BIOMEDICAL APPLICATIONS OF A HYBRID FLUORESCENCE MOLECULAR TOMOGRAPHY/MAGNETIC RESONANCE IMAGING (FMT/MRI) SYSTEM FOR SMALL ANIMAL IMAGING

A dissertation submitted to the
ETH ZURICH
for the degree of
Doctor of Sciences

presented by
AIKATERINI DIKAIOU
MSc ETH Biomedical Engineering
born December 31st, 1982
citizen of Greece

accepted on the recommendation of
Prof. Dr. Markus Rudin, examiner
Prof. Dr. Thomas Rösgen, co-examiner
Prof. Dr. Vasilis Ntziachristos, co-examiner

2013
## Contents

1 Introduction 1

1.1 Aims of the thesis 3

1.2 Outline 4

1.3 Contribution of the Thesis 5

2 Biomedical imaging modalities 7

2.1 A brief presentation of biomedical imaging modalities 8

2.1.1 Structural, functional, metabolic and molecular information 9

2.2 Magnetic Resonance Imaging 12

2.2.1 Nuclear Magnetic Resonance 12

2.2.2 Spatial encoding 13

2.2.3 Pulse sequences and contrast generation 14

2.3 Fluorescence-based optical methods 15

2.3.1 Light propagation in the body and contrast 15

2.3.2 Fluorescence reflectance imaging (FRI) 17

2.3.3 Fluorescence molecular tomography (FMT) 18

2.4 Hybrid imaging – combining FMT with MRI 22

3 FMT/MRI: Hardware components and development of improved reconstruction tools 25

3.1 A more compact FMT system 25
5.1 Preliminary remark ........................................... 63
5.2 Introduction .................................................. 63
5.3 Materials and Methods ....................................... 65
  5.3.1 Hybrid FMT/MRI system design ......................... 65
  5.3.2 Optical phantom preparation ............................ 67
  5.3.3 Animals ................................................... 67
  5.3.4 NIRF dye .................................................. 68
  5.3.5 In vivo FMT/MRI imaging .............................. 68
  5.3.6 Ex vivo optical imaging .................................. 69
  5.3.7 Data processing and analysis ........................... 70
5.4 Results ......................................................... 72
  5.4.1 FMT and MR performance assessment ................... 72
  5.4.2 In vivo FMT and MR imaging ........................... 73
5.5 Discussion ..................................................... 83

6 Application of a hybrid FMT/MRI system to study tumor vascularity 87
6.1 Preliminary remark ........................................... 87
6.2 Introduction .................................................. 87
6.3 Methods ....................................................... 89
  6.3.1 Hybrid system setup ..................................... 89
  6.3.2 Animals ................................................... 89
  6.3.3 Hybrid in vivo imaging .................................. 89
  6.3.4 Immunofluorescence and immunohistochemistry ....... 92
  6.3.5 Microscopy ............................................... 92
  6.3.6 Analysis of MR data ..................................... 92
  6.3.7 Reconstruction and analysis of FMT data ............... 95
  6.3.8 Statistical analysis ..................................... 96
  6.3.9 Analysis of immunofluorescence data .................... 96
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4</td>
<td>Results</td>
<td>96</td>
</tr>
<tr>
<td>6.4.1</td>
<td>DCE-MRI results</td>
<td>96</td>
</tr>
<tr>
<td>6.4.2</td>
<td>High resolution MRI results</td>
<td>99</td>
</tr>
<tr>
<td>6.4.3</td>
<td>FMT results</td>
<td>101</td>
</tr>
<tr>
<td>6.4.4</td>
<td>Histology results</td>
<td>101</td>
</tr>
<tr>
<td>6.5</td>
<td>Discussion</td>
<td>103</td>
</tr>
<tr>
<td>7</td>
<td>Conclusions and Outlook</td>
<td>107</td>
</tr>
<tr>
<td>7.1</td>
<td>Conclusions</td>
<td>107</td>
</tr>
<tr>
<td>7.1.1</td>
<td>FMT applications</td>
<td>107</td>
</tr>
<tr>
<td>7.1.2</td>
<td>FMT/MRI: hardware and software</td>
<td>108</td>
</tr>
<tr>
<td>7.1.3</td>
<td>Biological applications of the FMT/MRI system</td>
<td>109</td>
</tr>
<tr>
<td>7.2</td>
<td>Outlook</td>
<td>112</td>
</tr>
<tr>
<td>7.2.1</td>
<td>Exploiting the potential of MRI for metabolic information</td>
<td>112</td>
</tr>
<tr>
<td>7.2.2</td>
<td>Improving the FMT component</td>
<td>113</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Improving the FMT reconstruction with MRI prior information</td>
<td>114</td>
</tr>
<tr>
<td>A</td>
<td>Mathematics of light propagation in tissue</td>
<td>117</td>
</tr>
<tr>
<td>A.1</td>
<td>The diffusion equation for light propagation in tissue</td>
<td>117</td>
</tr>
<tr>
<td>A.2</td>
<td>The Helmholtz equation for a homogeneous medium</td>
<td>119</td>
</tr>
<tr>
<td>A.3</td>
<td>The diffusion equation for an inhomogeneous medium</td>
<td>120</td>
</tr>
<tr>
<td>A.4</td>
<td>The non-homogeneous Helmholtz equation for a non-infinite medium</td>
<td>121</td>
</tr>
<tr>
<td>A.4.1</td>
<td>Formulation of boundary conditions for photon diffusion</td>
<td>122</td>
</tr>
<tr>
<td>A.4.2</td>
<td>Solution of equation</td>
<td>123</td>
</tr>
<tr>
<td>A.5</td>
<td>The Kirchhoff approximation for arbitrary geometries</td>
<td>124</td>
</tr>
<tr>
<td>A.6</td>
<td>Measurements in free space</td>
<td>124</td>
</tr>
<tr>
<td>B</td>
<td>Green’s functions</td>
<td>127</td>
</tr>
<tr>
<td>B.1</td>
<td>Definition and usage of Green’s functions</td>
<td>127</td>
</tr>
<tr>
<td>B.2</td>
<td>Solution of the non-homogeneous Helmholtz equation</td>
<td>129</td>
</tr>
</tbody>
</table>
C Developed software
  C.1 MR Surface and registration to whitelight image of FMT .............. 131
    C.1.1 Main script ........................................ 132
    C.1.2 Important functions .................................. 135
  C.2 Use of surface in FMT reconstruction .............................. 149
  C.3 Display of coregistered FMT and MRI datasets .................... 151

