synomag-D (50 nm), Synomag-D (70 nm). Labeling efficiency was confirmed through the PPB stains shown in Figure 4.4A, where cancer cells appear pink, and the intracellular iron appears blue within the cell. For MPIO2 and PMAO2 the iron cores appear rust coloured, the PPB staining process appears to have not penetrated the coatings of these particles. Labeling was classified as successful if ≥ 80% of cells were labeled with iron (Figure 4.4B). Cells could not be successfully labeled with Perimag with simple-coincubation (0% labeled) or TAs (~28% labeled), therefore, this particle was eliminated from further study. The only study which has successfully used Perimag for MPI of cells did so in erythrocytes via a hypotonic dialysis and isotonic resealing procedure [39]. VivoTrax also did not successfully label cells using TAs (~12% labeled), this is consistent with our previous published observations [37], and so VivoTrax was also eliminated from further study.
Figure 4.4 Assessment of iron labeling of MDA-MB-231BR cells.
A) PPBs of MDA-MB-231BR cells labeled with MPIO1, MPIO2, MPIO3, Molday-G, PMAO2, Synomag-D (50 nm) and Synomag-D (70 nm). B) Labeling efficiency quantified from microscopy images, with successful labeling trials shown to have >80% positively labeled cells.
4.3.3 MPI Relaxometry of Iron Labeled Cells

We next used MPI relaxometry to investigate how the MPI signal and resolution would be affected for particles that efficiently labeled the 231BR cells. For Synomag-D (50 nm) ($P \leq 0.01$), Synomag-D (70 nm) ($P \leq 0.001$), and PMAO2 ($P \leq 0.0001$) there was a significant reduction in the MPI signal following cell labeling compared to each agent alone. There was no decrease in the MPI signal after cell labeling for the MPIOs or Molday-G (Figure 4.5).

When assessing resolution, we determined that Synomag-D (50 nm) ($P \leq 0.001$), Synomag-D (70 nm) ($P \leq 0.05$), PMAO2 ($P \leq 0.01$), MPIO2 ($P \leq 0.05$), and MPIO3 ($P \leq 0.05$) experienced a significant decrease after cell labeling (Figure 4.6). MPIO1 and Molday-G did not show worse resolution after cell labeling.
Figure 4.5 Comparison of the average peak signal for free SPIO (grey bars) for each particle to SPIO-labeled cells (blue bars).

There was a significant reduction in MPI signal following cell labeling for Synomag-D 50 nm (P ≤ 0.01), Synomag-D (70 nm) (P ≤ 0.001), and PMAO2 (P ≤ 0.0001). There was no decrease in the MPI signal after cell labeling for the MPIOs or Molday-G. (Unpaired t-tests, ns - P > 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, **** - P ≤ 0.0001).
Figure 4.6 Comparison of the average FWHM values for free SPIO (grey bars) for each particle to SPIO-labeled cells (blue bars).

There was a significant increase in the FWHM, representing resolution, for Synomag-D (50 nm) (P ≤ 0.001), Synomag-D (70 nm) (P ≤ 0.05), PMAO2 (P ≤ 0.01), MPIO2 (P ≤ 0.05), and MPIO3 (P ≤ 0.05) after cell labeling. (Unpaired t-tests, ns - P > 0.05, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001).
We then compared the change in MPI signal from each particle alone to each particle after cell labeling (Figure 4.7A). This shows that although there was a substantial loss in signal for both Synomag-D (50 nm) and PMAO2 following cell labeling, they still provide higher MPI signal compared to MPIO1, MPIO2, Synomag-D (70 nm), and Molday-G (P ≤ 0.0001). However, this resulted in Synomag-D (50 nm) and PMAO2 no longer retaining their initial gain in signal as free SPIO compared to MPIO3. With respect to Synomag-D (70 nm), the signal loss is such that it no longer provides more sensitivity than all MPIOs.

When assessing resolution between particles following cell labeling (Figure 4.7B), we saw that Synomag-D (50 nm) still provided superior resolution compared to MPIO1 (P ≤ 0.001), MPIO3 (P ≤ 0.001), MPIO2 (P ≤ 0.0001), Synomag-D (70 nm) (P ≤ 0.0001) and Molday-G (P ≤ 0.0001) following cell labeling. For Synomag-D (70 nm), resolution decreased from 7.4 mT to 42.8 mT, and its initial advantage in resolution as a free particle was lost, with MPIO1 and MPIO3 now providing significantly better resolution following cell labeling (P ≤ 0.01).
Figure 4.7 Comparison of the changes in peak MPI signal and the FWHM values following cell labeling for each particle.

A) Average peak signal for particles as free SPIO (grey) and SPIO after cell labeling (blue). (*) represents a significant decrease in MPI signal between free SPIO and SPIO after cell labeling (unpaired t-test). B) Average FWHM values for particles as free SPIO and after cell labeling (*) represents a significant increase in the FWHM value between free SPIO and SPIO after cell labeling (unpaired t-test).
4.3.4 Effect of Transfection Agents on MPI Signal and Resolution

Transfection agents are often used to enhance cellular uptake of SPIOs, they do this by forming TA-SPIO complexes and therefore cause SPIO aggregation [45]. Since the MPI signal decreased after cell labeling for all Synomag-D particles and this SPIO required TAs for cell labeling, we compared the MPI signal for these particles alone and after mixing with TAs (Figure 4.8A). The MPI signal was significantly reduced for particles plus TAs compared to particles alone. The average peak MPI signal decreased by ~17% for Synomag-D (50 nm) (P ≤ 0.01) and ~23% for Synomag-D (70 nm) (P ≤ 0.01). Since the MPI signal for Synomag-D (50 nm) decreased by ~55% with cell labeling, ~17% of the decrease can be attributed to TAs, and ~38% may be due to effects of cell labeling. For Synomag-D (70 nm), we saw an ~81% decrease in MPI signal following cell labeling, and therefore, ~23% of the decrease can be attributed to TAs, and ~58% may be due to effects of cell labeling.

