PHYSICAL CO-REGISTRATION OF MAGNETIC RESONANCE IMAGING AND ULTRASOUND \textit{in vivo}

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

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2012

The use of complementary non-invasive imaging modalities has been proposed to track disease progression, particularly cancer, while simultaneously evaluating therapeutic efficacy. A major obstacle is a limited ability to compare parameters obtained from different modalities, especially those from exogenous contrast agents or tracers. We hypothesize that combining Magnetic Resonance Imaging (MRI) and Ultrasound (US) will improve characterization of the tumour microenvironment. In this study, we describe a co-registration apparatus that facilitates the acquisition of *a priori* co-registered MR and US images *in vivo*. This apparatus was validated using phantom data and it was found that the US slices can be selected to an accuracy of ± 100µm translationally and ± 2° rotationally. Additionally, it was shown that MRI and US may provide complimentary information about the tumour microenvironment, but more work needs to be done to assess repeatability of dynamic contrast enhanced MRI and US.
Dedication

To my parents - whom I love above all.

To my fiancée...
Acknowledgements

My supervisor Dr. Greg Stanisz has truly been an inspiration in the journey for my Masters degree. I would like to acknowledge him for all of his help and support over the past three years and more importantly, for giving me the opportunity of a lifetime and unprecedented research freedom - thank you. My committee members Drs. Peter Burns, Greg Czarnota, and David Goertz have also been instrumental in guiding me along the degree with regular committee meetings and progress reports - thank you for your insightful comments and feedback. At the beginning of my degree, Dr. Peter Bevan collaborated with me on pilot experiments in rats. He was critical in helping me get up to speed with ultrasound theory and equipment and initially proposed and developed the idea of the fiducial marker shape. Dr. John Hudson’s experience with microbubble US was very valuable to me and he has always been available to support this project and advance it. Dr. Hudson also provided code for fitting his model to our data and assisted in optimizing the model for use in animals.

It was a true pleasure to be in Dr. Stanisz’ group, as all members have become extremely close personally and professionally. I would like to thank Rafal Janik, initially a research engineer but now a PhD student in the lab for everything that he has done for me over the past three years - he was a great person to bounce ideas off. He has generously donated his time to help me better understand MR physics, build surface coils, learn more about MR hardware, and for anything else that I have needed. Also from the Stanisz lab, Colleen Bailey, Wendy Oakden, and Kim Desmond were also a great help in getting me up to speed with imaging and analysis techniques. I would also like to thank Anoja Giles, Adrienne Dorr, Krista Holdsworth, Denise Wright, and Michelle Martin for their expertise in animal handling and tail vein catheterization. To Ahmed El-Kaffas and Melissa Yin: thank you for collaborating with me, helping me and
supporting an environment where we could all explore our research interests freely. To David Green, Michael Pozzobon, and Mo Kazem from the SRI Machine Shop, thank you for providing your technical expertise and creativity, it was a great help in building the equipment necessary for this study.

Finally, I would like to thank all of the friends I made in Toronto over the past three years. Your encouragement, sociability, and goodwill truly made the past three years of my life memorable beyond all reason, I truly appreciate your friendship: thank you all.
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Acronyms and Abbreviations

- **AIF** Arterial Input Function
- **BOLD** Blood Oxygen Level Dependent
- **CEST** Chemical Exchange Saturation Transfer
- **CD31** Cluster of Differentiation 31
- **CT** Computed Tomography
- **2CXM** Two Compartment Exchange Model
- **DCE** Dynamic Contrast Enhanced
- **DTI** Diffusion Tensor Imaging
- **T_E** Echo Time
- **EES** Extravascular Extracellular Space
- **FDG-PET** Fluoro-deoxy Glucose Positron Emission Tomography
- **FGF** Fibroblast growth factor
- **FID** Free induction decay
- **FSE** Fast Spin Echo
- **FOV** Field of View
- **FWHM** Full Width Half Maximum
- **GUI** Graphical User Interface
• **GD-DTPA** Gadolinium Diethylene Triamine Pentaacetic Acid

• **IAUC** Initial Area Under the Curve

• **ITK** Insight Segmentation and Registration Toolkit

• **LLC** Lewis Lung Carcinoma

• **MHz** Mega Hertz

• **MRI** Magnetic Resonance Imaging

• **MRS** Magnetic Resonance Spectroscopy

• **NC** Negative Control animals

• **NEX** Number of Excitations

• **NIfTI** Neuroimaging Informatics Technology Initiative

• **PC-3** Prostate Cancer 3 cell line

• **PE** Profile Efficiency

• **RARE** Rapid Acquisition with Relaxation Enhancement

• **RF** Radiofrequency

• **ROI** Region of Interest

• **T_R** Repetition Time

• **SNR** Signal to Noise Ratio

• **SPECT** Single Photon Emission Computed Tomography
• **SPGR** Spoiled Gradient Echo

• **SPL** Spatial Pulse Length

• **TGC** Time Gain Compensation

• **TTP** Time to Peak

• **TUNEL** Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

• **Tx** Treatment animals

• **US** Ultrasound

• **VEGF** Vascular Endothelial Growth Factor
Introduction and Motivation

At least 50% of all cancer patients receive some form of radiation, either curative or palliative [1]. To supplement and complement radiation therapy, cytotoxic substances (chemotherapies) were developed to achieve maximum tumour cell kill. Numerous advancements in chemotherapies over the past decade have sparked an urgent clinical need for non-invasive methods to assess treatment efficacy. There is a knowledge gap in the use and synergistic combination of therapies, and many treatment regimens expose patients to higher toxicity to normal tissue than necessary. Despite known extremes in patient response to treatment - even in cancers of the same type and grade - doses are generally prescribed by the success of patient populations exhibiting similar disease manifestations. Each class of drugs has unique modes of action and still, there are no reliable and reproducible methods to assess synergistic benefits of combined therapy regimens [2].

Traditionally, a common measure of treatment response has been to track tumour shrinkage following treatment [3]. However, it has been shown that tumour shrinkage can take weeks and sometimes even months to manifest [4] and in some cases may not occur at all [5]. For instance, anti-angiogenic agents typically arrest tumour growth by...
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disrupting existing vasculature or inhibiting new vessel growth. Despite a positive effect, these agents may not necessarily lead to tumour shrinkage. This mode of drug action is better characterized by assessing tumour vascular function rather than gross changes in physical size. Development of quantitative and non-invasive methods to assess tumours following therapy continues to be a burgeoning field.

The benefits of developing early, non-invasive measures of treatment efficacy are clear for both patients and health care systems. For patients, if a standard treatment regimen is prescribed and deemed ineffective early in the treatment regimen, the treatment can be altered and patients can directly benefit from personalized care. As potentially ineffective and expensive therapies are recognized and screened for early, the average cost of treatment will decrease. As such, there has been considerable interest in predictive biomarkers for early assessment. Several candidates appear and a seminal paper catalogued a vast array of cancer cell genotypes into “six essential alterations in cell physiology that collectively dictate malignant growth in tumours” [6]. These six aberrations are: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of programmed cell death, 4) limitless replicative potential, 5) sustained angiogenesis and, 6) tissue invasion and metastasis [6]. For this study, we identify the 5th hallmark, angiogenesis, as our biomarker of choice to non-invasively assess tumour changes following treatment.

1.1. Angiogenesis

Angiogenesis in tumours is the process by which new blood vessels are recruited from the existing vascular network to promote tumour growth [7]. Normally, this process is regulated by several angiogenic and antiangiogenic factors such as αβ integrin, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [8]. In tumours
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however, this process is completely deregulated and excess production of growth factors from rapidly proliferating tumour cells leads to a drastic increase in vasculogenesis. These newly formed vessels are unstable and must mature with the addition of pericytes, cells that surround the endothelium providing structural support. Pericytes are often malformed and poorly distributed in solid tumours contributing to a malfunctional vascular network. Vessel growth patterns in tumours are generally accepted to be abnormal with a defective and leaky endothelium [9]. Irregular diameters of tumour vessels, abnormal branching patterns and leaky vessel walls all contribute to an increase in vessel permeability. It is estimated that a single hole larger than 0.5\(\mu\)m in diameter would alter the permeability of a vessel significantly enough to result in solute extravasation to be limited by blood flow [9]. Disorganized and inefficient blood flow also limits the delivery of macromolecules, such as chemotherapeutic agents via the blood. Poor perfusion in the tumour due to a disorganized vascular network impairs the delivery of systemic drugs to the whole tumour and ultimately, reduces efficacy. Several strategies have been proposed to maximize cell kill including the combination of different therapies (such as radiotherapy and chemotherapy) and agents that “normalize” the tumour vasculature and prime them for receiving chemotherapies [10]. Tumour angiogenesis is extremely important in tumour growth, progression and metastasis and is a promising target for novel therapies [11]. For instance, “measuring” tumour angiogenesis has the potential to serve as a highly predictive prognostic marker for disease outcome and treatment. Histology remains the gold standard for angiogenesis detection (microvessel density) but has several critical limitations. Histology requires biopsy samples and patient comfort aside, biopsies only sample a small fraction of the potentially affected organ. The lack of functional information from biopsies as well as the practical challenges of obtaining longitudinal biopsy samples make non-invasive imaging a promising technique to complement and potentially reduce unneeded biopsies. Animal models of cancer are often
used in imaging studies to assess therapy efficacy and provide a platform for exploring novel imaging techniques.

1.2 Animal models of cancer

Animal models of cancer, particularly mouse xenograft models, are extremely useful in drug development as well as assessing efficacy of existing drugs. Failure of novel chemotherapy drugs is often not determined until significant investments of time and money have been made designing and implementing clinical trials. It is beneficial to develop models of human cancers in mice that predict clinical outcomes [12]. Most drugs that prove efficacious in animals do not prove efficacious in humans. However, drugs that have tested positively in humans also prove efficacious in mice [12]. According to Céspedes et al., an ideal cancer model should have the following characteristics [13]:

1. Share histopathological features with the human tumour,

2. Progress through the same stages and result in the same physiological and systemic effects,

3. Share the same genes and biochemical pathways in both tumour initiation and tumour progression,

4. Reflect the response of the human tumour to a particular therapy and

5. Predict therapeutic efficacy in human clinical assays.

Clearly there is a long way to go in obtaining clinically relevant animal cancer models but in the meantime, animals models provide researchers with a valuable platform for developing novel agents for potential targets. Animal models are also excellent for developing novel imaging techniques because they are non-invasive and thus, significantly
easier to translate to the clinic. Confounding many preclinical animal treatment efficacy studies is the variability of response. Tumours are extremely heterogeneous and the biological variability between tumours is simply immense [14]. Within a specific tumour, there exist populations of cells that differ in their morphology, growth rate, receptor status and sensitivity to therapeutic procedures [14][15]. Regional differences in pH, degree of oxygenation, nutrient concentration cause pockets of hypoxia, apoptosis and necrosis scattered throughout the tumour [14][16]. Vascular reorganization, particularly following treatment[17][18], leads to an irregular and shifting tumour microenvironment, a moving target for imaging modalities [8]. However, measuring these changes functionally, longitudinally, and non-invasively may result in a better understanding of the tumour microenvironment.

1.3 Non-invasive imaging

Non-invasive imaging methods are proving indispensable for studying angiogenesis in vivo [19] as they provide researchers with quantitative information about blood flow, vascular permeability, vessel density, vessel function and blood volume. Imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) and ultrasound (US), have all been proposed for studying angiogenesis [8]. Each modality is optimal for probing a particular set of biomarkers. For instance, intravascular ultrasound microbubbles can be used to characterize the vasculature, FDG-PET can characterize glucose metabolism, and MRI or CT are often used to probe the rate of tracer leakage in tumours. However it has become increasingly clear that a single imaging modality can not provide all the information required to make consistently accurate diagnoses [20][22]. We posit that complementary imaging modalities must be used to-
gether to more accurately assess the complex tumour microenvironment.

To study angiogenesis and its effects on tumour growth and treatment response, the tumour environment needs to be probed using tools that assess both the interstitial tumour volume as well as the tumour vasculature. Nuclear medicine techniques such as PET and SPECT employ radiotracers that can be measured at picomolar concentrations but at a significantly lower spatial resolution. DCE-MRI and DCE-CT offer similar perfusion measurements (rate of leakage and leakage space) as both rely on the administration of a contrast agent that diffuses from the vasculature. DCE-CT is advantageous as it has a direct linear relationship between the contrast agent concentration and the image intensity (attenuation numbers, given by Houndsfield Units) \[23\]. The disadvantage of CT however is that it requires ionizing radiation and iodinated contrast agents used in CT have been shown to have lower safety profiles compared to MR contrast agents \[24\]. MRI can also be used to measure additional information such as diffusion (DTI-MRI), tissue oxygenation (BOLD-MRI), and various biochemical tissue properties (CEST and MRS). A key limitation of DCE-MRI and DCE-CT is the unavailability of intravascular contrast agents. Contrast agents in US are large, intravascular, and have the potential for providing reliable estimates of blood flow and blood volume - parameters that are available in DCE-MRI and DCE-CT models but are difficult to measure accurately due to parameter coupling. If MRI and US are used to image the same region in a tumour, the combined information of blood flow, blood volume and vessel permeability can be used to more completely assess the tumour microenvironment. MRI offers excellent soft tissue contrast at high spatial resolution deep in the body while US offers cheap and portable systems that feature real-time imaging of many structures in the body. In this project, we hypothesize that MRI and ultrasound (US) will improve non-invasive detection of tumour changes following therapy and aim to identify the advantages and limitations of these modalities in turn.
1.3.1 Principles of Magnetic Resonance Imaging