D Preliminary studies for the tumor vascularity study .............. 157
  D.1 Study design ........................................... 157
  D.2 Choice of NIRF agent ..................................... 158
    D.2.1 Materials and methods ................................ 158
    D.2.2 Results .............................................. 159
  D.3 Choice of in vivo MRI protocol .................................. 161
  D.4 Choice of ex vivo validation approach .......................... 162

Bibliography 163
Abstract

Hybrid imaging systems, combining two biomedical imaging modalities in a common bore, have recently been developed for clinical and preclinical applications. Hybrid systems have the potential to unveil structural, functional and molecular alterations in disease and thus improve the diagnostic accuracy and development of therapeutic targets.

In this work, we have utilized a novel fluorescence molecular tomography/magnetic resonance imaging (FMT/MRI) hybrid system for small animal imaging. FMT allows quantitative assessment of the distribution of fluorescent agents in small animals. By employing targeted fluorescent probes, we can study molecular processes in vivo noninvasively. FMT has high sensitivity, but it suffers from low spatial resolution due to the diffuse light propagation in tissue. MRI is a highly versatile biomedical imaging modality which is widely used in the clinics and preclinical research to study morphological, functional and metabolic aspects of disease processes. We have focused on the incorporation of prior information from MRI into the FMT reconstruction and on the assessment of the increased diagnostic potential provided by the FMT/MRI system on a series of biomedical applications.

We first employed a standalone FMT system, constructed during this work, on two collaborative studies employing novel fluorescent probes which targeted epitopes on the surface of pancreatic cells and molecular anchors indicating signaling activity in tumor cells. On several occasions, the quantitative FMT readouts were found to be consistent with nuclear imaging methods and superior to commercial fluorescence reflectance imaging (FRI) systems. Yet when low signal to background ratios ratios were found to be low, caused either by unbound probes or by increased absorption/autofluorescence in tissue, the FMT quantification accuracy was compromised.

We contributed to the design and assessment of the first FMT/MRI system prototype designed for a small animal MRI scanner and to its subsequent improvement utilizing a
Abstract

low-noise, MR-compatible CMOS camera. We calculated the animal surface from MRI images and registered it to the reference image of the FMT dataset. Subsequently, we used the registered surface in the FMT reconstruction, eliminating the need to approximate the animal with a rectangular slab, thereby improving the quality of reconstructed data.

We employed the FMT/MRI system on a transgenic mouse model of Alzheimer's disease which overexpresses amyloid beta (Aβ) plaques in the brain. Using different MR contrasts, we assessed two morphological changes, ventricular enlargement and cerebral microbleeds, associated with this mouse model. We also performed magnetic resonance angiography (MRA) to identify brain vessel structures. Using dynamic FMT measurements with a targeted fluorescent probe which has been shown to bind to Aβ in vivo, we could follow the probe kinetics allowing the assessment of the specific tracer enrichment in the transgenic animals as compared to the age-matched wildtype mice. The in vivo FMT findings were confirmed ex vivo with FRI and confocal microscopy.