There was no significant change in resolution when comparing free particles to particles mixed with TAs for Synomag-D (Figure 4.8B). We estimate that TAs are not contributing significantly to the loss of resolution observed following cell labeling for Synomag-D.
Figure 4.8 MPI relaxometry showing A) the average peak signal and B) the average FWHM values for Synomag-D (50 nm) and Synomag-D (70 nm) as free SPIO, with TAs, and after cell labeling.

(Ordinary one-way ANOVA, ns - P > 0.05, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001, **** - P ≤ 0.0001).
4.3.5 Effect of PEG on MPI Signal and Resolution

Polyethylene glycol (PEG) coatings are known to prolong the circulation time of intravenously administered SPIOs and have been investigated as a strategy to reduce SPIO aggregation by decreasing the surface energy of particles [50]. As aggregation can modulate the magnetic performance of particles [35,51], we evaluated the effects of PEGylation on MPI signal for SPIOs compared to particles that were non-PEGylated. Therefore, Synomag-D (50 nm and 70 nm) with PEG added to the surface were purchased from micromod Partikeltechnologie GmbH (Rostock, Germany).

As free SPIO, Synomag-D (50 nm) produced significantly more signal than Synomag-D-PEG (50 nm) (P ≤ 0.05), however, Synomag-D-PEG (70 nm) produced significantly more signal than the Synomag-D (70 nm) (P ≤ 0.05) (Figure 4.9A). When comparing resolution, Synomag-D (50 nm) also demonstrated significantly superior resolution compared to Synomag-D-PEG (50 nm), however, Synomag-D-PEG (70 nm) outperformed Synomag-D (70 nm) (6.77 mT to 7.44 mT, P ≤ 0.01) (Figure 4.9B).
Figure 4.9 MPI relaxometry comparing Synomag-D Plain and Synomag-D-PEG particles as free SPIO.

A) Average peak MPI signal was higher for Synomag-D (50 nm) Plain vs. Synomag-D-PEG (50 nm) (P ≤ 0.05) but lower for Synomag-D (70 nm) Plain vs. Synomag-D-PEG (70 nm) (P ≤ 0.05). B) Average FWHM value was lower for Synomag-D (50 nm) Plain vs. Synomag-D-PEG (50 nm) (P ≤ 0.01) but higher for Synomag-D (70 nm) Plain vs. Synomag-D-PEG (70 nm) (P ≤ 0.05). (Unpaired t-tests, * - P ≤ 0.05, ** - P ≤ 0.01).
We then labeled cells with either Synomag-D-PEG (50 nm) (~85% labeled) or Synomag-D-PEG (70 nm) (~82% labeled) using TAs, with the successful labeling confirmed through the PPB stains shown in Figure 4.10A and 4.10B. When comparing the MPI signal after cell labeling for plain Synomag-D versus Synomag-D-PEG we saw that the MPI signal and resolution were both significantly higher for the PEG version (Figure 4.10C, D).
Figure 4.10 PPB and MPI relaxometry for Synomag-D-PEG (50 nm) and Synomag-D-PEG (70 nm) after cell labeling.
A) Synomag-D-PEG (50 nm) (~85% labeled) and B) Synomag-D-PEG (70 nm) (~82% labeled) efficiently labeled 231BRs using TAs. C) Average peak MPI signal was lower for cells labeled with Synomag-D (50 nm) Plain vs. Synomag-D-PEG (50 nm) (P ≤ 0.01) and lower for cells labeled with Synomag-D (70 nm) Plain vs. Synomag-D-PEG (70 nm) (P ≤ 0.01). D) Average FWHM value was higher for cells labeled with Synomag-D (50 nm) Plain vs. Synomag-D-PEG (50 nm) (P ≤ 0.001) but higher for cells labeled with Synomag-D (70 nm) Plain vs. Synomag-D-PEG (70 nm) (P ≤ 0.05). (Unpaired t-tests, * - P ≤ 0.05, ** - P ≤ 0.01, *** - P ≤ 0.001).
4.3.6 Evaluating Iron Per Cell

Of the particles with efficient labeling, quantitative measurements with MPI determined the average pg of Fe/cell for each SPIO particle, shown in Figure 4.11A, with associated numerical values shown in Figure 4.11B. These values are consistent with the range of values reported in the literature [37,47,52,53]. Synomag-D (70 nm) was determined to have the highest iron content per cell, with an average of 9.5 pg Fe/cell. Synomag-D-PEG (50 nm) and Synomag-D-PEG (70 nm) had the lowest iron per cell content, with an average of 2.4 and 1.2 pg Fe/cell respectively.
Figure 4.11 A) Average pg/iron per cell for MDA-MB-231BR cells labeled with each SPIO. B) Numerical values for each sample.
4.4 Discussion