The phenomenon of nuclear magnetic resonance arises in atoms with an odd number of protons and/or an odd number of neutrons. In biological specimens, water is the most abundant molecule in the body and the hydrogen atom in water, carbon and many other sources contribute to the overall signal. Other MR-active atoms include $^{19}$F, $^{23}$Na and $^{31}$P. These atoms possess a property known as spin angular momentum (a quantum-relativistic phenomenon), which arises from the odd number of protons and/or neutrons. An ensemble population of atoms possessing angular momentum are referred to as a population of spins. In the absence of an external magnetic field, the angular momentum vectors of each spin is expected to point in all directions and the net magnetic dipole moment of the population sums to zero. If the spin population is exposed to an external magnetic field $B_0$, the spins will precess about $B_0$ and the nuclei will emit energy at a resonant frequency described by the Larmor equation,

$$\omega = \gamma B$$  \hspace{1cm} (1.1)

where $\gamma$ is the gyromagnetic ratio, a known constant characteristic for each nucleus. Despite the introduction of a strong magnetic field, the energies associated with the orientation of individual spins in $B_0$ are much smaller than their thermal energies, so the spins only have a slight tendency to point along the direction of the field \cite{25}. Due to the large number of spins the slightly increased tendency of spins to point along the field results in the formation of a longitudinal equilibrium magnetization, $M$. $M$ can be decomposed into two components, the transverse component ($M_{xy}$, initially zero) and the longitudinal component ($M_z$, initially a constant $M_0$). $M$ is the signal that can be (indirectly) measured in MRI and can be manipulated to generate different contrast between species.
The net magnetization vector $\mathbf{M}$ arising from spins is many orders of magnitude smaller than the external magnetic field so the MR signal cannot be measured when it is aligned with the external field. Application of an RF pulse $\mathbf{B}_1$ can be interpreted as a torque applied to $\mathbf{M}$, causing it to ‘tip’ down into the transverse plane so it can be measured. In the transverse plane, interacting spins exchange energy with both the surrounding spins, as well as the surrounding environment (lattice), and $\mathbf{M}$ relaxes back to its equilibrium value. Relaxation of the longitudinal component (also called spin-lattice relaxation) of $\mathbf{M}_0$ from 0 back to the equilibrium value is characterized by the time $T_1$,

$$M_z = M_z(1 - e^{-t/T_1})$$ (1.2)

Similarly, decay of $\mathbf{M}_{xy}$ from $\mathbf{M}_0$ to 0 is characterized by the time $T_2$ (also called spin-spin relaxation).

$$M_{xy} = M_0 e^{-t/T_2}$$ (1.3)

Signal detection in MRI takes advantage of several principles from electromagnetism. If a coil is placed near a changing magnetic field (precessing magnetic moment), the flux results in an induced voltage in the coil by Faraday’s Law. As the spins lose coherence, the rotating magnetic moment decreases in amplitude and the induced voltage also decays. The decaying signal is referred to as a free induction decay (FID) and is refocused using gradients and pulses to generate measurable echoes. These echoes are then used to form images.

At a particular magnetic field, $T_1$ and $T_2$ values are intrinsic to each tissue type and depend on local environmental factors such as temperature, proton concentration, and molecular mobility. Differences in $T_1$ and $T_2$ values are used to generate contrast.
between different tissues. For example at 3T, brain white matter has a $T_1$ of 1084 ms and gray matter has a $T_1$ of 1820 ms [26]. In a $T_1$ weighted image of the brain, imaging parameters are selected such that the $T_1$ recovery curves of the white matter and gray matter are maximally separated and the $T_1$ decay curves are minimally separated. The resulting image will distinguish white and gray matter based on their respective $T_1$s, with white matter hyper-intense and gray matter hypo-intense. On the other hand, in a $T_2$ weighted image of the brain, white matter is hypo-intense and gray-matter is hyper-intense as white matter $T_2$ decays faster (69 ms) than gray matter $T_2$ (99 ms). Contrast agents in MRI are typically $T_1$ shortening and are designed to increase $T_1$ contrast.

**Dynamic contrast enhanced MRI (DCE-MRI)**

Tracer kinetic models are often applied to DCE-MRI data to extract physiologically relevant information about the microcirculation. DCE-MRI is frequently used in the clinic to quantitatively assess the kinetics of a contrast agent bolus passing through the body. The presence of this agent, typically a paramagnetic species such as Gadolinium, results in a decrease of the longitudinal relaxation time ($T_1$) proportional to the contrast agent concentration. In a $T_1$ weighted image then, presence of a Gadolinium-based agent (for e.g., Gd-DTPA) yields an increase in signal intensity.

GD-DTPA is a small molecule that readily traverses the endothelium but not the cell membrane [27]. This property provides a mechanism by which the dynamics of vascular leakiness (exchange between capillary bed and the tissue) can be evaluated. Choosing a kinetic model to fit the data requires some prior knowledge about the organ or system in question. For instance, the presence of the blood brain barrier in the brain dramatically alters the contrast agent kinetics. Similarly, in leaky tumours the extravascular contrast agents typically used in DCE-MRI leak out (and back in) of vasculature considerably faster than other tissues. Sourbron et al. indicate that choice of a tracer kinetic model
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should provide a link between relevant physiological parameters and measured data [28].

A number of models have been proposed and the most general of these is referred to as the standard two compartment exchange model (2CXM) [29] summarized in Fig. 1.1.

Figure 1.1: Graphical description of the two compartment exchange model. An arterial input function (AIF) governs the introduction of the tracer in the vascular compartment via a bolus intravenous injection. The two transfer constants - $K_{\text{trans}}$ and $k_{\text{ep}}$ - describe the rate at which the tracer is exiting and re-entering, respectively, the vascular component.

A principal assumption of the 2CXM is that the system of interest can be modelled as two compartments, the vasculature (blood plasma) and the extravascular extracellular space (EES). A bolus intravenous injection delivers the contrast agent (or tracer) and is characterized by the arterial input function (AIF). This input function is used to calculate the tracer concentration in the blood plasma over time. Because the tracer is small, exchange between the two components occurs readily. The rate constant describing the transfer of the tracer from the blood plasma to the EES is termed $K_{\text{trans}}$ and the rate constant in the reverse direction (EES to blood plasma) is referred to as $k_{\text{ep}}$. $K_{\text{trans}}$ and $k_{\text{ep}}$ are related by the volume of the EES,

$$k_{\text{ep}} = K_{\text{trans}} / v_e$$  \hspace{1cm} (1.4)

where $v_e$ is the volume of the extravascular extracellular space per unit volume of tissue. The generalized kinetic model of the tracer in the tissue ($C_t$) is given as a differential
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equation,

\[ \frac{dC_t}{dt} = K_{trans}C_p - k_{ep}C_t, \]  \hspace{1cm} (1.5)

where \( C_p \) is the tracer concentration in the blood plasma and \( C_t \), \( K_{trans} \), \( k_{ep} \) are as described above. Solving this differential equation requires an initial condition and the AIF provides the tracer concentration at the arrival time, before any exchange in the system occurs (\( t=0 \)). Thus, modelling of DCE-MRI data to produce quantitative and physiologically relevant values of \( K_{trans} \), \( k_{ep} \) and \( v_e \) requires an accurate calculation of the AIF. In clinical studies, determining the AIF on a per-patient basis is already accepted as a clinical research standard. Direct measurement of the AIF is typically performed in an artery within the imaging field of view but such a vessel is not always present and when it is, partial volume, in-flow and signal saturation effects all contribute to confounding AIF measurements. Direct measurement of the AIF is notoriously difficult in small animals using both invasive techniques (small total blood volume prohibits sampling) and non-invasive methods (requires high temporal and spatial resolution to accurately resolve the AIF). Current AIF measurement methods include shunting blood from the carotid artery through a receiver coil and then back into the animal [30] and using a separate receiver coil around the chest [31] or tail [32]. Alternate strategies include using a published population AIF from the literature or assuming a mathematical function that models the physiology [33]. Unfortunately, these strategies are wrought with pitfalls as the inter-subject variability of the AIF may be significant and parameters may vary up to 30\% depending on the selected AIF [34]. In particular for pre-clinical studies, published AIFs are not representative of all mouse models as considerable physiological changes exist between species [34]. Needless to say, these techniques are all complex, invasive, time consuming and resource intensive and ultimately, add another dimension to the project.
In this study, we have chosen a model-free approach to analyzing DCE-MRI data. The leading candidate for model-free analysis is the initial area under the enhancement curve (IAUC). This parameter can be interpreted as a measure of the amount of contrast agent delivered to and retained by the tumour in a specific time period \[35\]. An example of a typical DCE-MRI enhancement curve with the area under the first 60 seconds of the curve (IAUC60) shaded in red is shown in Fig. 1.2.

![DCE-MRI Signal Enhancement Curve](image)

Figure 1.2: DCE-MRI data from an ROI in a mouse tumour xenograft are shown. Contrast agent concentration increases rapidly upon bolus administration and then equilibrates. The red shaded region indicates the IAUC60 value for this tumour. The IAUC is a mixed parameter containing information about both the blood flow (slope of initial enhancement) as well as the blood volume (area under the curve once enhancement plateaus).

### 1.3.2 Principles of Ultrasound

An acoustic compression wave consists of a repeating pattern of high and low pressure regions propagating through a medium. A particle in a medium with a wave propagating through will experience alternating regions of compression and rarefaction due to the energy of the wave. An acoustic compression wave can be described by its spatial period \(\lambda\) and temporal period \(\tau\). The temporal and spatial periods respectively refer to the elapsed time \(\tau\) and distance \(\lambda\) between two successive peaks (or troughs). A wave
can be characterized by,

\[
\lambda = c\tau = \frac{c}{f}
\]  \hspace{1cm} (1.6)

where \(\tau\) is the temporal period, \(f\) is the temporal frequency, \(\lambda\) is the spatial period and \(c\) is the speed of the wave. Conventionally, acoustic waves are described using the inverse temporal period, the frequency. Sound waves emitted at a frequency above the limit of hearing for healthy young adults (approximately 20 kilohertz or kHz), is defined as ‘ultra’-sound. Typical diagnostic imaging using ultrasound is conducted with operating frequencies ranging from 2 to 10 MHz. The speed of sound travelling through a medium is characteristic of several parameters, including temperature, compressibility and density. Formally, the speed of sound in a material is given by,

\[
c = \sqrt{\frac{1}{\rho \kappa}}
\]  \hspace{1cm} (1.7)

where \(\rho\) is the material density and \(\kappa\) is the material compressibility, an intrinsic, measurable quality of the material. Speed of sound measurements in vivo have produced values ranging from 1400 to 1650 ms\(^{-1}\) in various biological tissues at body temperature. The acoustic impedance of a material - analogous to electrical impedance - is a measure of how ultrasound propagates through that material and is given by,

\[
Z = \rho c
\]  \hspace{1cm} (1.8)

where \(\rho\) is the density of the material and \(c\) is the velocity of the acoustic wave through the medium. Generation of an ultrasound image relies upon the production
and detection of echoes, which are reflections of sound produced at the boundary of two materials with different acoustic impedances. A portion of the acoustic energy from the propagating wave is transmitted to the second material, some portion is reflected back towards the transducer and a certain amount of energy is lost, absorbed by the surroundings (for e.g., converted to heat). Image intensity corresponds directly to the strength of the echo from a particular interface, and the strength of the echo corresponds to the difference in acoustic impedances of the two materials. In other words, large impedance mismatches will result in strong echoes and small mismatches will produce weak echoes. This introduces an interesting problem with imaging deep structures: because the energy of the initial ultrasound beam is decreasing with depth due to reflection, refraction, scattering and absorption, structures that are further away from the transducer will result in weaker echoes. The loss of sound energy due to all mechanisms is termed attenuation. To account for depth dependent ultrasound signal attenuation, all manufacturers employ time-gain compensation (TGC) during beam-forming to amplify the signal coming from deeper in the tissue.

Transducers are used to generate ultrasound and work on the piezoelectric principle. If mechanical stress is applied to a piezoelectric material, the material will produce an electric field that restores its shape. Conversely, if an electrical pulse is applied, the material will physical deform, generating a pressure wave. The type of piezoelectric material, as well as the amount of electrical energy used to stimulate the material will result in a pressure wave of a particular power and frequency. A transducer with an array of elements, each capable of producing a pressure wave by piezoelectric excitation is called an array transducer. A phased array transducer contains additional time-delays to offset the pressure waves from each element so all transmit signals passing to the elements are adjusted based their location in the array to ensure all the waves converge at the focal point at the same time (Fig. 1.4). Echoes produced by the scattering object in the imaging
Figure 1.3: A schematic of the resulting US beam from a typical phased linear array transducer. The focal point (red dot) is shown at the point individual pulses from each element in the array are directed.

plane are also received by the individual transducer elements and adjusted to account for element spacing. In this study, a phased linear array transducer was used with 64 active elements and 256 total elements distributed linearly over the transducer face \[36\]. An image is constructed by combining together sequential acquisitions of “amplitude lines (or A-line)” along the axial direction. For each acquired A-line, a different set of adjacent 64 transducer elements are utilized for ultrasound generation and echo reception. To acquire each A-line, 64 active elements are stimulated and a pressure wave is generated with a characteristic spatial pulse length given by,

$$SPL = \lambda N_{cycles}$$ \hspace{1cm} (1.9)

where \(N_{cycles}\) are the number of cycles in the pulse. As this wave propagates through a medium, it diffracts and the effective beam width increases with depth. The beam
width has implications for both the axial and azimuthal (or, lateral) resolutions of the image. Axial resolution is defined as the minimum distance that two point scatterers positioned along the long axis of the beam and still be imaged as separate objects. For a given SPL, the axial resolution $R_{\text{axial}}$ is given by,

$$R_{\text{axial}} = \frac{SPL}{2} = \frac{\lambda N_{\text{cycles}}}{2}$$  \hspace{1cm} (1.10)

Conceptually, this pulse will only produce two distinct echoes at two interfaces positioned $x$ mm apart along the axis if $x$ is larger than half the spatial pulse length. The azimuthal resolution of a particular position on an image is simply the beam width at that point. The beam width is a parameter dependent on the transducer design, including the element size, the fixed lens as well as the electronic focusing ability. A fixed concave lens focuses the beam in the elevation direction and azimuthal focusing is done electronically.