In a next step, we performed functional measurements with the FMT/MRI system to assess tumor vascularity on two subcutaneous tumor models. Kinetic modeling of dynamic contrast-enhanced MRI (DCE-MRI) upon administration of two MR contrast agents, we could detect structural (tumor blood volume, extracellular volume) and functional (vascular permeability) differences in the vascularization pattern of the examined tumors, which were consistent with histological validation. With FMT, we quantified the distribution of a NIRF blood pool agent on two distinct timepoints which are believed to correspond to the behavior of the two MR contrast agents employed. FMT results showed the same tendencies as MR measurements, but with a lower discrimination potential.

In conclusion, we have found that the combination of FMT with MRI in one hybrid system can provide improved FMT reconstruction accuracy. The high versatility of MRI, enabling assessment of multiple morphological and functional parameters, and the high sensitivity of FMT make FMT/MRI a promising tool in biomedical research.
Zusammenfassung

Hybridsysteme, welche zwei (oder mehr) biomedizinische Bildgebenderfahren in einem einzigen System kombinieren, wurden kürzlich für klinische sowie präklinischen Anwendungen entwickelt. Hybridsysteme bergen das Potenzial funktionelle, strukturelle als auch molekulare Veränderungen bei Krankheiten aufzuzeigen, und dadurch die diagnostische Genauigkeit zu verbessern, was auch für die Entwicklung von therapeutischen Ansätzen attraktiv ist.


Wir haben ein separates FMT System konstruiert und es für zwei Kollaborationsstudien angewendet, wo neuentwickelte Fluoreszensproben gegen Epitope von Pankreaszellen und gegen Markern, die die Signalaktivität in Tumorzellen anzeigen, evaluiert wurden. Die quantitativen FMT Resultate waren in bestimmten Fällen konsistent mit nuklearen Bildgebungsmethoden denen, die mit einem kommerziellen Fluoreszensreflexionstomographiesystem (FRI) gemessen wurden, überlegen. Die Genauigkeit der FMT Quantifizierung wurde jedoch beeinträchtigt, wenn das Signal-zu-Hintergrund Verhältnis tief war, verursacht entwe-
Zusammenfassung

der durch ungebundene Proben oder durch erhöhte Absorption/Autofluoreszenz im Gewebe.


Zusammenfassend konnten wir zeigen, dass die Kombination von FMT mit MRI in ein Hybridsystem eine verbesserte FMT Rekonstruktionsgenaugkeit ermöglicht. Die Kombination der hohen Einsatzflexibilität des MRI, die die Untersuchung von verschiedenen morphologischen und funktionellen Parametern ermöglicht, kombiniert mit der hohen Sensitivität des FMT, macht das FMT/MRI zu einem vielversprechenden Gerät für die biomedizinische Forschung.
Chapter 1

Introduction

The wish to see the inner workings of the body we inhabit is nothing new; already 5,000 years ago, humans made wall paintings of animals such as the one shown in Figure 1.1(a). To today’s observer, they present an eerie similarity with X-rays. In fact, it was the discovery of X-rays by Roentgen in 1895 that initiated the field of biomedical imaging. The diagnostic potential of the new method was immediately recognized; very soon after Roentgen’s announcement, European surgeons used it to detect bullets in soldiers’ bodies [2]. Within two months after the first report, X-rays of animals were first presented (Figure 1.1(b)).

![Figure 1.1: (a): Wall painting in complex X-Ray Style in Nawurlandja, Arnhem Land. Image from [3]. (b): Roentgen picture of a newborn rabbit made by J. N. Eder and E. Valenta of Vienna, 1896. Image from [4].]

Since the discovery of X-rays, the field of in vivo biomedical imaging has evolved in leaps. It is now an important tool for diagnosis and therapy follow-up in the clinics. X-ray and ultrasonography shall belong to every medical setting and all levels of health care
1. Introduction

(World Health Organization Guidelines, [5]). In countries of the developed world, magnetic resonance imaging (MRI) and X-ray computed tomography (CT) examinations are also a standard, with an average of 46.3 and 123 examinations respectively per thousand of inhabitants in 2010 [6]. All these techniques, along with nuclear tomographic methods such as single-photon emission tomography (SPECT) and positron emission tomography (PET), have allowed clinicians to estimate the structure, function and metabolism of tissue in all fields of neurology, cardiology and oncology.