The most important finding of this work is that the MPI-tailored SPIOs we tested that were designed to have high particle sensitivity lose this advantage after cell labeling. This was true specifically for Synomag-D (50 nm and 70 nm) and PMAO2, which had the highest MPI signals as free particles. PMAO2 was tailor made for MPI in the lab of Carlos Rinaldi. The original version of the PMAO particles we tested was called RL-1 and was described in 2021 [40]. In Liu et al., they compared RL-1 to Synomag-D and ferucarbotran (VivoTrax). Electron microscopy showed RL-1 (PMAO) to consist of single cores with a narrow size distribution. VivoTrax appeared as small, aggregated cores and Synomag-D consisted of heterogenous nanocrystal clusters (Supplementary Figure 1). RL-1 particles had core diameters between 20.7 and 22.6 nm. Ferucarbotran had average core diameters of 9.6 nm, and Synomag-D particles had core diameters of 28.6 nm. The magnetic properties of all three SPIOs were evaluated by SQUID magnetometry, and results suggested that all had two populations of particles with different size distributions. For RL-1, most of the particles (90%) had mean magnetic diameters between 17.1 and 18.4 nm with the remaining having a small mean core diameter between 2.5 and 3.1 nm. For ferucarbotran, 81% had a mean core diameter of 7.6 nm and 19% were clusters with a mean diameter of 22 nm. For Synomag-D 84% had a mean diameter of 19.3 nm and 16% has a mean diameter of 8.2 nm. RL-1 and Synomag-D had similar, and superior, MPI signal compared to ferucarbotran. This result agrees with our data. The core size is known to be an important factor affecting the magnetic performance of SPIOs. To preserve a high magnetic moment within a single particle a single magnetic domain is best. Above a certain particle size (∼50 nm) it is energetically more efficient to form multiple domains, which
decreases the total magnetization [54]. In addition, relaxation effects increase with particle size and cause blurring artifacts and a decrease in MPI signal [55]. Studies have shown that the MPI signal increases with core size up to approximately 25-30 nm. RL-1 and Synomag-D had similar particle distributions, both with most of the particles close to the optimal core size which likely contributed to their high MPI signal as free particles. This paper and others have shown the importance of monodispersity. Ferguson et al. showed that selecting or synthesizing particles with monodisperse magnetic cores can optimize MPI signal produced for a particular size [23]. More specifically, they describe that MPI signal for polydisperse particles will be dominated by cores near optimal size, and “off-sized” particles will not significantly contribute, therefore reducing sensitivity.

Despite the high MPI signal produced by free PMAO and Synomag-D, the signal decreased after cell labeling. This finding agrees with several studies which show that the magnetic response of SPIOs is strongly influenced by their environment, and more specifically that cellular binding and internalization, particle aggregation, clustering and/or interactions in cells, can lead to changes in the MPI signal [25,33,56–58]. Teeman et al. reported a 20% decrease in the magnetic performance of single core SPIO synthesized in their lab (21-28 nm) after internalization by cancer cells. Transmission electron microscopy (TEM) and confocal microscopy were used to investigate the localization of particles within cells, to better understand the mechanisms by which cell labeling could affect magnetic performance. SPIOs were only found in cytoplasmic vesicles (endosomes and lysosomes), as expected for endocytosis. TEM ruled out a decrease in MPI signal due to changes in the particle size after internalization which might have occurred due to degradation. They also found no change in the magnetic response of SPIOs with increasing viscosity. However,
magnetostatic interactions between particles were observed to significantly and negatively impact magnetic performance. This work showed that SPIOs in this size range interact with each other and limit their ability to interact with the applied magnetic field [56].

Cabrera et al. studied the effect of cell labeling on the magnetic performance of two commercial SPIO with different core sizes and hydrodynamic sizes; 11 nm core with 50 nm hydrodynamic size and 21 nm core with 67 nm hydrodynamic size. The MPI signal was reduced in SPIO labeled breast cancer cells compared to SPIO dispersed in solution. TEM showed that SPIOs were clustered within intracellular vesicles with significant aggregation of particles compared to SPIO in solution. This was more pronounced for the larger SPIO. Like Teeman et al., they found no change in the magnetic response of SPIOs with changes in viscosity which led to particle immobilization. This work showed that clustering of particles, which leads to magnetostatic interactions, contributes to an altered magnetic response [58]. We believe these mechanisms are likely to be responsible for the decrease in MPI signal observed for PMAO2 particles after cell labeling.

We, and others, have shown that Synomag-D is not taken up by cells without the use of TAs [47,59]. Many previous MRI cell tracking studies have used ‘magnetofection’, a method where TAs are mixed with SPIOs to encourage the endocytosis of the SPIO-TA complex [60]. The SPIO-TA complex leads to SPIO aggregation. Our data comparing free Synomag-D, Synomag-D mixed with TAs and cells labeled with Synomag-D and TAs shows that TAs reduce the MPI signal and that this is magnified once in cells. In our experiments, the reduction in MPI signal for cells labeled with Synomag-D and TAs (> 50%) was much greater than the decrease in signal reported in other papers where other SPIOs were studied which did not require TAs for cell labeling. We believe this is related
to increased hydrodynamic diameter, more substantial SPIO aggregation and particle interactions. We observed a greater reduction in the MPI signal after cell labeling with the Synomag-D, which has a hydrodynamic diameter of 70 nm, compared to the Synomag-D with a 50 nm hydrodynamic diameter. This makes sense since a larger hydrodynamic size is known to lead to greater relaxation effects which attenuate the SPIO response to the excitation field. Lowa et al. performed fractionation of ferucarbotran to separate the particles based on hydrodynamic size [24]. The fractions ranged from 30 – 100 nm in hydrodynamic size. An increase in the MPI signal was found for fractions up to ~50 nm, for larger hydrodynamic sizes the MPI signal decreased. This result fits well with our observations of reduced MPI signal for Synomag-D 70 nm compared to 50 nm.