**Contrast mode imaging**

Discussion of generating an US image has so far been limited to B-mode, or brightness mode where image intensities correspond directly to the strength of the received echo from interfaces. B-mode imaging works well enough for many anatomic imaging situations. However, if the desired application is to provide contrast between vascular regions and soft tissue, because the echogenicity of blood is very low compared to muscle and tissue, contrast agents can be used. US contrast agents are gas filled lipid shells called microbubbles that resonate at a particular frequency when driven by a mechanical wave. The resonance behaviour is non-linear and generates additional pressure waves and can be
Figure 1.4: Schematic of an 11-element phased array transducer is shown. Piezoelectric elements are excited with an electrical stimulus (transmit pulse) to produce a pressure wave. Each element is stimulated with a delay $\tau_n$, depending on its position in the array and the resulting wavefront after the central element has been stimulated is shown. For the pressure waves to converge at the focal point simultaneously, delays for the outer elements $\tau_5$ and $\tau_{-5}$ (furthest from the focal point) are set to zero and the delay for the central element ($\tau_0$, closest to the focal point) is the maximum[37]. This process is one component of beam forming on transmit but when the echoes are received a similar process called ‘dynamic focusing’ occurs. The basic idea of dynamic focusing on reception is to adjust the time delays such that as each scattered wave arrives at the receive element, the detected signal is delayed to focus where the signal originated. This image has been adapted from Richard Cobbold [37]
1.3. NON-INVASIVE IMAGING

detected by the transducer. Strong echoes are also generated due to the large impedance difference between the blood and the gas-lipid interface. Contrast mode imaging takes advantage of the non-linear signal generation from bubbles. In the absence of bubbles, if two successive ultrasound pulses are transmitted, the corresponding echoes and image should be identical. If however, the second pulse is twice the amplitude of the first pulse, the images will look very similar except that the second image will be twice as bright as the first. If the two images are subtracted and weighted by their pulse amplitude (i.e., \(2 \times \text{Image1} - \text{Image2}\)), the resulting image should be zero. In the presence of non-linearly behaving microbubbles however, this weighted subtraction will not be zero. All of the linearly-behaving signal will get cancelled out but any of the non-linear signal from micro bubbles will remain. This type of processing is called 'Amplitude Modulation', and is a powerful technique that enables US scanners to remove signal contribution from linearly behaving tissue and interfaces and isolates the signal from strongly scattering microbubbles. Microbubbles have previously been shown to be intravascular in normal vasculature and several variations have been approved for clinical use.

**Dynamic contrast enhanced Ultrasound (DCE-US)**

DCE-US measurements are typically made by selecting an imaging plane, injecting a bolus of microbubbles (typically with a diameter of up to 10 µm) and characterizing the contrast agent kinetics. Parameters such as the time-to-peak (TTP), wash-in, wash-out rates as well as regional blood volume can be extracted. Unfortunately, the use of boluses presents several problems in obtaining quantifiable and repeatable DCE-US parameters. This technique works well for imaging a single plane but becomes time consuming if data from multiple planes is required as a second bolus cannot be injected until the first has cleared from the body. Furthermore, physiological variability in clearance rates between animals of the same species and xenografted tumours of the same type
make bolus injections a difficult technique to standardize. An alternative to this technique, first proposed \cite{42} in 1998 by Wei et al. and extended by others \cite{43,45}, sought to measure perfusion by introducing a “negative” bolus. First, a constant concentration of microbubbles is established in the blood stream by constantly infusing a relatively low concentration of microbubbles at a very slow rate. Then, a short burst of high intensity ultrasound (‘burst pulse’) disrupts microbubbles in plane. Microbubbles continue to circulate systemically and replenish the disrupted microbubbles. The signal intensity change resulting from the negative bolus can be quantified to meter perfusion \cite{45}. This technique is already available in the clinic \cite{46} and has been attempted by others in preclinical tumour models as well \cite{47,48,49}. In this study, a high frequency US system designed for small animal imaging \cite{36,50} is used and a variation on the general disruption-replenishment protocol was developed.

Attempts at modelling the replenishment of microbubbles in-plane have been made \cite{41,51,52}, fitting data to the mono-exponential mathematical function remains the most common \cite{45}. The mono exponential model assumes systemic circulation facilitates a perfect mixing chamber where microbubbles arriving following disruption are immediately and uniformly present everywhere in the tissue. This assumption is clearly invalid and in 2009, Hudson et al. proposed a model for microbubble replenishment in a vascular network with a distribution of vessel sizes and associated flow rates \cite{45}. A general representation of the replenishment signal intensity over time is given by,

\[
S(t) = \int_V B(x, y, z) \cdot F(x, y, z),
\]

where the signal intensity at time \( t \) is determined by the number of microbubbles in the imaging field governed by a flow function \( F(x, y, z) \) that is spatially weighted by the elevation beam profile \( B(x, y, z) \) \cite{45}. The elevation beam profile term is required
because of the non-uniform slice profile (discussed and measured in Chapter 2). The flow function $F(x,y,z)$ was derived by modelling a vascular network (assuming a fractal branching pattern) as a single vessel with spatially varying blood velocities following a log normal distribution. Quantitative model-fitting of DCE-US data has the potential to produce parameters such as the regional blood flow rate and the regional blood volume. In this study only replenishment data averaged over the whole tumour ROI were fitted to the model as the signal to noise ratio (SNR) for DCE-US data were insufficient for model-fitting. The initial area under the replenishment curve (IAUC) was used to construct tumour parameter maps preserving spatial heterogeneity. In DCE-US IAUC is interpreted as a measure of the amount of microbubbles present within the tumour vasculature and a sample enhancement curve with the IAUC60 shaded in blue is shown in Fig. 1.5.

**Figure 1.5:** DCE-US data showing a constant intensity from the tumour ROI prior to application of the burst pulse. Following the burst pulse occurring just before the blue shaded region begins, microbubbles replenish the imaging region and steady state is again reached after about 40 seconds. Replenishment data has been fit to the Hudson model and the fit result is shown in red. The blue shaded region indicates the IAUC for the first sixty seconds (IAUC60) of this tumour following the burst pulse.
1.4 Combining DCE-MRI and DCE-US

It has been acknowledged that with DCE-MRI perfusion models, it is not always possible to decouple the effects of blood flow and vascular permeability as both effects contribute to overall signal change [28, 53]. MR contrast agents, also called tracers, are typically small molecules that readily extravasate out of the vasculature. In the highly permeable tumour vessels, diffusion of the tracer out of the vascular space is fast, which leads to a consistent overestimation of the volume transfer constant ($K_{\text{trans}}$) and an underestimation of the blood volume fraction ($v_B$) [15]. Attempts to develop fully intravascular bio-compatible T$_1$ contrast agents have been met with variable success [54, 55]. Without intravascular contrast agents and reliable measures of the arterial input function, DCE-MRI should be used carefully when attempting to characterize parameters such as blood flow or blood volume [28]. However, extravascular tracers can effectively measure the size of the extravascular extracellular space ($v_E$), the space into which small contrast agents leak.

Ultrasound (US) contrast agents (gas filled microbubbles) are assumed to be intravascular [38-40] as they range in size from 1-10µm in diameter. US imaging can be performed at a significantly higher temporal resolution (up to 60 Hz) than MRI (less than 1 Hz). Higher temporal resolution allows for more precise perfusion estimates, particularly for time sensitive parameters such as time to peak, mean transit time and blood flow. There are significant practical obstacles to overcome when acquiring and processing US data: a non-linear depth-dependent slice profile, reproducibly imaging a 3D volume using 2D slices and signal attenuation by highly scattering structures such as bones. US also typically requires significant tissue compression to couple ultrasound from the transducer to the body and this can result in physiological changes such as vascular compression or an increase in the interstitial tumour pressure. Furthermore, registration of US images...
with many other modalities using existing image registration algorithms is difficult due to image distortions from a non-linear beam profile \[45\] so in this study, we explore a fiducial marker based method to acquire *a priori* co-planar US and MRI data.

Using a fiducial marker to facilitate combined imaging has been explored recently in rabbits \[56\] with a clinical US imaging system and 1.5T MRI system. While this approach is suitable for considering the challenges of translating this technology to the clinic, a different set of obstacles need to be considered when working at high frequency US and high-field MRI for small animals. There is a need to consider these obstacles as high-field MRI and high frequency US provide dramatic improvements in the temporal and spatial resolution. Novel analysis methods have also been developed recently for DCE-US \[45, 47\] and compared to parameters derived from DCE-MRI in small animal tumour models \[47, 57, 58\]. The biggest pitfall in combined MR and US imaging is that it is unknown if the same region is being imaged in both modalities. Many groups typically use DCE-US in a single plane with the largest cross-sectional tumour area \[47\] and thus, small shifts in positioning the US transducer or prescribing the MR slice may result in comparison of two heterogeneous regions confounding data interpretation \[49\]. Furthermore, nonlinearities in the US beam profile become more pronounced at higher frequency, and at high field MR, bore sizes decrease drastically in size and gradient non-linearity may become a factor in imaging. In this study, the contribution of these effects is considered in context for imaging tumours xenografted in small animals.

When MRI and US are used to image the same region in a tumour, blood flow and blood volume from US as well as vessel permeability \((K^{\text{trans}})\) and cellularity \((v_E)\) can be used to more completely assess the tumour microenvironment (Fig. 1.6). In DCE-US, it is expected that if microbubbles are intravascular, there should be no signal enhancement in avascular regions of the tumour. Conversely, signal enhancement in the same region using an MR contrast agent may show a gradual enhancement as the agent
1.4. COMBINING DCE-MRI AND DCE-US

Figure 1.6: Schematic of the aberrant tumour vasculature with some DCE-MRI and DCE-US parameters. DCE-MRI measurements were made by injecting a bolus of the MR tracer and model the signal intensity as a function of time to obtain the vessel leakiness ($K_{\text{trans}}$) and the leakage space ($v_e$). DCE-US measurements are made after microbubbles in-plane are disrupted by a burst pulse. Because the contrast agent is being continually infused, the replenishment curve can be characterized to provide regional blood flow and blood flow. Combining these parameters may provide a more complete picture of the tumour microenvironment.
slowly diffuses from nearby leaky vessels. In vascular rich regions however, it is expected that both US and MRI results in signal enhancement. Due to the different mechanisms of contrast generation in MRI and US, and because the tracers are vastly different (e.g., size, weight, diffusibility), the kinetics of the tracer varies and may provide additional information about the tumour microenvironment. Parametric maps of DCE-US and DCE-MRI in the same tumour at the same location may also provide complementary information of tumour response and ultimately, further insight into the mechanisms of therapy effect.

1.5 Summary of research objectives

The overarching goal of this project was to develop a pre-clinical platform for assessing changes to the tumour microenvironment following therapy using a DCE-MRI and DCE-US protocol. In addition to designing and constructing an apparatus to facilitate combined MR and US imaging, a key component of this work was to investigate potential sources of error, implement corrections where necessary and ultimately, validate the platform for use in collaborative studies. The thesis is divided into two chapters, validation of the apparatus and preliminary results from a pilot in vivo experiment. The first chapter is composed of a set of phantom experiments and analyses to assess contributions of potential sources of error. The second chapter describes a pilot in vivo DCE-MRI and DCE-US experiment with six animals, four treated with 8Gy and two controls. In addition to several analysis methods, challenges in data interpretation and study design are considered and potential experiments are proposed for further study into the area. In the fourth chapter, some interesting observations from our DCE-US studies, ideas to improve robustness of the apparatus and some challenges that need to be overcome are described.
Design and Validation of Co-registration Apparatus

This chapter contains details for designing and building an apparatus to acquire \textit{a priori} registered images. Several challenges associated with combined MR and US imaging are considered and experiments measuring the MRI and US slice profiles are detailed. Results of a phantom study to validate the accuracy of the apparatus are described and suggestions are proposed to improve accuracy further.
2.1 Introduction

In this study, we have chosen to acquire images registered a priori with a physical co-registration apparatus to demonstrate the potential for co-registered MR and US imaging. An apparatus was designed and constructed to facilitate co-planar image acquisition. It is important to consider the acquisition volumes when attempting multi-modality imaging due to partial volumes and slice profile effects. In ultrasound imaging for instance, linear array transducers have a characteristic beam profile highly dependent on the geometry of the individual elements as well as the acoustic lens that focuses the beam in the elevation direction (z-axis). In MRI the acquisition volume is often assumed to be a slab (3D slice) with perfect edges, but this assumption fails with imperfect excitation pulses and gradient non-linearities. The slice profiles of both MR and US images were characterized in phantoms using scan parameters similar to our in vivo studies. Potential sources of error in the acquisition such as the ultrasound beam profile and the MRI slice profile are also presented. Gel phantoms were used to validate the limitations of the apparatus assuming six degrees of freedom (translations along the x,y and z dimensions as well as rotations $\alpha$, $\beta$, $\gamma$).

This study is separated into three sections discussing first the MR slice profile, then the US slice profile followed by the validation experiment conducted to describe the accuracy of selecting co-planar imaging slices using the apparatus. A schematic of the MRI slice geometry, direction conventions and US beam geometry are shown in Fig. 2.1. The coordinate axes x,y, and z conventionally used in MRI correspond to azimuthal, axial and elevation directions conventionally used in US.
2.2 Co-registration apparatus

To enable co-planar imaging with MRI and US, an apparatus that minimized animal motion and facilitated the acquisition of images registered a priori was designed (Fig. 2.2). Principal components of this apparatus are: a) a long platform with four poles to mount a water tank, b) a water tank with an acoustically transparent membrane (McMaster Carr; Los Angeles, USA) and c) a 3D-printed fiducial marker insert. The apparatus was designed so an anaesthetized mouse could be placed on the platform and moved between the MRI and US systems. For phantom imaging, a block of agar with inclusions was placed on the platform and US gel (Sonotech - LithoClear; Bellingham, USA) was applied generously to couple the ultrasound from the water tank to the phantom. Positioning of the US transducer involves adjusting pitch and roll angles to minimize skew in the fiducial marker, as described in Fig. 2.3. Adjusting the yaw angle (γ) was rarely required as the water tank was constructed so the MS-250 transducer sat flush against the sides. Prescribing the MRI slice was significantly easier as the MRI system allows for oblique
2.2. CO-REGISTRATION APPARATUS

angle prescriptions parallel to the fiducial marker (Fig. 2.4).

![Photograph of the physical co-registration apparatus with its constituents. The animal was placed prone on the platform and the water tank with membrane was lowered onto the tumour-bearing hind limb. US gel was applied to the tumour to allow for ultrasound transmission.](image)

**Figure 2.2:** Photograph of the physical co-registration apparatus with its constituents. The animal was placed prone on the platform and the water tank with membrane was lowered onto the tumour-bearing hind limb. US gel was applied to the tumour to allow for ultrasound transmission.