In preclinical research, biomedical imaging has a different role. Apart from diagnosis, it supports the basic understanding of disease-associated processes in the intact organism and assists in the development and evaluation of new therapeutic targets. Imaging is used to evaluate discoveries made in isolated molecular and cellular systems in the context of the intact living organism. Given the macroscopic spatial resolution provided by biomedical imaging modalities, results obtained from such in vivo studies have then to be validated ex vivo by histological or immunohistochemical analyses. Being non-invasive, imaging allows monitoring biological processes as a function of time (dynamic measurements) and repeated studies in an individual. Evaluating alteration with regard to an initial condition typically increases the statistical relevance of the results and at the same time can reduce the number of employed laboratory animals, thus contributing to animal welfare. At the same time, integration of biomedical imaging in preclinical research is associated with a high cost; to justify their usage, imaging modalities must provide relevant readouts, which are both sensitive and quantitative. The development of preclinical imaging systems faces increased technological demands compared to their clinical counterparts; due to the small dimensions of the typically employed mice and rats, both the spatial resolution and sensitivity must be increased. Physiological parameters such as breathing and heart rate are also faster in laboratory animals; therefore, the temporal resolution of methods must also be increased. At the same time, the smaller dimensions of animals also mean that methods that are limited with regard to tissue penetration can be employed to probe the intact organism.

A notable example is the use of imaging techniques based on visible light. Despite light’s high absorption by tissue, the smaller path lengths involved in mice allow its use for in vivo imaging. In particular in the wavelength range 650 to 900nm (red and near infrared, NIR, domain) absorption of light photons by tissue is relatively weak and signals may be detected originating from a depth of a few centimeters. Optical imaging lends itself to combination with fluorescence; exogenously administered fluorescent probes or genetically encoded fluorescent reporter systems can be sensitively detected when absorbing and emitting in the
1.1. Aims of the thesis

The method of fluorescence molecular tomography (FMT) emerged ten years ago [8, 9] and has shown potential for recording molecular information in vivo quantitatively. In the past years, it has been employed in a series of applications, mostly in the field of oncology. A major limitation of FMT is its low spatial resolution – this problem is inherent to the method, as it is caused by the high light scattering in tissue. How can we combine the lacking high spatial resolution with FMT’s high sensitivity and quantification potential?

Such problems in biomedical imaging have been solved by combining modalities. In its most technologically advanced version, this combination is performed in fully integrated systems, termed hybrid systems [10]. Recently developed clinical hybrid systems (PET/CT and PET/MRI) have shown an improved potential for diagnosis compared to standalone systems [11]. Preclinical hybrid systems (PET/CT, FMT/XCT, PET/MRI) have also been recently developed, with promising results. However, the field is still young; whereas hardware integration without artifacts or other major decreases in performance has been been solved to a large extent [12], software integration to improve the reconstruction of the low resolution component is still under way [13–15]. Finally, so far animal studies in hybrid systems have been performed exemplarily to demonstrate proof of principle – large scale studies which address biological problems have not yet been presented.

1.1. Aims of the thesis

The objective of this work was (a) to improve the performance of a hybrid FMT/MRI system recently developed at the Animal Imaging Center, Institute for Biomedical Engineering, University and ETH Zurich [1] by utilizing complementary information provided by the two modalities to improve the quality of the FMT reconstruction, and (b) to demonstrate the potential of the FMT/MRI combination with selected biomedical applications. The combination of FMT with MRI was chosen due to the high soft tissue contrast and the high flexibility of MRI for structural, functional and metabolic measurements.
1. Introduction

To assess the FMT advantages and limitations, a standalone FMT system was constructed according to an existing prototype and utilized in two different biological studies. Assistance was provided to construct a first FMT/MRI prototype and a second, improved version of it. A software tool has been developed for co-registering MRI and FMT data, and using the surface information derived from MRI data in the FMT reconstruction. Finally, the improved FMT/MRI prototype was employed for two biological applications, in the fields of neurology and oncology. All results were validated \textit{ex vivo}.

1.2 Outline

A brief overview of biomedical imaging methods, with focus on MRI and FMT, is presented in Chapter 2. It is shown that although diagnosis and drug development undoubtedly would benefit from characterizing a disease state using various parameters including structural, functional, metabolic and molecular information, no imaging method can provide insight into all these aspects at once. Therein lies the motivation behind multimodal imaging, and more specifically behind hybrid systems, which combine one or more imaging techniques. Recent attempts at constructing hybrid imaging systems for research will be presented.

In Chapter 3, tools and methods developed in the course of this thesis are presented. These include the construction of an additional standalone FMT system, contribution to constructing two prototypes of a hybrid FMT/MRI system, and a method for extracting the sample surface (phantom, mouse) and including this information in the FMT reconstruction.