VivoTrax also requires TAs for effective cell labeling [37]. Suzuka et al. studied the MPI signals from Resovist (VivoTrax), with and without TAs, and from colon cancer cells and macrophages labeled with Resovist. Cancer cell labeling required the use of TAs, macrophages did not because of their intrinsic phagocytic activity. The MPI signal was significantly reduced, and the hydrodynamic diameter was significantly increased for Resovist mixed with protamine, or heparin and protamine, compared to Resovist alone. This agrees with our results for Synomag-D, and it is likely that the signal loss observed when a SPIO is mixed with TAs is due to an increased hydrodynamic diameter. Cell labeling caused a reduction in MPI signal for both cell types, but this was much more pronounced for cancer cells. TEM showed that there was much more SPIO aggregation in cancer cells compared to macrophages. These results suggest that the requirement for TAs to label cancer cells leads to particle aggregation and causes a greater reduction in the MPI signal [35].
Paysen et al. have shown that the unique signals for free versus intracellular SPIO can be incorporated into the image reconstruction to possibly minimize these negative effects or to separate these signals with a technique known as multi-colour MPI [57]. Multi-colour MPI involves assigning a unique colour to the different signal sources and has been used to separate signals from different particle types and aggregation states [30]. This may be useful for differentiating between two cell types or between live and dead cells.

Of particular interest was our finding that the MPIOs tested in this study did not show a loss in MPI signal following cell labeling. There are no other examples of iron particles which have been used for MPI which have shown no change in MPI signal after cell labeling. MPIOs are microspheres with small iron oxide crystals dispersed throughout a polymer matrix. As free particles, we found that MPIOs produce significantly less MPI signal, and had much lower resolution, than Synomag-D particles and PMAO2. This is likely due to their very large hydrodynamic size relative to most SPIO used for MPI. Our results showed that MPIO3 produced more MPI signal than MPIO1 and MPIO2. MPIO1 and MPIO2 are known to have a broad size distribution whereas MPIO3 are highly uniform. This monodispersity may have contributed to the better magnetic performance. Our observations that cell labeling with MPIOs did not result in lower signal compared to free MPIOs agrees with Melo et al. and Makela et al., who also employed MPI relaxometry and found that MPI signal was not significantly reduced for cells labeled with MPIO1 [1] and MPIO2 [61] compared to free MPIO. It is possible this difference is due to the composition of MPIO particles, which may limit aggregation and interactions between particles compared to other SPIOs.
Coating particles with PEG is known to prevent the aggregation and opsonization of particles, reduce macrophage uptake and increase the blood circulation time \[40,62\]. The MPI signal for PEG coated Synomag-D particles that we tested was also reduced after cell labeling. However, the reduction in signal was less than that observed for the plain Synomag-D particles. This may be related to less particle aggregation. Feng et al. compared polyethylenimine (PEI) and PEG coated SPIO particles for labeling macrophages and cancer cells. Like our findings, PPB staining showed that there was low cellular uptake with PEG coated particles. Particle aggregation was dramatically reduced for PEG coated particles compared to PEI \[62\]. We believe that when labeling cells with the PEG coated Synomag-D there was less particle aggregation compared to labeling with plain Synomag-D and that this lessened the signal loss after cell labeling.

For MPI cell tracking we are most interested in cellular detection limits and therefore optimizing free particle and intracellular particle sensitivity. However, we have also studied resolution, which will impact our ability to resolve the MPI signal from cells \textit{in vivo}. Both the core size and the hydrodynamic diameter have a strong impact on the particle resolution. The Langevin model of superparamagnetism predicts a cubic improvement in spatial resolution with magnetic core diameter. However, this does not take relaxation effects into account. Tay et al. found that resolution improves with core size up until approximately 25 nm \[18\]. Above this size the response, or relaxation, of the SPIO particles is slower and leads to blurring. This opposes the expected Langevin behavior causing resolution to stop improving after 25 nm. Resolution also deteriorates with increasing hydrodynamic diameter of SPIO particles \[18\]. The aggregation of particles in cells after labeling or, due to the use of TAs to enhance cell labeling, can lead to clustering and an
increase in hydrodynamic size. We observed a significant reduction in resolution after cell labeling for all particles other than MPIO1 and Molday-G. There was no significant difference in resolution after cell labeling for MPIO1, possibly due to the variability of the measurements. There was a significant improvement in resolution for Molday-G after cell labeling, this might be due to an increase in size caused by aggregation, bringing these small particles closer to the optimal size for MPI.

4.5 Conclusion

Many groups have shown exciting progress towards the design and synthesis of SPIOs tailored for MPI. However, this work, and that of others, has revealed that the MPI signal strength and resolution of SPIO particles can change dramatically after cell labeling. This may limit the use of some SPIOs for cell tracking. This work provides some insights into the choice of SPIO for MPI cell tracking.