![Images showing pitch (α) and roll (β) angles.](image)

**Figure 2.3:** The fiducial marker was used to position the transducer to minimize the pitch (α) and roll (β) angles to ensure parallel (to the marker) imaging slices are acquired. Adjusting the transducer pitch angle was relatively straightforward as its effect (skewed image) was easily visible (top left and bottom left). Adjusting the transducer roll angle requires sweeping the transducer back and forth over the edge of the fiducial marker. If there was a significant roll angle (top right), it will become manifest with a difference of intensity at the edges of the image (arrows). When the transducer has a minimal roll angle, the intensities at the edge of the fiducial marker should be very similar (bottom right).
2.3 Ultrasound beam profile

2.3.1 Methods

The focus of an ultrasound transducer is fixed and determined by an acoustic lens in the elevation direction (z-axis). Thus, the ultrasound beam converges at the focus and then diverges, so the elevation width of the beam varies as a function of distance from the transducer (Fig. 2.1). The transducer (MS250 - VisualSonics; Toronto, Canada) was characterized using a needle hydrophone with a 40 µm active element diameter (Precision Acoustics; Dorchester, UK) and calibrated to 60 MHz by National Physical Laboratories (NPL; Teddington, UK). A water tank was filled with degassed and deionized water with the transducer positioned relative to the hydrophone using a motion-control system with micrometer resolution (U511 - Aerotech; Pittsburgh, USA). The beam width in the elevation direction was estimated by measuring maximum positive or negative acoustic
2.3. ULTRASOUND BEAM PROFILE

pressure at coordinates of interest in the 3D imaging field. To increase the signal-to-noise ratio, 32 repeat measurements were averaged at each location. The transducer was set to transmit at a frequency of 21 MHz with a pulse repetition frequency (PRF) of 10 kHz. The hydrophone was used to measure the pressure response at each point in the y-z plane, y = 0 mm to y=29mm in the axial direction relative to the transducer face and z= -2.5mm to z=+2.5mm in the elevation direction relative to the transducer midline (Fig. 2.5A). At each coordinate, the received voltage signal was acquired over 25µs at a sampling frequency of 500MHz (Fig. 2.5B). Peak positive voltage values were determined at each coordinate by taking the Hilbert transform and selecting the maximum value of the resulting envelope. Peak positive voltages were normalized to the maximum received voltage (on-axis at the natural focus of the transducer) to construct an US beam field map (Fig. 2.5D). Because the voltage data were normalized, absolute pressures were not calculated but NPL provides calibration data to convert voltage values to pressure. Normalized voltages acquired along the elevation direction were plotted and the full width at half maximum (FWHM) value was calculated and reported as the elevation beam width of the transducer (Fig. 2.5C) at each axial position.

The square of the normalized voltage signal is proportional to the amount of energy received by the transducer if a point scatterer was placed at a spatial coordinate within the beam field. Analysis and processing of this data leads to the point spread function (shown in Fig. 2.5C). The profile efficiency in US, PE\textsubscript{US}, was defined as the ratio of two calculated quantities: 1) the total energy from an ideal 1mm thick slice within the region of interest and 2) the total energy from the entire region of interest (i.e. the portion of the field of view that contained echo-producing structures). Essentially, the PE\textsubscript{US} was used to estimate the signal contribution of a 1mm thick slab to the final image.
2.3. ULTRASOUND BEAM PROFILE

Figure 2.5: A) Schematic of the beam-profile in the axial-elevation (y-z plane) plane. To measure the profile, a transducer was set up to emit a pulse and a hydrophone was used to record the received pressure wave (in the form of a voltage) at each coordinate in the plane. B) Sample voltage-time data shows the arrival of the pressure wave indicated by a change in the recorded voltage. The peak amplitude of the voltage signal at each coordinate was used to represent the maximum signal recorded at that coordinate. C) Plot of the normalized peak amplitude vs. elevation (z-axis) distance from the centre of the transducer for a sample line in the axial-elevation plane. As expected, the peak amplitude at the transducer midline produces the largest recorded signal. The FWHM is reported as the slice thickness. D) Normalized beam geometry map, equivalent to a point spread function in the axial-elevation plane with red representing maximum voltage (1) and blue representing zero voltage.
2.3.2 Results

The elevation (z-axis) beam width of the transducer, defined as the full width at half maximum (FWHM), was calculated and plotted as a function of distance from the transducer. Measurements were taken with the adjustable azimuthal (x-axis) focus set every 2 mm from 4 mm to 28 mm to assess its effect on the US slice thickness. Based on the measured beam widths as well as the size of tumours, a phantom was constructed with the axial (y-axis) focus set at 18, 20 or 22 mm below the transducer. Figure 2.6 shows the mean slice thickness of the values obtained by setting the axial focus at these positions with an assumed 5% error in hydrophone positioning. Error bars on the y-axis of the plot represent the standard deviation in the three measurements used to calculate the mean slice thickness at each axial position. The elevation beam width was largest 1mm away from the transducer face at $2.35 \pm 0.02$ mm and smallest 11mm away from the transducer face at $0.289 \pm 0.002$ mm. The profile efficiency $PE_{US}$ for this experiment was $0.84 \pm 0.02$.

2.4 MRI slice profile

2.4.1 Methods

The Hermite pulse is set as the default slice select pulse for many MRI scans on the Bruker 7T system as it represents a fair compromise between low power requirements (enabling lower $T_E$ and thus, faster imaging) and a good slice profile. The Sinc10h pulse (Hanning-windowed sinc with ten zero crossings) is often used when a better slice profile is required, at the cost of slightly higher power requirements. While other slice select pulses are available, they were deemed unsuitable for this study. The profiles of both the Hermite and Sinc10h slice select pulses (shown in Fig. 2.7) were measured by imaging a
Figure 2.6: Slice profile of US plotted against the distance from the transducer. Based on typical tumour sizes, beam geometry, fiducial marker size and apparatus geometry, the ‘tumour imaging area’ - defined as the region within an US beam where the tumour was located - was selected to be between 13 and 22m from the transducer face.
2.4. MRI SLICE PROFILE

50mL tube filled with deionized water using a spoiled gradient echo sequence (TR/TE = 10000ms/15.3 ms) repeated for several flip angles between 5 and 90 degrees.

Acquisition parameters were altered to turn off the phase encode gradient and the read and slice-select gradients were set to be applied in the z-direction (along the bore of the magnet). With the slice prescribed to a thickness of 5mm (spatial resolution 0.1 mm/px), centred at z=0mm, the x-axis of the resulting one dimensional intensity image ranged from -5mm to +5mm. Images were then imported into MATLAB to normalize the intensity data (to the maximum intensity value in the image) and the normalized image intensity was plotted against distance to obtain the pulse profile. An ideal pulse profile has the shape of a rect function with a sharp rise, constant amplitude over the slice followed by a sharp fall (Fig. 2.7). The central 5mm integral area of each pulse profile was calculated and divided by the integral area of the ideal pulse to determine the profile efficiency, PE_{MRI}. This quantity provides an estimate of the actual imaged slice contribution relative to a hypothetical prescribed slice.

2.4.2 Results

Fig. 2.7 shows the slice profiles of the Hermite and Sinc10h pulses. Imaging with the default Hermite pulse yielded a PE_{MRI} value of 0.79 and the Sinc10h pulse significantly improved the slice profile with a PE_{MRI} value of 0.95. Furthermore, the Hermite pulse took longer to ramp up (and down) so the resulting image contained more signal from outside the prescribed slice than when using the Sinc10h pulse. To maintain slice fidelity, the Sinc10h pulse was selected for further in vivo animal studies.
Figure 2.7: Slice profiles of the Hermite (blue), Sinc10h (green) and a theoretical perfect rect pulse (red) are shown for the flip angle used in this experiment (15°). While the Hermite pulse results in a variable slice profile over the imaging region of interest, the sinc10h pulse was fairly uniform. The area under the pulses were integrated and normalized to the theoretical perfect pulse. \( PE_{MRI} \) values for the Hermite and Sinc10h pulses were 0.79 and 0.95 respectively.

2.5 Validation of co-registration accuracy

2.5.1 Methods

A gel phantom mould with inclusions was designed to validate the co-registration apparatus (Fig. 2.8). Two parallel cylinders and a square pyramid were used as inclusions. The gel body was composed of 6% (by weight) agarose and 1.5% silicon dioxide to increase contrast in US between the highly scattering gel body and the inclusions. Once the gel body set, inclusions were removed, filled with room equilibrated deionized, degassed water. Images generated from this phantom are shown in Fig. 2.8. The phantom was designed to have its features appear in the field of view at approximately the same location as the tumour during animal imaging. Acquisition parameters were matched with
animal imaging in both MRI and US. The goal of validation was to assess the degree to which the apparatus allowed co-planar imaging, so data were acquired at increased spatial resolution to reduce the contribution of the voxel size to mis-registration.

**Phantom imaging**

![Image of phantom mould and imaged slice](image)

**Figure 2.8:** A) 3D drawing of the mould used to create the gel phantom for validation. With the inclusions in, the mould was filled with agar. After the agar has set, the inclusions are removed and filled in with room-equilibrated deionized and degassed water. B) MR image of slice through the phantom in the $x$-$y$ plane. C) Corresponding US slice of the same slice through the phantom.

MRI was performed on a 7T scanner (Bruker Biospin; Bruker, Germany) with the BGA12-S gradient insert and an 86 mm volume coil used for both transmit RF excitation pulses and to receive MRI signal. A 3D fast spin echo (TR/TE =2000/4.8 ms) with an acceleration (RARE) factor of 50 and 10 acquisitions (NEX) was used to acquire
2.5. VALIDATION OF CO-REGISTRATION ACCURACY

100µm isotropic images of the phantom. Ultrasound imaging was performed using a high frequency pre-clinical imaging system (Vevo 2100, VisualSonics; Toronto, Canada) with the MS-250 transducer operating at a centre frequency of 21 MHz. To acquire high spatial resolution images, a stack of 2D images were acquired sequentially every 100µm. Acquired images were imported into MATLAB (The MathWorks; Natick, MA, USA) cropped, processed and exported as NIfTI volumes (data format by Neuroimaging Informatics Technology Initiative). Intensity based automatic segmentation was applied to mask out everything except the two cylinders and square pyramid. The two volumes were imported into the open source software Paraview and a centre of mass method was used to automatically estimate initial conditions for a rigid transformation. A custom implementation of the Insight Segmentation and Registration Toolkit (ITK) was created and utilized for the registration process (Martel Lab, SRI; Toronto, ON). The Euler 3D transform with a fixed centre of rotation and a simple mean squares metric used with the Regular Step Gradient Descent optimizer was used to register the volumes in 3D. The fixed volume was the ultrasound dataset and the MRI dataset was the moving volume. The moving volume was iteratively transformed to match the fixed volume until the metric was minimized. The final transformation of the moving volume to the fixed volume included translations ($T_x, T_y, T_z$) and rotations ($\alpha, \beta, \gamma$). The translations $T_x$, and $T_y$ were not relevant for validation since the co-registration apparatus was designed to ensure complete coverage of the slice in-plane ($x$-$y$ axis). The four remaining parameters - $\alpha, \beta, \gamma,$ and $T_z$ - constitute the error in plane prescription using this apparatus. Fixed and moving volumes were imported into Paraview to confirm successful registration.
2.5. VALIDATION OF CO-REGISTRATION ACCURACY

2.5.2 Results

Three independent trials were used to assess the repeatability of the setup process. Registration results are summarized in Table 2.1. The standard deviation of each parameter is an estimate of the error in the apparatus facilitating co-planar imaging, but it is recognized that the standard deviation is ill-defined for only three values. While pixel-by-pixel registration of the two imaged volumes was possible with this apparatus, the goal of this initial study was to investigate and minimize errors in the three rotation angles $\alpha, \beta, \gamma$ as well as $T_z$, the translation in $z$ (through-plane). The final registration result is shown in Fig. 2.9.

<table>
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<th></th>
<th>$\alpha$ (°)</th>
<th>$\beta$ (°)</th>
<th>$\gamma$ (°)</th>
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Table 2.1: Results for rigid registration of MRI and US volumes repeated three times.
2.5. VALIDATION OF CO-REGISTRATION ACCURACY

Figure 2.9: Data from both MRI and US were rendered in 3D as a surface and displayed on the same axis. On the left (A), the red structures from US appear offset in the z-direction (elevation) compared to the blue structures from MRI. There is also an x-y axis offset that can be calculated to centre the images (the field of view in MR is larger than the field of view in Ultrasound). On the right (B), the data is shown after image registration and includes the x-y adjustment to centre the images. In this case, we can see that features of the two datasets (MRI in blue and US in red) match more closely (the parameters yielding this transformation are shown in Table 2.1).
2.6 Discussion

An apparatus was designed and built to facilitate coplanar MR and US imaging for mouse tumour xenograft models. Phantoms were constructed to: determine the accuracy in the selection of imaging planes and validate the limitations of the apparatus. We have reported that the US slice profile was non-uniform and measured it to ensure the imaged region was contained within the MRI slice. The US beam geometry analysis discussed above was only valid under the specific imaging conditions under which the data were acquired. More specifically, the US beam field map was estimated using a single hydrophone (acting as a point scattering source) moving through the imaging field. During actual imaging conditions, the signal source is significantly more complex as there are many structures and features with radically different echogenicities that combine together to produce signal for the final image. Measuring the US beam field under actual imaging conditions was impractical and technically challenging and the best available option was selected.

The MRI and US beam profiles were used to verify that the prescribed slice actually contributed to the final images. Indeed, $\text{PE}_{US}$ and $\text{PE}_{MRI}$ were 0.84 and 0.95 respectively, indicating that a significant majority of the prescribed slices contributed to the final images in both modalities. By virtue of the larger slice thickness in MRI, it has been shown that 100% of the US slice was contained within the MR image. An estimate of the fractional volume that was imaged with both MRI and US can be made by taking the ratio of $\text{PE}_{US}$ and $\text{PE}_{MRI}$ and for the phantom study, that ratio was 0.88. In other words, 88% of the region of interest was imaged with both MRI and US. Furthermore, results from the phantom validation experiment suggest that the mean pitch, roll, yaw angles are less than 1.5° each and the total translation error through-plane (z-direction) was less than 0.1mm. Large PE values and small errors in pitch, roll, yaw and translation in
2.6. DISCUSSION

z direction suggest strongly that this apparatus was sufficient for use in situations where the limiting factor of co-registration accuracy are the voxel sizes of the coarsest imaging modality. If slightly higher accuracy is required, the slice thickness in the MR image can be reduced to overlap more closely with the US slice at the region of interest. Reducing slice thickness has other consequences including lower SNR and a decrease in temporal resolution to maintain full tumour coverage, but can be compensated for with a custom receive coil, reducing spatial resolution or increasing inter-slice spacing.