The developed standalone FMT system was used in two collaborative biological studies, which are presented in Chapter 4. The studies involve custom-made molecular fluorescent probes based on antibodies targeting epitopes expressed at the surface of pancreatic cells and molecular anchors indicating the intracellular signaling activity in tumor cells, respectively. By comparison of the FMT results with results from both other \textit{in vivo} imaging modalities and \textit{ex vivo} histology, the increased quantification accuracy of FMT and its limitations due to tissue absorption, especially when measuring outside the near-infrared region (NIR), could be demonstrated.

The first \textit{in vivo} results using a hybrid FMT/MRI system for a neuroimaging application are presented in Chapter 5, where simultaneous structural MRI and FMT using an amyloid plaque-binding fluorescent probe are described using on a mouse model of Alzheimer’s dis-
1.3 Contribution of the Thesis

Using a custom-made standalone FMT system, we were able to assess the expression of transmembrane protein (TMEM27), which is highly expressed on β cells, using a fluorescently labeled antibody. The quantification provided by FMT was found to be equivalent to assessment with PET studies (using a radiolabeled antibody) and superior to fluorescence reflectance imaging (FRI). However, when performing FMT with a molecular reporter with a fluorescent label excitable at 594nm, i.e. not in the NIR spectral domain, we found that the quantitative FMT results were inferior to FRI and nuclear imaging methods. This demonstrated the great importance of measuring in the NIR spectral range, ensuring enough light penetration and thus the collection of depth information, when performing FMT. Additional measures, such as spectral unmixing, might be required so that this problem is overcome.

I contributed to the development and characterization of a first FMT/MRI hybrid imaging prototype and its subsequent improvements towards a second generation system. My principal contribution was the development of software tools in order to co-register the two datasets, to allow the extraction of the animal surface from MRI and to incorporate this information into the FMT reconstruction.

The potential of this hybrid system for neuroimaging applications was demonstrated exemplarily using a genetically engineered mouse model of Alzheimer’s disease. Using MRI, no obvious morphological differences could be detected overall between wild type and trans-
genic mice, with the exception of focal hypointensities that could be attributed to microbleeds. Repeated measurements with FMT in 20 minute intervals using a fluorescent probe binding specifically to Alzheimer’s plaques revealed that differences between wild type and transgenic animals could be detected in vivo by virtue of the different clearance kinetics of the fluorescent probe. *Ex vivo* analysis confirmed that these differences indeed came from the dye’s binding onto Alzheimer plaques. These results illustrate the complementarity of the FMT and MRI readouts. They further suggest that the hybrid FMT/MRI system can be used in neuroimaging applications and that it is capable of unveiling molecular processes occurring at low speed (with a time scale of minutes or longer).

In a second application, the FMT/MRI system was employed to study tumor vascular features in subcutaneously implanted tumors in mouse using two different mouse cell lines. This study was carried out to demonstrate the flexibility of MRI as a reference method: it allows complementing structural data with dynamic/functional information. It was found that, in addition to standard structural MRI, dynamic MRI could also be performed with this FMT/MRI setup. The equivalence of the standard DSC- and DCE-MRI quantitative measures and of FMT with a non-targeted vascular probe for assessment of the tumor vascular system was investigated. It was found that DCE- and DSC-MRI offers superior results to FMT in this case, which suggests that the advantages of FMT are greater when it is used with targeted probes. At the same time, the results suggest the increased potential of the FMT/MRI system compared to other hybrid FMT systems due to the high versatility of MRI.
Chapter 2

Biomedical imaging modalities

Biomedical imaging strives to create maps of the body by exciting it with a known input and detecting the measured output. From this perspective, biomedical imaging methods belong to the class of inverse problems [16]. In this chapter, basic principles of biomedical imaging in research will be presented. Magnetic resonance imaging (MRI) and fluorescence molecular tomography (FMT) will be explained in more detail due to their relevance in this thesis. It will be shown that by using imaging it is possible to unveil disease mechanisms on different levels; structural, functional, metabolic and molecular, and that it is not possible to access them all with a single biomedical imaging modality. Therein lies the motivation behind hybrid imaging systems, combining two or more imaging modalities. A review of recent work in the field of hybrid imaging, with a clear focus on animal imaging, is presented.