Our initial enthusiasm for the observations of high particle sensitivity for Synomag-D and PMAO particles was somewhat diminished after finding that the MPI signal decreased significantly once these particles were inside cells. Ultimately, the intracellular MPI signal for MPIO3 was approximately the same as that for Synomag-D (50 nm) and PMAO2 because the signal for MPIO3 did not change after cell labeling. If faced with choosing between these three particles for experimental MPI cell tracking, there are a number of considerations. The first should be cell labeling. The amount of SPIO taken up by cells has a large impact on MPI sensitivity. This is affected by SPIO uptake efficiency and the amount of iron per particle. Using standard cell labeling protocols established in our lab all three particles had efficient cell labeling (>80%) and Synomag-D (50 nm) and MPIO3 had higher iron per cell than PMAO2. Since we did not try to optimize iron uptake, we can’t
conclusively say which particle would result in the highest amount of iron per cell. The various coatings and sizes of the SPIOs we tested will both impact iron uptake levels. We do know, however, that the MPIO particles contain more iron than other SPIO, they are often ~50% iron oxide by weight [52] and optimizing labeling with MPIO3 may result in further improvements to its MPI sensitivity for cell tracking.

Biocompatibility and stability *in vivo* are also important to be concerned about. For Synomag-D the particles are coated with dextran to allow biodegradation, increase colloidal stability, prevent aggregation in physiological buffers and reduce redox-reactive effects improving biocompatibility. MPIO particles are inert, so not biodegradable, which precludes their clinical use. Because they are inert, MPIO can allow the detection of cells for longer periods of time. This has been demonstrated with MRI cell tracking. Long term tracking of proliferative cells is challenging with the smaller SPIO because the particles are diluted in daughter cells and eventually the amount of iron per cell is too low to detect by MRI. However, Shapiro et al. have shown that even after MPIOs are diluted to a single particle per cell that cell can still be detected by MRI [63]. This should hold true for the detection of low numbers of MPIO labeled cells as well, for example monitoring of stem cell retention and survival in transplant models. The inert nature of MPIO3 may also be of benefit for long term MPI cell tracking. The custom synthesized PMAO particles are currently being tested in our lab with various surface modifications to optimize biocompatibility, stability, and cell uptake. Another consideration is the method of cell labeling. Cell labeling with Synomag-D required the use of TAs whereas MPIO3 and PMAO2 did not. TAs cause clumping and aggregation of particles which changes their magnetic performance. Other SPIOs requiring TAs to enhance cellular uptake can be
expected to behave in a similar way. The PEG-coated Synomag-D particles show less aggregation, which likely limits particle interactions, and as a result the magnetic performance after cell labeling was not affected as dramatically as for other non-PEG particles. However, cell labeling with PEG-coated Synomag-D was not as efficient as other SPIO.

In summary, the magnetic behavior of SPIOs is strongly influenced by their microenvironment and the process of cell labeling with SPIO for cell tracking with MPI can lead to signal loss. It is important to evaluate SPIO for MPI cell tracking prior to undertaking *in vivo* studies to understand the extent of the alterations in magnetic performance and to choose the most ideal SPIO for any application.
4.6 References


Chapter 5

5 Summary and Future Work

In this thesis, we present the first study to compare the metastatic growth of the MDA-MB-231BR cell line in both the nude and NSG mouse using longitudinal, noninvasive iron-based cellular MRI. Further, we demonstrate the first iron labeling of a patient-derived brain metastatic breast cancer cell line and longitudinally track its growth with MPI and BLI. Lastly, we compare different iron oxide particles and evaluate their magnetic performance alone and following labeling breast cancer cells with the particles.

5.1 Discussion and Conclusions

Despite the poor prognosis following a diagnosis of breast cancer brain metastasis, there are limited experimental models and methods for studying this disease. The majority of studies have relied on in vitro systems or ex vivo endpoint histology. This thesis employed numerous experimental imaging techniques to noninvasively monitor the growth of brain metastatic breast cancer and compare imaging agents for cancer cell tracking.

5.1.1 Chapter 2 – Comparing the fate of brain metastatic breast cancer cells in different immune compromised mice with cellular magnetic resonance imaging

In Chapter 2, we used iron-based cellular MRI to track the fate of iron-labeled brain trophic breast cancer cells (MDA-MD-231BR) in nude and NSG mice. This work demonstrates for the first time the use of in vivo longitudinal MRI based cell tracking to compare murine models of brain metastatic breast cancer by describing differences in the arrest, clearance, proliferation, and retention of cells between strains. The main findings were:
1. While the initial arrest, clearance, and retention of iron-labeled cells was similar between strains, significantly more brain metastases developed at an earlier timepoint for NSG mice compared to nude mice.

2. Post-mortem examination revealed visible tumour burden in the livers of NSG mice, with nude mouse livers appearing healthy. MR images obtained on ex vivo livers showed similar arrest of iron-labeled cells for both strains, but numerous regions of high signal intensity associated with liver metastases in NSG mice.

3. The duration of each model substantially differed between NSG and nude mice. Nude mice in this study reached an experimental endpoint of day 32 and NSG mice could only be studied until day 21 due to significant weight loss and liver tumour burden.