The MRI slice profile was also measured with the default excitation pulse (Hermite) but excitation using the Sinc10h pulse provided a significantly better slice profile and was selected for the in vivo animal study. There are potential disadvantages of using the Sinc10h pulse, such as increased specific absorption rate (SAR) and decreased temporal resolution (due to a longer pulse duration) but we do not anticipate these to be significant obstacles in a preclinical study. Gradient non linearities were considered as a potential confounding factor in MR image acquisition, but the magnet specifications indicate a 4% peak-to-peak linearity deviation of the gradients (BGA12-S, Bruker, Germany) over a diameter spherical volume of 80 mm. The sample volume (animal and apparatus) are well within this range of linearity.

Another potential source of mis-registration may arise with the assumed speed of sound on US scanners. Amongst many complex and proprietary post-processing steps performed after data acquisition, each US scanner creates an image by assuming a constant value for the speed of sound and the Vevo 2100 assumes a value of 1.50 mm/µs. While it is true that the speed of sound through tissue and water are similar, it is important to ensure whether the difference in speeds results in a significant shift in acquired images. Literature values of the speed of sound through non muscular soft tissue such as breast range from 1.48 to 1.53 mm/µs [59]. This difference corresponds to a shift of approximately 4µm within our imaging field of view, one order of magnitude smaller than
the ultrasound image voxel size and nearly two orders of magnitude smaller than the MR image voxel size. Due to the decreased penetration depth of high frequency ultrasound, it was not expected that the speed of sound differences would significantly affect image interpretation or analysis in the *in vivo* study.

The purpose of the co-registration apparatus was to minimize the error in selecting co-planar images. Quantitative voxel-by-voxel correlation of MR and US images was not an objective as there are significant additional challenges associated with interpretation, particularly with a depth-dependent spatial resolution. There was inherent error associated with the selection of coplanar imaging slices and some potential sources were discussed in the preceding sections. Assessing the true error was difficult without a gold standard but our approach in estimating the errors was to construct and image, at high resolution, a phantom with known dimensions using the apparatus. The preparation of the phantom for imaging was nearly identical to the preparation for animal imaging and because the acquired phantom volumes were assumed to be the same in physical space for both modalities, the volumes could be registered to each other using a 3D rigid registration algorithm. The resulting transformation parameters are an indication of the combined error in the apparatus to acquire coplanar MR and US images. A limitation of this approach to validating the apparatus was that the transformation was restricted to be rigid as there was insufficient information to place appropriate bounds on deformations. There are significant deformations in the ultrasound acquisition as the beam profile varies with depth. If greater accuracy is required, it may be important to carefully consider other sources of deformations and assess whether the errors can be improved with deformable registration. Ultimately however, deformations in the acquired images are inevitable and in the case of US images inherent so it appears the most practical workaround is to set up the experiment and analysis in a way that do not confound interpretation.
2.7 Conclusions

Despite the many challenges associated with combining MRI and US imaging, it is clear that there is potential for a synergistic combination of the two modalities. Intravascular microbubbles are an important tool in assessing vascular function not only in tumours, but also in cardiovascular systems, interventional and therapeutic work [39]. At the preclinical level, high frequency ultrasound is showing considerable promise for assessing the tumour microvasculature [40, 47, 60, 61]. In this study, we have explored some of the technical aspects of combining MRI and US and shown that with some initial efforts, a priori coplanar imaging is possible. The apparatus described in this chapter has been shown to be sufficient for co-planar imaging with MRI and US at a maximum spatial resolution of 100µm isotropic. With this condition, it is stipulated that tumour heterogeneities occurring at length scales larger than the pixel dimensions of the coarsest imaging modality (or 100µm, whichever is larger) cannot be studied.

In the next chapter, the apparatus will be used to assess changes in the tumour microenvironment following a therapy. Many chemotherapies appear to result in tumour regression and control at the preclinical level but fail in clinical trials [62]. The preclinical imaging platform described here has the potential to be used for assessing novel cancer therapies and combinations in a controlled environment. A controlled environment not only allows for assessing drug potential, but also facilitates understanding of the underlying mechanisms of actions. For instance, assessing the microvasculature network of a tumour using US and the surrounding extracellular extravascular volume using MRI provides different information. Ultimately, piecing together different information from the same tumour may lead to a more complete understanding of the tumour microenvironment and new techniques can be translated to the clinic with ease as US systems are available and accessible in many hospitals and most research centres.
3

Pilot *in vivo* Experiment

The focus of this chapter is to present the pilot animal experiments performed using the co-registration apparatus on an animal model of cancer. A significant portion of this chapter is also dedicated to obstacles that were overcome, outstanding challenges and potential solutions. An attempt was made to adapt the clinically available constant infusion protocol in small animals to permit multi-slice imaging with DCE-US. Tumours were imaged at baseline and 24 hours after a single large dose of radiation (8Gy) delivered to the tumour. Potential data analysis methods are also proposed and applied to an accurately co-registered set of MR and US images and compared to histopathology.
3.1 Introduction

The purpose of this pilot *in vivo* study was to gain some experience using the co-registration apparatus described in Chapter 2 to assess effects of a therapy. While it has been demonstrated that the apparatus is suitable for acquiring co-planar DCE-MRI and DCE-US data, this study aims to show the practical benefits of combining MRI and US *in vivo*. Furthermore, questions remain about the best way to analyze and interpret the acquired data and here we explore several analysis methods including parametric maps, voxel-based distributions, and ROI-averaged DCE-MRI and DCE-US curves.

3.2 Methods

The methods consist of a description of the experimental design and tumour implant procedure, set-up of the mouse in the apparatus, imaging considerations in MRI and US, data analysis techniques and histology.

3.2.1 Experimental design

Mice used in this study were 4-6 week old hairless females with the severe combined immunodeficient (SCID) phenotype, referred to as SHO (Crl:SHO-Prkdc<sup>scid</sup>H<sup>Hr</sup>) mice (Charles River; MA, USA). SHO mice were used because the SCID phenotype allowed us to also implant xenograft tumour cell lines (such as MDA-MB231, LS-174T, PC-3). The hairless phenotype eliminated depilatory chemicals for hair removal to reduce US scatter. Six SHO mice were injected with 5x10<sup>5</sup> Lewis Lung Carcinoma (origin: C57BL mice) cells (ATCC; VA, USA) subcutaneously in the hind limb and allowed to grow to a diameter of approximately 1cm (8-10 days). Tail veins were catheterized for contrast agent injections under isofluorane with a 27G needle and glued to the animal.
Figure 3.1: Experimental design of the in vivo experiment with four (4) treated animals (Tx) and two (2) control animals (NC).

for the duration of the imaging session. Anaesthesia was achieved with 95% oxygen and 5% isofluorane, maintained at 1.5-2% at a flow rate of 2mL/min. After catheterization, isofluorane was slowly weaned off and an intraperitoneal injection of a combined ketamine (150 mg/kg) and xylazine (15 mg/kg) mixture was given. All six mice were imaged at baseline and four were treated with 8Gy radiation for 8.0 minutes at 160kV and 6.3 mA using a small animal irradiator (Faxitron CP-160; Tuscan, USA). The mouse was entirely covered with a sheet of lead to prevent body irradiation and there was a small hole cut out of the sheet so only the tumour was irradiated. Treated animals were labeled T1 through T4 and negative controls were labeled NC1 and NC2. A schematic of the experimental outline is shown in Fig. 3.1. Experiments conducted in this thesis were approved by the Sunnybrook Research Institute Animal Care Committee (Animal Use Protocol 411).

3.2.2 Animals in the apparatus

Anaesthetized animals were placed prone on the platform (Fig. 3.2) and a rectangular piece of absorbing material (e.g., rubber) was used to elevate the tumour-bearing hind
3.2. METHODS

limb to dampen the effects of respiratory motion and reduce US scattering. Room-equilibrated deionized water was used to fill the water tank and couple the ultrasound from the transducer to the tumour. Acoustic transmission gel (Lithoclear - Sonotech; Bellingham, USA) was applied over the tumour and the surrounding area to couple ultrasound traversing through the water tank to the tumour. Depending on the size of the tumour, an appropriate fiducial marker was chosen and inserted into the water tank. The walls of the fiducial marker defined the imaging region and the entire tumour volume was contained between the fiducial marker walls. The ultrasound transducer was mounted on a motorized stage (VisualSonics, Toronto, Canada), aligned (as described in Chapter 2, Fig. 2.3) relative to the fiducial marker and manual adjustments were made to the transducer to minimize pitch, roll and yaw angles. The mouse was immobilized and neither the tank nor the fiducial marker insert moved relative to the tumour for the duration of the imaging session. DCE-US was conducted first and following it, the entire apparatus with the mouse was moved to the MR scanner for DCE-MRI. During US imaging, mice were kept warm with a heat lamp and during MR imaging, a heated saline bag was used to maintain the temperature.

3.2.3 Ultrasound imaging

US imaging was performed using a high frequency pre-clinical imaging system (Vevo 2100 - VisualSonics, Toronto, Canada) using the MS-250 transducer operating at a centre frequency of 21 MHz and data were acquired at 5Hz. Since this system was not capable of acquiring true 3D volumes, pseudo-3D imaging was accomplished by combining together contiguous 2D slices. A major limitation of imaging contiguous slices was that the acquisition time was cumulative. If multiple planes within a single tumour need to be imaged to account for tumour heterogeneity, each plane must be imaged separately.
Figure 3.2: Photograph of a mouse being set up in the apparatus. The mouse was placed prone on the platform with the hind and fore limbs taped down. The tumour bearing hind limb was elevated on a piece of rubber positioned to be within the walls of the fiducial marker. The poles on the platform were removed during set-up and once the mouse was positioned, ultrasound gel was applied on the tumour and the water tank was lowered onto the mouse (not shown).
and this time accumulates. The central slice as well as two immediately adjacent slices (± 1.0 mm from central slice) were selected for a disruption-replenishment experiment. Anaesthetized mice were continuously infused with a commercially available US contrast agent MicroMarker (VisualSonics; Toronto, Canada) diluted 1:20 in saline (median diameter in volume 2.3 - 2.9 µm according to the manufacturer), at a rate of 50µL/minute continuously for 30 minutes. The diluted concentration of the bubbles was approximately 1x10^8 microbubbles per mL and a total volume of approximately 1.5mL was infused.

Perfusion measurements were made recording US signal before and after disrupting microbubbles in the imaging plane using a high mechanical index ultrasound pulse (burst). Following the burst, microbubbles reperfused the imaging region and the replenishment curve was calculated on the whole tumour ROI as well as voxel-by-voxel. Three repeat measurements were made in each plane and a regional parameter map of IAUC was constructed by taking the difference of voxel intensities immediately following a burst and after 60s of reperfusion.

### 3.2.4 MR imaging

MR imaging was performed on a 7T scanner (Bruker Biospin; Bruker, Germany) with the BGA12-S gradient insert and an 86 mm volume coil used for both pulse transmission and signal reception. A high contrast anatomic image with spatial resolution of 0.2 x 0.2 x 0.5 mm was acquired with a 2D spin echo sequence (repetition time [TR] 3200ms, echo time [TE] 11.6ms with an acceleration factor of 8, 15 NEX (number of excitations). Despite known problems with 2D multi slice imaging techniques such as a variable slice profile, there are benefits to acquiring MR images in 2D. For instance, a spin echo sequence can be set up to produce multi-slice T₂ weighted images with full tumour coverage with reasonably high SNR and spatial resolution in under five minutes. The T₂ contrast is
necessary for picking out features in the image and a quick scan is needed to reduce the total time the mice are under anaesthesia.

For DCE-MRI, a 3D spoiled gradient echo sequence was used (TR/TE 7.8/3.8 ms, flip angle 15° and spatial resolution of 0.2 x 0.2 x 1.0 mm). A 0.2 mL bolus of gadodiamide (Omniscan, GE Healthcare, Milwaukee, WI) diluted to 0.05 mM/mL was injected manually and data acquired at a temporal resolution of 8.7s with full tumour coverage.

3.2.5 Data analysis

Tumour volume

In addition to a fast SPGR scan for DCE-MRI, prior to bolus administration, a higher spatial resolution (0.2 x 0.2 x 0.5 mm) T2 weighted fast spin echo anatomic scan (TR/TE = 3200/11.6 ms) was also acquired to quantify the tumour volume. Regions of interest were drawn manually on each slice by the same operator to maintain consistency. Voxel counts were converted to volume by using the voxel size.

DCE-MRI enhancement curves

Enhancement curves for DCE-MRI were calculated by manually drawing an ROI around the tumour boundary on Gd-enhanced images. Each dataset was normalized and signal enhancement was calculated according to the convention proposed by Galbraith et al. [63],

\[ E(t) = \left[ \frac{S(t) - S_0}{S_0} \right] \]  \hspace{1cm} (3.1)

where \( S(t) \) is the time varying signal intensity of an ROI and \( S_0 \) is the mean baseline signal intensity of the ROI (prior to bolus administration). The contrast agent injections were performed manually (i.e., without a syringe pump) and considerable variability in
Figure 3.3: Sample anatomic MR dataset showing tumour slices 0.5 mm apart (top) as well as the manually segmented tumour ROIs (bottom). Each box in the 4x4 grid is a single slice.
3.2. METHODS

the curves was observed. Further experiments are necessary to determine if the variability is due to physiology, injection rates or a combination of the two.

DCE-US enhancement curves

Replenishment curves for DCE-US were calculated by manually drawing an ROI around the tumour boundary avoiding the highly scattering layers of skin on tumour images immediately following the burst pulse. Each dataset was normalized for comparison by subtracting the signal from the ROI immediately following the burst pulse and then dividing by the mean plateau intensity prior to the burst. Normalized data for a whole tumour ROI were fit to the Hudson model (Fig. 1.5), but some optimization is required to improve SNR before it can be applied robustly voxel-by-voxel. Normalization and data fitting techniques are only suitable with sufficient smoothing (temporal or spatial - spatial in this case from the whole tumour ROI) and thus, was not applied to the generation of IAUC60 parameter maps.