Limitation of scope and terminology

In the following, a general presentation of biomedical imaging techniques is included. For reasons of clarity and cohesion with the rest of this work, only applications in oncology will be used to illustrate the principles discussed here. The words “body”, “tissue” or “samples” are used; they always refer to imaging of a body or a part of it in vivo and noninvasively. Finally, only biomedical imaging methods based on electromagnetic (EM) radiation will be presented in this chapter – the use of pressure waves for imaging, as performed in ultrasound and optoacoustics, is outside the scope of this work.
2. Biomedical imaging modalities

2.1 A brief presentation of biomedical imaging modalities

Only certain regions of the EM spectrum can be used for biomedical imaging; the limiting factor is the degree of the radiation’s attenuation by the body. Attenuation should be existent, so that contrast is generated, but low enough, so that enough signal is detected upon excitation. A list of biomedical imaging modalities and the corresponding regions of the EM spectrum they use is shown in Figure 2.1.

![Figure 2.1](image1.png)

**Figure 2.1:** Attenuation of EM radiation by biological tissue. \"Windows\" to the body, which are suitable for biomedical imaging, occur at different energy levels (left axis). The biomedical imaging modalities operating in these windows are shown on the right column. Figure adapted from [7].

Important considerations in imaging are the spatial resolution, the temporal resolution and the sensitivity. A trade-off exists between those quantities; for a given imaging setup, longer acquisition times are necessary to acquire high resolution images with a signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) useful for diagnostics. Additionally, small dimensions of a voxel correspond to less detectable signal, and thus lower sensitivity. No imaging modality can cover all three aspects, as shown in Figure 2.2. The selection of the suitable imaging modality for a given biomedical application is performed based on the most defining factor of the three. In practice, additional considerations such as the cost, the type of radiation (ionizing/non-ionizing) also play a role in this selection.
2.1. A brief presentation of biomedical imaging modalities

Figure 2.2: Position of biomedical imaging modalities in the space defined by sensitivity and spatial resolution. Each modality is represented as a sphere, whose diameter is proportional to the cost of the modality. Note that the modalities are arranged along the axes and not in the diagonal – i.e., they have a clear advantage either with respect to sensitivity or with respect to spatial resolution. Figure adapted from [17].

2.1.1 Structural, functional, metabolic and molecular information with biomedical imaging

The most obvious application of biomedical imaging is structural imaging, where a map reflecting the spatial distribution of a tissue parameter (e.g., the X-ray attenuation coefficient) is computed. The oldest example of this is X-ray radiography. Diagnosis is based on detection of tissue with different properties than its surroundings, and the quantifiable properties regard tissue morphology (as described by volume, cross-section and others).

However, disease does not only manifest itself in structural changes, but also in changes in tissue function and metabolism. It is conceivable that physiological/functional tissue parameters are more sensitive indicators of tissue state than those of tissue morphology [18]. Moreover, disease is typically triggered by aberrant molecular processes in tissue. Often, a series of such alterations is necessary for a phenotype to appear; this is illustrated in Figure 2.3 for the case of cancer, as introduced in [19] and updated in [20]. Figure 2.3 shows how the more comprehensive understanding of a disease process can help identify drug targets; in the same manner, it can also assist in identifying imaging biomarkers, i.e. parameters.
yielding information on a critical path in the development of a pathology that is modulated by therapeutic intervention [21].

Figure 2.3: The hallmarks of cancer represent biological capabilities acquired during the multistep development of tumors, arranged into a logical framework. According to those hallmarks, therapeutic targets can be identified, shown here in frames. Additionally, imaging targets can be defined. Figure from the Supplementary Material of [20].

Schematically, biomedical imaging illuminates the "black box" that is the body. Structural imaging lets us see its components without having to open it; but obviously, seeing the components of a computer is not enough to understand how it works.

**Functional imaging**

Using the above analogy, functional imaging probes how "current" flows into the body during operation. What is probed here is the blood; either directly, e.g. in the case of per-
fusion, or as a proxy for neural activation via the neurovascular coupling as known from fMRI [22]. Typically, tissue perfusion is assessed using the tracer dilution principle, i.e. it is analyzed how labeled blood passes through the tissue of interest. The label is typically an exogenous compound (X-ray or MRI contrast agent, fluorescent dye, PET tracer). Functional measurements including perfusion measurements are always carried out in a dynamic fashion, signal changes are analyzed as function of time. Time resolution is governed by the physiological process itself and for perfusion measurement is typically of the order of seconds, requiring high temporal resolution of the imaging method. Quantitative analysis of the signal profiles as a function of time yield then information on the perfusion status such as on tissue blood volume tissue blood flow, or on the permeability of the vascular wall.