These findings demonstrate that more immune compromised mouse models may be more permissive for tumour growth and eventual metastasis. The early endpoint and quicker development of tumours in the NSG mouse brain and liver suggest that differences in immunity can influence cancer progression. A goal for this work was to evaluate the NSG mouse as a model for studying breast cancer brain metastasis, and therefore, we compared the growth of the 231BR cell line in this strain to the well characterized nude mouse. Cellular MRI provided a noninvasive method imaging strategy to acquire measurements of cancer cell arrest, clearance, and tumour burden. We predict that understanding the NSG mouse model for cancer progression will provide a platform for studying patient-derived xenografts and evaluating therapeutic efficacy.
5.1.2 Chapter 3 – A method for the efficient iron-labeling of patient-derived xenograft cells and cellular imaging validation

In Chapter 3, we successfully used the NSG mouse as a platform for studying the growth of a patient-derived xenograft breast cancer cell line and developed a method to iron-label these cells for the purpose of cell tracking. While previous literature has focused primarily on human metastatic breast cancer cell lines, there is momentum towards implementing PDX models to better reflect the histopathology, tumour behavior, and metastatic properties observed in the original tumour. We were the first to report successful iron-labeling of the triple-negative F2-7 breast cancer cells and perform imaging with MPI and BLI. The main findings were:

1. Perl’s Prussian Blue staining revealed that iron labeling of F2-7 cells with MPIO was most efficient at a concentration of 25 μg Fe/mL and labeling while cells were in suspension. Increasing the iron concentration and using a magnetic plate was found to be ineffective.

2. F2-7 mammary fat pad tumors were monitored out to day 42 with BLI. BLI signal increased over time, indicating that the PDX cells had successfully engrafted and proliferated over time.

3. Iron-labeled mammary fat pad tumours were monitored out to Day 28 with MPI. MPI signal from the MFP decreased over time, suggesting the clearance of iron from the tumour.

These findings show that luciferase expressing PDX cells can be labeled with iron oxide particles to allow for the in vivo, noninvasive, longitudinal cell tracking with both MPI and
BLI. While other studies have used either BLI or MPI to detect iron-labeled immortalized cancer cells *in vivo*, we demonstrate that these modalities are complementary. We show that BLI was able to monitor the viability of these cells longitudinally, however, it is limited by tissue attenuation of emitted light. This work demonstrates that MPI can address this as iron content can be directly quantified but does not provide measures of viability. As PDX models continue to become more utilized in cancer research, we predict that the work demonstrated here will provide a method to determine the fate of these cells *in vivo*.

### 5.1.3 Chapter 4 – Evaluating superparamagnetic iron oxide particles for cell tracking with magnetic particle imaging (MPI)

In Chapter 4, we used MPI relaxometry to evaluate the magnetic performance of different iron oxide particles following cell labeling of breast cancer cells. Previous literature suggests that particle characteristics such as core size and shape, hydrodynamic diameter and surface functionalization have all been shown to affect their magnetic properties [1–3]. Therefore, we evaluated several SPIOs, free in solution and after cell labeling to further understand SPIO features which produce the highest sensitivity and resolution for *in vivo* cell tracking. The main findings were:

1. MPI tailored SPIOs we tested that were designed to have high particle sensitivity lost this advantage after cell labeling, specifically Synomag-D and PMAO2.

2. MPIOs tested in this study did not show a loss in MPI signal following cell labeling.

3. Particles that required the use of TAs for efficient labeling, once mixed with TAs, and after labeling, showed that TAs reduce the MPI signal and that this is magnified once in cells.
The findings of this work show that MPI signal strength and resolution of SPIO particles can change dramatically after cell labeling. Our findings support previous work that demonstrates that the magnetic performance of SPIOs, in addition to their characteristics, can also be influenced by the local environment which can lead to aggregation and changes in particle relaxation. As both MPI sensitivity and resolution are closely related to the type of SPIO, this may influence the choice of SPIO for future MPI cell tracking applications. Additionally, we recommend that future work should focus on the development particles with features that are optimized for MPI performance, prevent or minimize aggregation within cells, and can label cells efficiently. This could include synthesizing particles using methods that better control for particle monodispersity within the optimal core size range. We also recommend that the SPIOs in this work that produced higher sensitivity and resolution on their own could be modified with features such as particle coatings that reduce aggregation and clustering in the cellular environment. Lastly, optimizing labeling methods that increase the iron content per cell may further improve cellular sensitivity, which is described further in Section 5.3.4.

5.2 Challenges and Limitations

5.2.1 Limitations of Iron-Based Cell Tracking

While iron-based cell tracking has significant benefits that are discussed and demonstrated throughout this thesis, there are several limitations of this technique to consider. First, in Chapter 2, we use iron-based cell tracking with MRI to detect disseminated cancer cells within the brain. Quantification of these iron-labeled cells with MRI is challenging, and current approaches are semi-quantitative in nature. In this thesis, we calculated the percentage of black pixels throughout the mouse brain images, which is a method that has
been established previously in our lab [4,5]. MPI can be used to address this limitation by providing a quantitative measure of iron content and estimates of cell number [6]. Bystander cell uptake of iron-labeled cells following cell death is another potential limitation for iron-based cell tracking which can lead to misinterpretation of the source of the signal loss [7–11]. This is a more significant problem for studies which aim to track the survival and retention of therapeutic cells. While the dilution of iron particles during repeated cell division in proliferative cells can result in a loss of detectability by MRI [12,13], in Chapter 2 we could differentiate between proliferative cells and nonproliferative cells based on the loss or retention of iron particles. Reporter gene technology, such as cells engineered to express luciferase genes, can remain stably expressed over time and provide measures of cell viability with proliferation [4]. This was demonstrated in Chapter 3 where we used both MPI and BLI to track breast cancer cells over time.