IAUC60 parameter maps and distribution

While parameters determined from whole tumour ROI analyses provide some valuable insight about global tumour averages, tumours are characteristically heterogeneous and averaging the information leads to a loss of this heterogeneity. Voxel-by-voxel parameter maps retain tumour heterogeneity information, but interpretation was slightly more challenging as the information is generally presented visually and SNR is significantly lower [63]. In this study, DCE-MRI and DCE-US data were used to calculate IAUC60 maps on a voxel by voxel basis. DCE-US data were analyzed on linear data but log-compression was applied for visualization and interpretation purposes. The IAUC60 for DCE-MRI was calculated using a signal change ratio (Eqn. 3.1) and thus, the quantity was unit-less. IAUC60 values for DCE-US were calculated using non-normalized replenishment curves with arbitrary units. Single slice parameter maps were constructed for
DCE-MRI and DCE-US (Figs. 3.4A and 3.4B respectively). A histogram analysis was also performed on all three acquired slices to quantify the distribution of IAUC60 values over the imaged region (Figs. 3.4C and 3.4D).

Figure 3.4: IAUC60 parametric maps for DCE-MRI (A) and DCE-US (B) were overlaid on anatomic images of a tumour. Parameter maps were calculated as described in the methods on manually drawn ROIs. Red and blue voxels represent high and low IAUC60 values (respectively). The distributions of the IAUC60 values represented by total voxel counts per IAUC60 value (combined for all three adjacent slices) are shown for MRI (C) and US (D).

3.2.6 Histopathology

Following the second imaging session 24 hours after the baseline, animals were euthanized by cervical dislocation and tumours excised (maintaining orientation where possible) and frozen in liquid nitrogen. A cryostat was used to obtain duplicate serial sections of the tumour 10 microns thick, every 1mm for full tumour coverage. One set of serial sections
was stained with standard H&E and the other set was dual stained for apoptotic cells (TUNEL) and vasculature (CD31). Images were acquired at 40x magnification using an automated digital slide scanning system (MIRAX SCAN - Carl-Zeiss Microscopy; Göttingen, Germany).

3.3 Results

3.3.1 Tumour volume

Data from two imaging sessions separated by 24 hours allows for a comparison of tumour volumes to monitor growth and absolute size differences between subjects (Fig. 3.5). There was also a large variability in the tumour volumes between animals. Tumours NC1 and NC2 were randomly selected to act as controls and Tumours T1-T4 were placed in the 8Gy treatment group. Tumours from animals T1, T3, NC1 and NC2 appear to be at least 50\% smaller by volume compared to tumours from animals T2 and T4. Furthermore, tumour growth rates were highly variable with one tumour growing 33\% in volume over 24 hours and another growing only 6\% (Table 3.1).

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Condition</th>
<th>Baseline Volume (mm$^3$)</th>
<th>24h Post Volume (mm$^3$)</th>
<th>Volume Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC1</td>
<td>Control</td>
<td>142 ± 3</td>
<td>173 ± 4</td>
<td>21 %</td>
</tr>
<tr>
<td>NC2$^\circ$</td>
<td>Control</td>
<td>90 ± 2</td>
<td>89 ± 2</td>
<td>-1 %</td>
</tr>
<tr>
<td>T1</td>
<td>8Gy</td>
<td>114 ± 2</td>
<td>151 ± 3</td>
<td>33 %</td>
</tr>
<tr>
<td>T2</td>
<td>8Gy</td>
<td>235 ± 5</td>
<td>256 ± 5</td>
<td>9 %</td>
</tr>
<tr>
<td>T3</td>
<td>8Gy</td>
<td>317 ± 6</td>
<td>335 ± 7</td>
<td>6 %</td>
</tr>
<tr>
<td>T4</td>
<td>8Gy</td>
<td>132 ± 3</td>
<td>147 ± 3</td>
<td>11 %</td>
</tr>
</tbody>
</table>

Table 3.1: Summary of tumour volume growth. Growth rates in 24 hours of the six animals used in this study and the treatments applied.

$^\circ$ Tumour from animal NC2 was compressed by the water tank and thus, the volume measurement is inaccurate.
3.3. RESULTS

Figure 3.5: Tumour volumes shown for baseline and 24 hours post baseline, as measured by drawing ROIs on anatomic MR images. Error bars represent the percent error in segmentation determined by the same observer in a randomly selected test case. All tumours except NC2 showed a significant increase in volume over 24 hours.
° Tumour NC2 was compressed during imaging in the second session so the volume measurement was underestimated on the second imaging session.

3.3.2 Enhancement curves

Manual ROIs were drawn on the three slices for which both DCE-MRI and DCE-US data were acquired during the baseline and 24h post imaging sessions.

Data were averaged over the entire ROI to determine whole tumour average enhancement curves (MRI) and replenishment curves (US) for the control and treated animals. DCE-US replenishment curves were normalized to the pre-burst intensity for this particular analysis so the Hudson model could be fit to the data [45]. Treated tumours (T1-T4) showed a large spread in DCE-MRI enhancement curves at baseline but 24 hours following an 8Gy treatment, all four tumours showed a more uniform enhancement curve (Fig. 3.6). DCE-US data also showed increased variability at the baseline imaging session compared to the 24h post imaging session (particularly at the initial reperfusion stage). The initial (first 60 seconds) area under the enhancement curve parameter, IAUC60, was calculated for the control and treated animals and is shown in Table 3.2.
3.3. RESULTS

Figure 3.6: Summary of DCE-MRI and DCE-US curves for treated animals (T1-T4). The top two panes show the DCE-MRI data averaged over the ROIs of three slices to assess the whole tumour response. The bottom two panes show the DCE-US data averaged over the three corresponding slices. It is seen that the enhancement curves are more heterogeneous in the baseline imaging session in both MRI and US.
### 3.3. RESULTS

#### MRI IAUC60

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Baseline (a.u.)</th>
<th>24h Post (a.u.)</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC1</td>
<td>36 ± 5</td>
<td>26 ± 4</td>
<td>-28%</td>
</tr>
<tr>
<td>NC2</td>
<td>30 ± 5</td>
<td>25 ± 4</td>
<td>-17%</td>
</tr>
<tr>
<td>T1 (8Gy)</td>
<td>37 ± 6</td>
<td>27 ± 4</td>
<td>-27%</td>
</tr>
<tr>
<td>T2 (8Gy)</td>
<td>41 ± 6</td>
<td>35 ± 5</td>
<td>-15%</td>
</tr>
<tr>
<td>T3 (8Gy)</td>
<td>13 ± 2</td>
<td>28 ± 4</td>
<td>+115%</td>
</tr>
<tr>
<td>T4 (8Gy)</td>
<td>19 ± 3</td>
<td>23 ± 3</td>
<td>+21%</td>
</tr>
</tbody>
</table>

#### US IAUC60

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Baseline (a.u.)</th>
<th>24h Post (a.u.)</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC1</td>
<td>50 ± 10</td>
<td>44 ± 9</td>
<td>-12%</td>
</tr>
<tr>
<td>NC2</td>
<td>50 ± 10</td>
<td>43 ± 9</td>
<td>-14%</td>
</tr>
<tr>
<td>T1 (8Gy)</td>
<td>48 ± 10</td>
<td>43 ± 9</td>
<td>-10%</td>
</tr>
<tr>
<td>T2 (8Gy)</td>
<td>55 ± 11</td>
<td>57 ± 11</td>
<td>+4%</td>
</tr>
<tr>
<td>T3 (8Gy)</td>
<td>50 ± 10</td>
<td>49 ± 10</td>
<td>-2%</td>
</tr>
<tr>
<td>T4 (8Gy)</td>
<td>53 ± 11</td>
<td>47 ± 9</td>
<td>-11%</td>
</tr>
</tbody>
</table>

Table 3.2: **Top - whole tumour ROI DCE-MRI IAUC60 Summary.** Mean AUC values of the enhancement curves shown in Fig. 3.6 are presented with the errors estimated from the standard deviation of the voxel-based AUC analysis as well as the uncertainty in the intensities after averaging. The baseline mean IAUC60 for control and treated animals is 33 ± 5 and 28 ± 6. Mean IAUC60 values in the 24 hours post session are 26 ± 4 and 28 ± 4 for the control and treated animals respectively.

**Bottom - whole tumour ROI DCE-US IAUC60.** Mean AUC values of the normalized DCE-US enhancement curves shown in Fig. 3.6 with errors estimated from the standard deviation of the voxel-based AUC analysis as well as the uncertainty in the intensities after averaging. The baseline mean IAUC60 for control and treated animals is 50 ± 10 and 52 ± 11. Mean IAUC60 values in the 24 hours post session are 44 ± 9 and 49 ± 10 for the control and treated animals respectively.
3.3. RESULTS

3.3.3 IAUC60

IAUC60 was reported as a whole tumour average (Table 3.3), as a distribution of individual voxels (Fig. 3.7 and 3.8) and in the form of parametric maps. Whole tumour averages of IAUC60 with DCE-MRI showed a similar trend as the enhancement curves: the mean IAUC60 (± standard error) of the treatment group at baseline was 3900 ± 530 and post treatment was 3200 ± 670. Mean IAUC60 values for the two controls at baseline and 24 hours post were 3400 ± 190 and 3900 ± 46. Results from whole tumour averages of IAUC60 in DCE-US also showed no significant difference between the treated and control groups. IAUC60 calculated using DCE-US yielded in a statistically significant change in the control groups over 24 hours as mean IAUC60 values increased from 3400 ± 190 to 3900 ± 46 for the two animals. The treated group did not show a statistically significant change in the IAUC60 values before treatment (3900 ± 530) compared to 24 hours post treatment (3200 ± 670). While it is common to report parameters averaged over the whole tumour, many studies also show representative parametric maps to indicate heterogenous regions within the tumour [64–66]. Thus, a histogram analysis of all voxels within the tumour provides a more representative summary of the tumour.

Distributions (plotted as connected points instead of histograms for easy comparison) of IAUC60 values at baseline and 24 hours later for the two control and four treated animals are shown in Figs. 3.7 and 3.8 respectively. Among the control group, tumour NC1 grew 21% in volume between the two sessions and consequently, there were far more voxels in the DCE-MRI distribution after 24 hours of growth. The DCE-US distribution also showed an increase in the total number of voxels but the shift was to the right, suggesting there were more voxels that had higher IAUC60 values in the second session. The distributions of IAUC60 in tumour NC2 were difficult to interpret as the tumour was slightly compressed during imaging. Of the treated group, tumour T1
Table 3.3: **DCE-MRI IAUC60 Summary.** Summary of mean and standard deviations in whole tumour DCE-MRI IAUC60 averages for each animal at baseline and 24 hours post treatment. Baseline mean and standard error for the control and treatment groups was 2.5 ± 0.3 and 2.3± 0.4 respectively; Session two averages were 1.9 ± 0.1 and 2.1 ± 0.3 for control and treated groups respectively.

**DCE-US IAUC60 Summary.** Summary of mean and standard deviations in whole tumour DCE-US IAUC60 averages for each animal at baseline and 24 hours post treatment. Baseline mean and standard error for the control and treatment groups was 3400 ± 190 and 3900 ± 530 respectively; 24h post treatment, the averages were 3900 ± 46 and 3200 ± 670 for the control and treated groups respectively.

grew the most increasing in volume by 33% and this change was clear from the IAUC60 distribution. In both DCE-MRI and DCE-US, the distribution of values showed marked increases in the total number of voxels and in DCE-US, there was a definite shift towards higher IAUC60 values. Tumours T3 and T4 grew the least over the two sessions at 6% and 11% volume growth and the DCE-US distributions indicate little change. This trend was not mirrored in the DCE-MRI distributions, as both tumours T3 and T4 changed considerably: the tumour T3 IAUC60 distribution shifted to higher values while the tumour T4 IAUC60 distribution shifting to lower values. Overall, the DCE-MRI for the tumours post treatment indicated the tumours changed but it is unclear if this change was due to physiological variability, tumour growth, treatment or a combination of factors.
Figure 3.7: Distribution of IAUC60 values at baseline and 24 hours post for control tumours are shown. In each pane, the distributions above and below are DCE-MRI and DCE-US, respectively.
Figure 3.8: Distribution of IAUC60 values at baseline and 24 hours post for treated tumours. In each pane, the distributions above and below are DCE-MRI and DCE-US, respectively.
3.3. RESULTS

3.3.4 Comparing regional ROIs in MRI and US

IAUC60 values from the intravascular microbubbles in DCE-US are assumed to be related to the blood volume (integral of the intensity vs. time curve) and IAUC60 from the extravascular Gd-based MR agent is a combined parameter with components of $K^{\text{trans}}$ (rate constant describing transfer of contrast from blood plasma to the extravascular extracellular space) and $v_E$ (volume fraction of the extravascular extracellular space). A representative example of MRI and US contrast agents with different contrast kinetics in the same region is shown in Fig. 3.9. The large necrotic region in the centre of the tumour is indicated by a signal-poor region in US but in MRI, the extravascular tracer likely accumulated in the same region. The right side of the tumour exhibited a hyper-intense region in MRI where malformed and abnormal vessels likely led to agent extravasation. In US, the regions are sparsely hyper-intense, possibly due to the distribution of blood vessels. While this was a very simple example of the complementary information that DCE-MRI and DCE-US provide, with further experiments using the methods described here, there is potential to acquire biological insight into the complex mechanisms of drug response in the tumour microenvironment.