Metabolic imaging

Metabolic imaging detects running processes by measuring what resources are heavily used. Diseases such as diabetes are linked to abnormal metabolism. In tumors, hypoxia causes switching of cells to the very glucose-inefficient anaerobic metabolism [23]. High cell proliferation increases the demand on choline, necessary in membrane synthesis [24]. Information on tissue metabolism is obtained by either by detecting the endogenous metabolites (e.g. using magnetic resonance spectroscopy, MRS) or by probing metabolic pathways through the administration of labeled substrates (e.g. [18F]-2-fluoro-2-deoxyglucose, FDG, which is used in combination with PET to probe local glucose utilization). As the concentration of metabolites in tissue is low, high SNR is necessary, which is commonly achieved at the cost of spatial resolution.

Molecular imaging

Molecular imaging tries to detect what commands are being executed. These "commands" involve activation of molecular pathways, enzymatic reactions and receptor interactions. For cancer, possible molecular targets are depicted in Figure 2.3. To perform molecular imaging, we need either exogenous specific probes, combining a targeting moiety and a signal-generating moiety, or transgenic animals/cell lines expressing reporter systems –the use of the latter is clearly limited to experimental animals. Requirements for reporter systems are stated in [18]. As molecular processes happen in low numbers, high sensitivity is essential.
Different types of information and imaging modalities

It is now clear that different types of biomedical imaging (structural, functional, metabolic and molecular) can help advance our insights into disease. They can be employed on longitudinal studies to monitor disease progression or to assess therapeutic efficacy. Some of the biomarkers identified in preclinical research can potentially be translated in the clinics; in any case, however, the noninvasive and longitudinal character of imaging can help reduce the number of animals used in experiments.

The suitability for various biomedical imaging modalities for structural, functional, metabolic and molecular information is shown in Table 2.1.

<table>
<thead>
<tr>
<th></th>
<th>Structural</th>
<th>Functional</th>
<th>Metabolic</th>
<th>Molecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Imaging</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>X-ray</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Optical imaging</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>MRI/MRS</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(yes)</td>
</tr>
</tbody>
</table>

Table 2.1: Biomedical imaging modalities – suitability for structural, functional, metabolic and molecular applications.

2.2 Magnetic Resonance Imaging

This section treats the basic principles of Magnetic Resonance Imaging, and practical aspects which are relevant to this work. For a thorough treatment of the topic, the reader is referred to the excellent Handbook of MRI Pulse Sequences [25]. The following description is largely based on [7] and [26].

2.2.1 Nuclear Magnetic Resonance

When nuclei with an odd number of protons and/or neutrons are placed in a static magnetic field of strength $B_0$ (assume $\vec{B} = B_0 \cdot \vec{z}$), their magnetization vectors $\vec{M}$ are aligned along to the field. The sample can be excited with an angular frequency $\omega_L$, called the Larmor frequency, which depends on the specific nuclei via the gyromagnetic ratio $\gamma$: 
\[ \omega_L = \gamma \cdot B_0 \]

For practical applications, the Larmor frequencies are in the radio frequency (RF) range of the EM spectrum. For example, hydrogen nuclei (protons) have the gyromagnetic ratio is \( \gamma / 2\pi = 42.577 \, \text{MHz/T} \). For a static magnetic field of 9.4T, the Larmor frequency is 400MHz.

Upon excitation, the net magnetization vector \( \overline{M} \) will be rotated by an angle \( \alpha \) and it will precess around the static magnetic field direction \( z \) with the Larmor frequency \( \omega_L \). This traverse magnetization will induce an oscillatory voltage in a pickup coil. This is the NMR signal.

As with any physical system, the net magnetization vector will return to its thermal equilibrium position \( M_z \). This is mathematically described by the Bloch equations. The rate with which the return to equilibrium occurs is given by the relaxation rates \( R_1 \) and \( R_2 \); \( R_1 \) describes the recovery of the \( M_z \) component of the net magnetization vector and \( R_2 \) describes the decay of the \( M_{xy} \) component of the net magnetization vector. Both components are shown in Figure 2.4(a, b). These processes are also called \( T_1 \), spin-lattice or longitudinal relaxation and \( T_2 \) or transverse relaxation, respectively. The transverse relaxation relates to the nuclei losing phase coherence due to slight differences in the local magnetic field to which they are exposed. For liquid samples, \( T_1 > T_2 \). In practice, the observed decay of the \( M_{xy} \) component occurs with a rate \( R_2^* \), where \( R_2^* > R_2 \), due to static magnetic field inhomogeneities. The signal that can be detected with an NMR coil in this simple experiment from the moment of excitation to the complete decay of transverse magnetization is called free induction decay (FID) signal. It is an exponentially decaying signal of rate \( R_2^* \) modulated by the Larmor frequency of the sample, as can be easily seen by Fourier transforming the FID (Figure 2.4(c)).