5.2.2 Challenges of PDX Models

In Chapter 3, we employed a patient-derived xenograft cell line called F2-7 which was developed from a triple-negative brain metastasis sample and established in NSG mice over two generations [14]. While PDXs have emerged as a clinically relevant platform for studying metastatic progression and therapeutic drug testing, there are several challenges associated with the establishment and maintenance of these models. First, since PDX models are developed to better recapitulate human disease, these cells can require several weeks to months to successfully grow out in culture [15,16]. In our experience, the F2-7 cells required 2-4 weeks in culture prior to use in in vivo experiments. This makes performing repeated and reproducible experiments challenging in a limited timeframe. Second, the incidence of metastasis are often low and may not occur spontaneously for
several weeks or months [17]. This also makes studying metastasis in a specific location challenging as any resulting tumour burden may not be specific to an organ of interest. Lastly, severe immune deficiency is required for the successful establishment of PDX cells in mice [18], and therefore, this platform is very limited for studies on cancer immunity. Due to these challenges, we did not continue with cell tracking studies using this PDX cell line moving forward.

5.3 Future Work

This thesis contains some of the first studies to use noninvasive molecular imaging to study the development and progression of brain metastatic breast cancer *in vivo*. Future work will be focused on applying MPI and BLI for longitudinal tracking of luciferase expressing iron-labeled cells disseminated within the brain. Further, development of new models of dormancy would provide additional platforms for interventional studies and characterization of this cell population. We also describe future work using MPI relaxometry to evaluate the magnetic performance of iron within immune cells would be valuable for advancing the field of immune cell tracking with MPI. Lastly, future work that focuses on optimizing labeling protocols with SPIOs would be valuable for improving cellular uptake and quantification for iron-based MPI cell tracking studies.

5.3.1 Longitudinal Tracking of Breast Cancer Brain Metastasis with MPI and BLI

In Chapter 3, we were able to detect the growth of an iron-labeled luciferase expressing mammary fat pad tumour over time with MPI and BLI. In future studies, we want to investigate whether we can visualize disseminated breast cancer cells in the brain with both modalities longitudinally. Iron-based MPI would enable quantification of iron content and
estimates of cell number, while BLI would provide a complementary technique to measure cell viability. This work would also be complementary to the longitudinal monitoring of breast cancer cells in the brain demonstrated in Chapter 2, as iron-based cellular MRI is limited by its inability to determine cell viability and semi-quantitative nature. Melo et al. demonstrated that we were able to detect MPI signal in the brains of mice injected with MPIO-labeled 231BR cells, however, this was only performed at one imaging time point in vivo [5]. Additionally, MPI images had poor resolution due to the use of MPIO. Using more optimal SPIO for MPI cell tracking, as explored in Chapter 4 and engineering breast cancer cells with a luciferase reporter gene, it would be interesting to track the retention of iron-labeled cells over time in the mouse brain to quantify the number of cells that persist and simultaneously monitor cells that proliferate with BLI. To implement this, preliminary experiments should be performed to develop an optimal MPI protocol for longitudinal brain imaging and determine the detection threshold for disseminated luciferase iron-labeled cells in the mouse brain.

5.3.2 Developing Models of Breast Cancer Dormancy

Discerning the factors that ‘awaken’ non-proliferative or dormant cancer cells are important for understanding the potential for future cancer recurrence, however, investigating these factors are limited by the scarce models of dormancy that exist. In Chapter 2, the cellular MRI results illustrate that a small quantity of non-proliferative cancer cells persisted in the brain until the experimental endpoint, which could then be no longer followed due to the overall tumour burden caused by the MDA-MB-231BR cell line in these mice. We believe the ‘nonproliferative’ iron-retaining cells represent dormant cancer cells based on previous evidence from our lab [19–21]. Our group has shown that it
is possible to use early WBRT to prevent tumour growth in a mouse model of HER2+ brain metastatic breast cancer while iron-retaining cells persisted for long periods of time as detected with iron-based MRI [20]. Future experiments could use this WBRT technique with the 231BR cell line to further understand the disseminated dormant cell population in a new model and implement additional imaging strategies such as BLI to observe if these cells are viable or can be awakened.

5.3.3 Evaluating Intracellular Iron in Immune Cell Lines

In Chapter 4, we used MPI relaxometry and imaging to assess the magnetic performance of a variety of iron oxide agents following cellular internalization by breast cancer cells. As MPI cell tracking is increasingly being used for studies of immune cell migration and immune cell therapies, understanding how SPIO are compartmentalized in immune cells and whether the MPI performance of SPIOs is altered in the same way in immune cells would be important to understand. Suzuka et al. studied changes in the MPI signal following labeling of a colon cancer cell line and macrophages with ferucarbotran (Resovist) [22]. In this study, they determined that the reduction in MPI signal after cell labeling was greater for cancer cells compared to macrophages and attributed this to differences in localization within the cellular compartments of cells seen with TEM. In macrophages, particles were located within the inner surface of cell compartments, while in cancer cells, particles were strongly aggregated within the center. They suggest that this difference may be due to different modes of endocytosis between cell types. Future work in our lab could continue to use MPI relaxometry to evaluate macrophages with other SPIO tested in Chapter 4, as well as other immune cell types, such as T-cells, dendritic cells, and NK cells. Preliminary experiments that focused on developing optimal labeling protocols
for each cell line with particles of interest would be a necessary and would provide a platform for iron-based cell tracking with each of these cell lines for additional studies. TEM should be employed following cell labeling to determine where iron is being compartmentalized in each cell type and how that might contribute to changes in MPI signal.