3.3.5 Histopathology

Maintaining orientation between imaging sessions, tumour excision, sectioning and staining was a challenging venture. It was unclear if deformations in the tissue during sectioning and freezing were significant enough to change the tumour shape, a feature we were hoping to rely on for matching imaging slices with histopathology. Subsequent attempts at maintaining the orientation have been far more successful and we hope to implement strategies to limit human error contributing to the process. While there were some regions within the tumour (particularly near the skin) where there were actually
3.3. RESULTS

Figure 3.9: The same tumour was imaged in both modalities and tumour ROIs were selected and the enhancement curves plotted. The red region appears to be a region of high vascularity and this was reflected in both DCE-MRI and DCE-US. In DCE-MRI, the cyan region showed a gradual enhancement suggesting that diffusion resulted in a gradual accumulation of the small agent. In DCE-US, the same region showed virtually no enhancement as the microbubbles were intravascular.
no cells, tear patterns in some tumour sections (e.g., T3) were consistent with sectioning and freezing artefacts. These can be prevented with faster freezing and more meticulous sample preparation during cryosectioning. Nevertheless, the histopathology data confirmed that the effects of the 8Gy treatment on this tumour cell line were not significant after 24 hours.
**Tumour Histopathology:**

CD31 (brown)
TUNEL (pink)

**Figure 3.10:** Histology sections of the largest available section through a tumour stained for vasculature (CD31, brown) and apoptotic cells (TUNEL, pink). These images were representative of the whole tumour and all images were scaled identically and scale bars represent 2mm. Qualitatively, the histology data suggested two trends: 1) The tumours vary considerably in size and morphology, 2) the treated tumours appeared to have slightly higher TUNEL staining.
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Sample DCE-MRI and DCE-US parameter maps of a treated (T3) and control tumour (NC1) compared to histology is shown in Fig. 3.11. The histology sections for tumours T3 and NC1 correspond to the imaging slice. In the case of T3, the second lobe of the tumour was missing due to a sectioning artefact (confirmed as none of the histology slices from this tumour contain the second lobe) but using the visible skin at the top of the image, we were reasonably confident that the slices correspond. Some trends identified from the parametric maps can be confirmed with this histology data. For instance, dark regions in the DCE-US IAUC60 map (Fig. 3.11A) correspond well to regions of high TUNEL (apoptosis) staining (Fig. 3.11C). Blood supply to patches of apoptotic regions is limited or non-existent and with a reduced blood supply. Thus, delivery of microbubbles to that region is also impaired and this leads to low IAUC60 values. In DCE-MRI, the dark regions were also in roughly the same locations, but there were fewer dark regions as the small MR contrast agent readily diffuses out of vessels and distributes throughout the tumour. TUNEL staining along the upper rim where the tumour connects to the skin was representative of all six tumours in this study and was consistent with typical staining patterns in the Lewis Lung Carcinoma cell line. Insets of the histology sections (Fig. 3.11D and H) show representative regions for a microscopic view of the tumour microenvironment. CD31 is a protein expressed on endothelial cells and from the macroscopic histology sections (Fig. 3.11C and G) it appears that the treated tumour T3 has a reduced vessel distribution compared to the control NC1. In the insets (Fig. 3.11D and H) however, the background tissue signal was higher for NC1 compared to T3.
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Figure 3.11: A,E) DCE-US IAUC60 map for T3 and NC1 respectively. B,F) DCE-MRI IAUC60 map for T3 and NC1, respectively. C,G) Macroscopic view of a histology slice corresponding to T3 and NC1, respectively. D,H) Microscopic view of an ROI (box) for tumour T3 and NC1, respectively.
3.4 Discussion

The primary goal of this pilot study was to demonstrate that DCE-MRI and DCE-US can provide complementary information about the tumour microenvironment based on the unique tracer kinetics in each modality. Figure 3.9 shows the potential for the apparatus detailed in Chapter 2 and used in this study. Coplanar imaging removes a crucial unknown variable in tumour perfusion studies and the question, “Are we imaging the same region when comparing results from two modalities?” has been addressed with other modalities [2, 67, 68] and now it is also feasible for small animal imaging with this work and several others [47, 49, 56–58, 69]. However, the more ambitious goal of this project was to show that combining DCE-MRI and DCE-US leads to improved insight into the tumour microenvironment following a treatment. A pre-requisite to this goal is an assessment of the repeatability of the methods and techniques described here. It was unfortunate that the treatment used in this study did not have a greater effect. In fact, the LLC tumours may have seemed to respond poorly to treatment simply because this cell line grows very aggressively and tumour growth may have muted treatment effects. Nonetheless, the process was instructive in establishing just how heterogeneous tumours were and more importantly, how there is no such thing as a ‘tumour control’; each tumour behaves and responds completely independently from others, even from amongst the same cohort, injected with approximately the same number and amount of cells. The nature of the chaotic and uncontrolled growth and development of the tumour microenvironment is extremely difficult to model and describe, but it is fundamentally important for understanding mechanisms of drug action and tumour response.

Heterogenous and chaotic vascular networks in these tumours - at different stages of growth as well as the size difference between the tumours likely contributed to the variable results from this study. All six animals were implanted on the same day with
approximately the same number of cells and tumour volumes were measured during the MR imaging sessions, about three weeks after implantation. One of the goals of this study was to acquire pilot data to design an extensive study assessing the inter-day repeatability of DCE-US and DCE-MRI. Preliminary indications are that changes in the tumour microenvironment of a fast growing and aggressive tumour cell line such as LLC (in vitro doubling time of 21 hours) vastly overshadows any repeatability issues such as MR contrast injection rate variability, US microbubble decay over time, and low signal to noise in MR. This trend is reflected in statistically significant growth of all but one tumour (NC2, see Fig. 3.5). Tumour NC2 was an anomalous result as both DCE-MRI and DCE-US distributions suggested there were fewer voxels in the second imaging session for tumour NC2. This is inconsistent with results from all other mice (treated or not), but is consistent with the observation on MR images (also noted during data acquisition) that showed tumour compression. Over 24 hours, even the tumours that grew in volume the least (volume of T2 and T3 increased by 9% and 6% respectively) still exhibited considerable change compared to the baseline imaging session in both the IAUC60 distributions (Fig. 3.8) as well as the enhancement and replenishment curves (Fig. 3.6).

3.4.1 Interpreting combined MR and US data

Parametric maps calculated voxel-by-voxel are a great tool to preserve information about tumour heterogeneity but are difficult to compare between animals and between imaging session. Thus, each parameter map can be represented by a number, typically the mean or median of the whole tumour average [63]. The disadvantage of this method is that unless the entire tumour is affected, regional effects of a therapy will be averaged out. For instance, consider a drug designed to selectively target hypoxic cells given to a tumour-
3.4. DISCUSSION

bearing mouse and imaged before and after treatment. If 30% of this tumour is hypoxic, when imaging data from the viable tumour regions are averaged with the hypoxic regions, the tumour effect will be indeterminable. IAUC60 distribution plots captured the spatial heterogeneity in the tumour but spatial localization was not retained as only the voxel counts were considered. Distribution plots provided a global summary of the tumour without averaging over the entire tumour. Voxels with higher IAUC60 values for DCE-MRI can be interpreted as regions where a relatively higher amounts of the tracer was delivered or was retained. Lower values are best interpreted as poorly perfused regions, not as accessible to the tracer either by diffusion or by delivery through the vascular network. With the assumption that microbubbles used in DCE-US are intravascular, the interpretation of IAUC60 values is slightly different: higher values indicate regions with large vessels carrying higher concentrations of microbubbles and lower values are regions with smaller capillaries and/or fewer vessels. A shift to the right of an IAUC60 distribution in DCE-US likely indicates the tumour is recruiting more vasculature and growing. Indeed this was the case for tumour T1 (Fig. 3.8) which showed a significant shift to the right and it grew 33% in volume over just 24 hours (Table 3.1). Increased vasculature in tumour T1 led to a shift up in the DCE-MRI IAUC60 distribution suggesting that the contrast agent concentration in the tumour increased.

Enhancement curves preserved only temporal information and were useful for a global picture of tumour behaviour. For instance, two similarly sized tumours from the same tumour cell line implanted in the same species should produce similar enhancement curves provided that the temperature, heart rate, injection rate, volume and concentration are constant. In practice however, it was difficult to maintain complete consistency in heart and breathing rates as anaesthesia has a significant effect on animals. Furthermore, each animal responds differently to anaesthesia and dose must be adjusted. In the absence of a “tumour control”, variability in enhancement curves averaged over the entire tumour
can be used as a marker of repeatability. From Fig. 3.6 it is immediately evident that the DCE-US appear to be more consistent than their DCE-MRI counterparts (before and after treatment). It is hypothesized that because the DCE-US replenishment curves are averaged over only the vascular component of the tumour microenvironment (microbubbles are intravascular), the inter-tumour changes are less pronounced compared to the DCE-MRI enhancement curves. Recall that the tracer in MRI extravasates out of vessels and likely accumulates in necrotic areas of the tumour - since the amount of necrosis in varies considerably between tumours, increased variability in DCE-MRI can be expected.

In this study, the six animals imaged at baseline had a high degree of variability in their enhancement curves, suggesting that the tumours were growing at different rates and thus, were affected by treatments heterogeneously. Tumour T3 showed large regions of apoptosis (Fig. 3.10) and while it is possible that this was due to the radiation treatment, the more likely explanation is that the tumour was large and poorly perfused. Increased distance of cells from the blood supply leads to regions of the tumour starving for nutrients (and undergoing apoptosis) as these regions are beyond the diffusion limits of, for example, oxygen and glucose. This was consistent with our findings from DCE-US as well as DCE-MRI. Potential effects of radiation treatment are discussed next.

### 3.4.2 Changes in the tumour microenvironment

Physiological variability, as well as rapid uncontrolled growth of tumours led to marked differences in the tumour microenvironments. It is also readily apparent from Fig. 3.10 that even within each tumour slice, there was significant variability from region to region. Patches of CD31 and TUNEL stains were scattered throughout the tumour with no distinguishable patterns and this was consistently observed in all of the histology data. However, a confounding factor in interpreting the histology data was the variable
background signal from imaged slides. For instance in Fig. 3.11, the histology insets from the two tumours T3 and NC1 indicate that the smaller tumour (NC1) has more CD31 staining and thus, is more vascular. The DCE-MRI and DCE-US data do not fully support this observation and a possible explanation is that the background tissue signal is higher in the smaller tumour (NC1). Considering this increased background tissue signal - likely an artefact, the vascular density does not appear to be significantly different.

Typical explanations for higher tissue background include sectioning artefacts leading to thicker sections or staining artefacts. Investigating this phenomenon is planned for future studies to optimize the staining and slide imaging protocol.

A leading hypothesis suggests that following some treatments, flow in tumours “improves” likely by pruning of smaller, less developed vessels that are more susceptible to damage from the treatment. Thus, poorly perfused regions may become completely apoptotic or necrotic if even a fraction of vessels supplying that region are damaged. In this study, microbubbles replenished the tumour region fairly uniformly in the imaging session 24 hours following treatment (Fig. 3.6). However, averages of the two groups yielded no statistically significant difference between the IAUC60 of either DCE-MRI nor DCE-US between the baseline imaging session and 24h post (Table 3.2). This suggests the variability between animals was too large and combining the data may have averaged out the trend seen in the enhancement curves.

In the baseline imaging session, considerable variability was observed in the animals. This observation was consistent with results from DCE-MRI as well: enhancement curves became more uniform following treatment (Fig. 3.6). While this is not a conclusive response to radiation treatment as histopathology was inconclusive in confirming the effect, it is worth discussing the expected effects of this treatment in context with this finding. Radiation treatments of single fractions at high doses (8Gy and above) have been shown to cause rapid endothelial apoptosis followed by days of tumour regression.
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At lower doses, microvascular damage is a key mechanism of tumour response \(^7\) and our hypothesis is that the tumour response shown may be due to early stage apoptosis (including mitotic arrest) in the tumour leading ultimately to microvascular damage. Response of vasculature to radiation doses is not well understood \(^1\), but it is known that single fractions of high dose radiation causes severe vascular damage and this may result in the smaller, less stable vessels becoming nonfunctional, which in the short term, leads to improved flow in the active tumour vessels. The near-uniform enhancement following treatment in the DCE-MRI data may be explained by these hypotheses as the contrast agent kinetics appear to have become more uniform in all four tumours. Further studies (outlined in Chapter 4) with higher statistical power, more replicates and histological data available at more time points is required to make definitive conclusions. Use of aggressive cell lines for xenograft experiments is a double edged sword: while tumours grow quickly (8-10 days for 1cm diameter) and reliably (100% success rate), there is considerable variability in the growth rate as well as the size. Scheduling experiments with tumours growing at different rates is a significant challenge and we have since learned that the optimal strategy is to implant more animals than needed and to stagger imaging sessions by immediately imaging tumours that reach the target size rather than waiting until all tumours are at least as the target size. Furthermore, to interpret histology data accurately a few tumours from each treatment group must be sacrificed after each session. Another outstanding issue is tumour orientation selection between sessions. Tumours tend to grow and shift and dramatically alter in shape and features that make landmarking difficult. As a potential solution to this problem, Bains et al. proposed implanting a tube filled with wax and saline prior to tumour cell implantation so the tumour grows around the tube \(^7\). In imaging sessions, MR slices were prescribed perpendicular to the tube. While this reduces the problem from a 3D localization to a 2D localization, the method needs to be extended further for use with this apparatus and
3.4.3 Optimizing DCE-MRI and DCE-US

There were a significant number of challenges with quantifying DCE-US data. In fact, except in a few select applications (for e.g., measuring velocity using Doppler techniques) quantifying US signal absolutely is intrinsically problematic, primarily due to nonlinear wave dispersion in the medium. Typically respiratory motion was a significant problem when acquiring data at high temporal resolutions without gating acquisition to the breathing rate. In this study, motion is an unlikely cause for confounding data interpretation as the tumours were elevated (1-2 cm) and isolated (pulled away from the body) leading to dramatic dampening of respiratory motion. Data were not corrected for cardiac motion but this was assumed to be constant inter-session and inter-animal. To accurately correlate image intensity with even surrogate physiological parameters is challenging without using complicated models and phantoms describing and validating wave propagation. Furthermore, accounting for signal attenuation and beam deformation over the imaging field of view is non-trivial. That being said, total control over data processing is critical in research studies so these issues can be mitigated. All US data analyzed in this study was obtained immediately after beam-forming and prior to any other post-processing steps, functionality that is often not available on non-research scanners. This is not generally an issue in the clinic however, as such quantitative information is rarely sought (or necessary) except in clinical trials where it is necessary to compare results between centres. Steps were taken to control the imaging conditions very strictly. For instance, care was taken to acquire data with consistent scanner settings with the transducer controlled mechanically, oriented in a fixed position to acquire sagittal images. All attempts were made to position the tumour close to the geometric
3.4. DISCUSSION

focus of the transducer and the adjustable azimuthal focus was placed just below the
tumour (between 19mm and 22mm, depending on tumour size). Despite this however, it
was not possible to control for tumour size when imaging and often tumours grew larger
than we felt comfortable imaging reproducibly given the measured beam profile.