5.3.4 Optimizing Iron Labeling Protocols

In Chapter 4, we labeled 231BR cancer cells with a variety of different SPIO using standard cell labeling protocols established in our labs. While these labeling protocols were successful, they were not specifically optimized for each SPIO with this cell type. In future work, a series of steps will be implemented as routine for each new SPIO we use, to optimally label cells without affecting cell viability, and to sort labeled and unlabeled cells to improve quantification accuracy of iron labeled cells. These strategies include using magnetic column separation to reduce the number of unlabeled cells included in samples and Ficoll-Pacque density gradient to remove unwanted, extracellular iron. Gevaert et al. demonstrated the use of both strategies for both VivoTrax and VivoTrax+ and found that these techniques reduced MPI signal that was attributed to the presence of extracellular iron [23].
5.4 References


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Curriculum Vitae

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World Molecular Imaging Congress (WMIC)

02/21  Student Travel Stipend Award ($100 USD)
World Molecular Imaging Congress (WMIC)

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Metastatic Breast Cancer Research Conference (MBCRC)

07/21  Magna Cum Laude Merit Award – International Poster Presentation
International Society for Magnetic Resonance in Medicine

06/21  CIHR Gold Award of Excellence – National Oral Presentation ($500 CAD)
Canadian Student Health Research Forum

05/21-24  Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarships – Doctoral Research Award (CGS-D) ($35,000 CAD/year)
Canadian Institutes of Health Research (CIHR)
05/21 Ontario Women’s Health Scholars Award ($35,000 CAD) (Declined)
Council of Ontario Universities

05/21 Ontario Graduate Scholarship ($15,000 CAD) (Declined)
Western University

04/21 Canadian Student Health Research Forum Sponsorship and Nomination
Selected as top 5% of PhD Students in Canada
Schulich School of Medicine & Dentistry

03/21 Trainee Educational Stipend ($250 USD)
International Society for Magnetic Resonance in Medicine

09/20 Translational Breast Cancer Doctoral Research Scholarship ($18,000 CAD)
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Course: Medical Biophysics 3501A/9501A: Biophysics of Transport Systems

MENTORSHIP EXPERIENCE
10/21-04/22  Supervisor/Mentor, Western University, London, ON  
Student: Tasnia Nabil  
Project: “Magnetic particle imaging of therapeutic cells”

10/19-04/20  Supervisor/Mentor, Western University, London, ON  
Student: Kyle VanBeek  
Project: “Developing a novel technique for the quantification of iron labelled breast cancer cells arrested in the brain using magnetic resonance imaging”

RESEARCH EXPERIENCE
09/18-04/23  Graduate Research Assistant, Robarts Research Institute, London, ON  
Supervisor: Dr. Paula Foster, PhD
07/18-08/18  **Research Assistant**, Robarts Research Institute, London, ON
Supervisor: Dr. Paula Foster, PhD

09/17-01/18  **Research Assistant**, University of Waterloo, Waterloo, ON
Supervisor: Dr. James Beck, PhD

11/15-04/16  **Research Assistant**, University of Waterloo, Waterloo, ON
Supervisor: Dr. Emiko Yoshida, PhD

09/15-12/15  **Research Assistant**, University of Waterloo, Waterloo, ON
Supervisors: Dr. Crystal Tse, PhD and Veronica Brown, MEd

**PEER-REVIEWED PUBLICATIONS**


**BOOK CHAPTERS**

PRESENTATIONS

International


**NN Knier**, PJ Foster. Tracking cancer cells in the mouse brain with magnetic resonance imaging (MRI) and magnetic particle imaging (MPI). *International Society for Magnetic Resonance in Medicine*. Virtual Conference. May 2021. **Poster Presentation. Recipient of the Magna Cum Laude Merit Award.**


**NN Knier**, AM Hamilton, PJ Foster. Characterizing the fate of a brain metastatic breast cancer cell line in the severely immune-compromised NSG mouse with MRI. *Future of


**National**


**NN Knier**, PJ Foster. Comparing magnetic particle imaging (MPI) to magnetic resonance imaging (MRI) for the detection of breast cancer brain metastasis. *Canadian Student Health Research Forum*. Virtual Conference. June 2021. **Oral Presentation. Awarded to top 5% of Canadian PhD students. Recipient of the Award of Excellence (Gold Category).**


**Regional**


NN Knier, PJ Foster. Tracking cancer cells in the mouse brain with MRI and magnetic particle imaging (MPI). *Oncology Research and Education Day*. June 2022. **Poster Presentation.**


NN Knier, PJ Foster. Comparing the detection of breast cancer brain metastasis with magnetic particle imaging (MPI) to magnetic resonance imaging (MRI). *Rising Stars of MPI eSymposium*. Virtual Conference. March 2021. **Power Pitch Presentation.**


NN Knier, PJ Foster. Comparing the detection of breast cancer brain metastasis with magnetic particle imaging (MPI) to magnetic resonance imaging (MRI). *Ontario Institute of Cancer Research Translational Research Conference*. Virtual Conference. March 2021. **Poster Presentation.**