Although the focus of this study was not to model DCE-MRI data to extract quantita-
tive DCE-MRI parameters, this discussion is relevant and applicable to future iterations
of this study. There exist many conventions and protocols for small animal imaging
at high field MRI. Limitations of a model-free approach to analysis methods have been
profiled extensively [28, 29, 73, 74]. Furthermore, repeatability studies have also shown
that intra-day inter-session repeatability in measuring $K_{trans}$ and $v_e$ is fairly good [33],
that the AIF is critical for compartmental modelling [34] and that model-free parameters
are as quantitative and as predictive as compartmental model parameters but less repro-
ducible between centres [27, 63, 75]. Samuel et al. published a comprehensive theoretical
study in 2006 to determine the relationship between IAUC and $K_{trans}$ using simulated
contrast agent curves [27]. The simulations concluded that while IAUC was a mixed
parameter intractably linked to $K_{trans}$ and $v_e$ (and the plasma volume $v_p$), it cannot be
used as a surrogate marker (or $K_{trans}$ or $v_e$).

Authors have also implemented dual surface coil systems to simultaneously measure
the AIF for each animal during tumour imaging [31] and proposed novel projection based
AIF measurements [76]. For this pilot experiment, a specialized surface coil compatible
with the apparatus was not available so the large volume coil used for both anatomic
imaging as well as DCE-MRI. The standard volume coil was more than adequate for
anatomic imaging in terms of sensitivity, spatial resolution as well as SNR-efficiency [77].
However, for dynamic contrast enhanced imaging, the requirements of full tumour cov-
erage at high spatial resolution as well as the need for high temporal resolution (which
precludes averaging) make it difficult to use the volume coil. The maximum temporal

75
resolution of DCE-MRI imaging was limited to about 8 seconds, and our goal was to reduce that to 3-4 seconds interleaved with AIF acquisition every 1 second. To perform more quantitative DCE-MRI studies pre-clinically, a small receive-only surface coil should be built with high sensitivity for the tumour region. There are several technical and practical challenges associated with this as the coil cannot be placed either above the tumour (US attenuation and tumour compression) or below it (hard surfaces scatter ultrasound). The coil must be encased in epoxy or another waterproof resin to avoid exposing components to the ultrasound gel, which was applied on the tumour to achieve contact with the water tank membrane. To ensure co-registered planes between MR and US, the coil must also be decoupled from the transmit/receive volume coil during large FOV anatomical imaging. For future studies, a dual coil system is outlined in Chapter 4 to maximize temporal and spatial resolution. Once the imaging protocols for DCE-MRI and DCE-US are optimized, a repeatability study to assess the sensitivity of measured parameters in the tumour cell line of interest is highly recommended. Similar studies have been conducted in DCE-MRI [33, 63, 75] as well as DCE-US [78] for different applications and should be used as templates. This repeatability study should be conducted longitudinally so the impact of tumour growth on perfusion measurements can be assessed and used as a reference for assessing the efficacy of treatments.

### 3.5 Conclusions

In conclusion, we have demonstrated that there is potential for combined DCE-MRI and DCE-US imaging, proposed analysis schemes for data interpretation, solved some existing challenges associated with combined MR and US imaging and proposed some solutions to outstanding issues. Ultimately the biggest limitation of this pilot study was the limited sample size and the variability in the tumours. However, this study
has produced several collaborations and spin-off projects and it is our hope that once the outstanding issues discussed above are addressed, this project has the potential to become a serious platform for pre-clinical researchers to assess therapies and provide useful biological insight to understanding mechanisms of therapy response.
This chapter provides suggestions for improving the apparatus, data acquisition, and data analysis methods. Key obstacles in DCE-MRI and DCE-MRI are discussed and some solutions are proposed.
4.1 Apparatus

A major drawback of non invasive imaging techniques in small animals to track tumour microenvironment changes is that it is often not possible to image the same regions over multiple imaging sessions. In normal tissues and organs such as the brain, this is not as hampering as structural landmarks remain fairly consistent. In rapid growing and heterogeneous tumours however, tumour morphology may change considerably and in our experiments, we have seen as much as a 33% growth in tumour volume over just 24 hours. It is interesting to consider whether or not it is even important to image the tumour in the same region and compare, or if it is enough to compare whole tumour averaged parameters. The answer boils down to the biological question being investigated. For example, if the goal is to assess whether there is a response after a treatment, whole tumour averages may be sufficient in describing bulk tumour response, but to assess and understand mechanisms of therapy response, tumour heterogeneity must be considered.

Ideally, tumour volumes would be acquired in 3D at a high isotropic resolution so the data can be reconstructed in any orientation so longitudinal tumour registration wouldn’t be an issue. The 3D volumes would be aligned so the slices are co-planar and compared directly. Unfortunately with MRI, increased spatial resolution is usually done at a cost of decreased temporal resolution and/or SNR. In ultrasound, isotropic 3D volumetric acquisition is not technically possible yet but 3D scanners are available (to acquire non isotropic 3D volumes). Until these technological challenges are overcome (for instance rapidly developing techniques such as parallel imaging, compressed sensing and improved surface coils may allow 3D isotropic DCE-MRI in the foreseeable future), co-planar imaging planes must be selected manually. An improvement to the next version of the co-registration apparatus could feature a circular water tank (as opposed to the current rectangular design). A circular water tank would allow for a fiducial marker insert that can be rotated clockwise or anticlockwise. The set-up time for the animal
in the apparatus would be slightly longer in this case as the fiducial marker insert must be rotated so the tumour slice from the previous imaging session is matched. Once the closest matching plane is selected, the fiducial marker can be locked in place and MR slices can be prescribed parallel to the fiducial marker insert. Alternative possibilities for landmarking include implanting a tube in the animal so the tumour grows around the tube and imaging slices can be prescribed with the tube as a landmark [72]. This method can be extended by implanting a cross instead of a tube and with that additional landmark, the cross can be used to define a plane and cross sections of this landmark can be used to adjust the prescribed volume.

4.2 DCE-US

In a previously published constant infusion study using Micromarker, it has been noted that it takes approximately 3-5 minutes for certain regions in the brain to achieve a steady state concentration in the brain [79]. While the kinetics of a small, diffusible agent are different in the brain due to the blood-brain-barrier, it is not expected that the kinetics of intravascular contrast agents will deviate from the brain to other tissue. However in all of our tumour experiments (in excess of 50 imaging sessions), we noted the time for tumours to achieve a steady state concentration of microbubbles exceeded 10 minutes, and approached 15 minutes for many. A possible explanation for this could be that the microbubbles aggregate and 'stick' to each other. The chemical composition of MicroMarker is fundamentally different from the clinically approved Definity. More specifically, MicroMarker can be altered slightly by the addition of a molecule to the lipid shell so the microbubbles bind to specific cell surface markers for research purposes. The lipid shell has to be manufactured in a way that supports the addition of these molecules and it turns out this feature results in microbubbles aggregating. A follow-
up to this observation is recommended as it must be determined whether this effect is tumour dependent microbubble type/size dependent.

Using a constant infusion imaging protocol in small animals runs the risk of the infusion volume affecting the physiology and ultimately, the perfusion measurements. Literature is scarce on the physiological effects of large infusion volumes at slow infusion rates, partly because these are extremely difficult measurements to make. Diehl et al. published an excellent guide to the administration of substances in many animals [80]. In mice, the reported mean blood volume to be 72 mL kg$^{-1}$, or 1.8 mL for a 25g mouse [80]. A typical mouse in this study will be infused with about 1.5mL of a diluted microbubble solution over 30 minutes (at a rate of 50$\mu$L min$^{-1}$. In 1980, Yoneda conducted an experiment to determine where injected fluid accumulates after varying bolus sizes [81]. With a large bolus injected quickly, cardiac burden increases tremendously because the systemic circulation is overloaded and the heart has to pump significantly harder. Furthermore, Yoneda found that 5 minutes after a 1.0 mL bolus injection in a 25g mouse, electron microscopy showed a widening of the peri-bronchiolar tissue and that some alveoli were filled with fluid [81]. In our experiments fluid was infused 20 times slower, potentially mitigating some of the problems associated with injecting large volumes quickly. While the infused volume is significant, it is below the 2.3 mL recommended infusion volume over 4 hours at 3.3$\mu$L min$^{-1}$ and well below the 4.8mL maximum volume mice can handle safely in a 24 hour period. In our experiments, before the disruption pulse, microbubbles are infused for 10 minutes to achieve a steady state concentration in the blood. Further studies are needed to assess whether the infusion rates can be reduced without increasing the length of the experiment by increasing the microbubble concentration.

It is unlikely that the blood volume is being doubled as most of the infused fluid will fill the bladder first and then collect in the interperitoneal space. Nevertheless, it is important to verify the physiological effects of the infusion. An experiment suggested by
my committee members to assess the extent of this issue was to infuse during an MRI scan to quantitatively assess where the volume is accumulating. If the infused volume is collecting in the interperitoneal space and the bladder, then it is unlikely that the circulation volume is changing appreciably so the measured parameters would not be affected of the infused volume.

4.3 DCE-MRI

Several improvements can be made with the DCE-MRI protocol used in this study including improving the SNR and reducing the total imaging time. The total imaging time for the study in Chapter 3 was approximately 70 minutes - this time can be reduced and used more efficiently as more time was spent on acquiring data, erring on the side of caution due to concerns of low SNR. For instance, the 2D anatomic scan acquired with a FSE sequence took about about 10 minutes due to averaging (NEX=15, acceleration factor 8). In subsequent phantom studies, the acceleration factor was increased to 50 with no noticeable distortions in the regions of interest and this feature may be useful for anatomic imaging provided that distortions in the image are minimized. With further optimization, anatomic scan length can be reduced to 4 minutes with adequate SNR and a reasonably high spatial resolution (0.2 x 0.2 x 0.5 mm).

4.3.1 Surface coil

A dedicated surface coil would drastically improve the imaging efficiency for functional imaging of the tumour. Surface coils optimize the SNR over a smaller volume of interest with reduced sensitivity to noise (and unwanted signal) from outside its sensitivity profile. By design, basic surface coils image smaller physical regions with higher SNR compared to larger volume coils. For this project, a simple moisture-resistant ring shaped surface
coil that fits around the tumour is sufficient. A ring shaped design is necessary because a traditional hard-backed design would prevent ultrasound from travelling to the tumour. Furthermore, the surface coil must be encased in a water-proof resin to prevent electrical components from shorting or performing off-spec (e.g., wet capacitors) when covered with ultrasound gel. Using a dual-coil system would allow the volume coil to be used for transmitting RF pulses and the surface coil to be used for receive. Engineering knowledge is required as the two coils must be decoupled to prevent interference in situations where the body coil is used for both transmit and receive (for e.g., localizing and anatomic imaging).

4.3.2 Quantitative modelling

Quantitative modelling of DCE-MRI data can yield physiologically relevant parameters that are predictive of, for example, therapy response. As discussed in Chapter 1, a prerequisite to quantitative modelling is a time-varying contrast agent concentration. The AIF (arterial input function) provides the input signal of how the contrast agent is delivered to the imaging region. To convert arbitrary MR signal intensity to a concentration, a reference $T_1$ map is required and can be obtained either with a typical inversion recovery experiment or a variable flip angle approach. While obtaining a $T_1$ map is trivial (relative to obtaining an AIF), extra scans acquired at the same spatial resolution as the DCE-MRI scan must be acquired at either variable inversion times or variable flip angles depending on the method. In protocol development experiments, $T_1$ maps were acquired with the inversion recovery sequence at 7 different inversion times each taking 4.5 minutes. Since each animal was already under anaesthesia for 2 hours due to US and MR imaging and there was no AIF available, $T_1$ maps were not acquired in the pilot experiment. With a surface coil, the imaging region can be reduced drastically and $T_1$
4.4 TUMOUR MODEL

maps with sufficient SNR should be possible to acquire in under 15 minutes.

4.4 Tumour model

In pre-clinical research studies with the goal of technique development, tumours should be imaged at roughly the same point in their growth to minimize the effects of tumour size on perfusion measurements. However it is well known that tumours, by definition, grow abnormally and cannot be controlled or regulated. Despite this, the start-point of initiation of imaging should occur when tumours first reach the target size and imaging sessions should be adjusted for each tumour. This was not possible in this study as the tumour model selected for this study was an extremely aggressive murine tumour model (Lewis Lung Carcinoma, LLC) because a well-vascularized tumour was required. Both the inter and intra-day spread in the tumour size was fairly large and contributed to variability in inter-tumour perfusion measurements with both DCE-MRI and DCE-US. With this experience, we recommend that a slower growing cell-line be selected for tumour implants as it will provide greater flexibility in deciding the optimal imaging time. For instance, the PC-3 cell line is a human prostate adenocarcinoma that may be well suited as its growth rate is approximately twice as long as the LLC cell line. Preliminary studies suggest that while PC-3 is less vascular than the LLC, tumours do contain enough vasculature to make it worth studying with the techniques presented here. Ultimately, multiple cell lines should be used to investigate both the repeatability of the perfusion techniques as well as the efficacy of therapies.
4.5 Potential for clinical Translation

A key motivation of this project was to determine whether US and MRI provide complimentary information to more completely assess the tumour microenvironment. While the particular application in this project was to develop a platform for assessing experimental treatments at the pre-clinical stage, it is clear that if successful, translating this technology to the clinic will quickly become a priority. Considerations for combining DCE-MRI and DCE-US in human patients are significantly different compared to the pre-clinical stage and indeed, the methods and protocols discussed in this study cannot directly be translated to the clinic. For instance, imaging time will need to be reduced drastically and an alternative system must be designed for ensuring co-planar imaging between MRI and US. On the other hand, after the analysis of potential sources of registration error in the pre-clinical case, we can predict with a high degree of confidence that accounting for the beam profile in standard clinical US transducers (at frequencies ranging from 2-10MHz) is not necessary. Furthermore in patients, there are significantly more features present within the body that can be used as landmarks for aligning MR and US slices. There are several groups working on this problem at the clinical stage and devices have been constructed that facilitate a priori registered images using optical methods to track the position of removable external fiducial markers placed on the patient [22, 82]. While an intriguing proposition, the technique’s use is limited to breast imaging and additional challenges remain for imaging tumours deep in the body and more importantly, the techniques remain unvalidated.
4.6 Summary

To improve the prospects of this project, three issues need to be addressed: 1) verify whether or not the DCE-US infusion protocol has measurable effects on the parameters, 2) measure the repeatability of DCE-MRI and DCE-US measurements, and 3) validate measured effects with histology. In general, the preclinical platform described here has the potential for improving our understanding of the tumour microenvironment. In many ways, it can be extended to become a more robust tool, useful for not just co-registration between US and MRI, but also MR and other modalities such as PET and CT.


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