### 2.2.2 Spatial encoding

The NMR experiment described above will give an ensemble signal from the whole sample. To separate the signal coming from different positions, we have to apply spatial encoding. This can be done by varying the static magnetic field according to position; in this way, the Larmor frequency in each position will also differ. By Fourier transforming the acquired FID, we can assign the signal to coordinates. This variation of the magnetic field is done
by linear magnetic field gradients. To measure in three dimensions, \( x, y \) and \( z \) gradients are applied which typically correspond to the frequency, phase and slice directions.

\[ \text{Figure 2.4: Calculation of the longitudinal (a) and transverse (b) magnetization components according to the Bloch equations. The static magnetic field was assumed to be along the } z \text{ axis. The initial state assumed only transverse magnetization. Values used for the calculation were } \omega_L = 60\text{Hz, } R_1 = 1\text{Hz, } R_2 = 5\text{Hz, } R_2^* = 6\text{Hz. The measured signal is the voltage induced on a pickup coil by the transverse magnetization component. Due to field inhomogeneities, the FID signal has a decay rate } R_2^*. The Fourier transform of the FID is shown in (c). It is centered around } \omega_L \text{ and shows some dispersion. This figure has recreated Figure 2.7 from [7].} \]

### 2.2.3 Pulse sequences and contrast generation

Once the net magnetization has returned to equilibrium, it can be re-excited identically with an RF pulse with the Larmor frequency \( \omega_L \). In this way, whole pulse sequences can be designed and run. The body contains different types of tissue, which differ in the content of magnetic nuclei (water, for the most commonly used case of 1H-MRI) but also in their relaxation rates \( R_1, R_2, R_2^* \). We can modify the experimental parameters in order to generate contrast dependent on the relaxation rates of tissue as follows:

- \( R_1 \): If we choose the time between excitations, termed repetition time \( T_R \), to be smaller than the necessary time \( 5T_1 \) to return to equilibrium, then the signal will be weighted by \( T_1 \).

- \( R_2 - R_2^* \): We can remove the effect of field inhomogeneities, which are static, by applying a so-called refocusing excitation pulse of \( 180^\circ \) at \( t = T_E/2 \), which flips the magnetization vectors around the \( y- \) axis. At \( t = T_E \), the magnetization vectors become co-aligned. This refocusing is called spin echo and the time \( T_E \) echo time. The echo recorded in this way is not \( T_2 \) weighted, but \( T_2 \)-weighted.
By selecting $T_E$ and $T_R$, a continuum of different contrasts can be achieved. This is the reason for the flexibility of MRI in optimizing the contrast for a specific application.

It must be noted here that the relaxation parameters of tissue $R_1$, $R_2$ and $R_2^*$ can be modified by administration of contrast agents (CAs). Contrast agents are paramagnetic or superparamagnetic contrast compounds with different numbers of unpaired electrons. As the magnetic moment of those electrons is much higher than that of protons, those compounds can induce strong local magnetic fields and thus increase the relaxation rates locally. The effect of this increase is dependent on the concentration of the CA in tissue, and mathematical modeling is necessary in quantification. Contrast agents are used in preclinical research and some of them have been approved for clinical use.

### 2.3 Fluorescence-based optical methods

#### 2.3.1 Light propagation in the body and contrast

The areas of the EM spectrum corresponding to visible light and its neighborhood have been used routinely in biology in the field of microscopy [27]. However, our everyday experience indicates that light cannot penetrate deep tissue. This is because light is heavily absorbed from the body [28]. The main absorbers in tissue are water and blood, with the defining components of the latter being hemoglobin and deoxyhemoglobin. Figure 2.5 shows that the absorption coefficient of tissue, $\mu_a$, is minimal in the near infrared (NIR) region. Even then, tissue penetration can only reach up to several centimeters [9]. Therefore, imaging with visible light is limited to small animal imaging or clinical applications where the tissue thickness is small or the tissue absorption is relatively low, such as in breast imaging.

An additional issue with using light to probe tissue is that it is heavily scattered – the effect of scattering is in fact so strong that light propagation in tissue can be reasonably approximated with diffuse propagation [30]; in other words, light loses its directionality in tissue. Thus, the mathematical methods used to solve the other medical imaging inverse problems cannot be applied. Reconstructing the distribution of $\mu_a$ and $\mu_s$ in tissue is the objective of diffuse optical tomography (DOT). For the mathematics underlying DOT reconstruction, the reader is referred to Appendix A. DOT has been applied to assess functional parameters, such as blood volume and oxygenation, by exploiting the known absorption coefficient of oxy- and deoxyhemoglobin. Clinical applications include neonatal brain imaging, optical
2. Biomedical imaging modalities

![Graph of the absorption coefficient of light in tissue as a function of wavelength. The main absorbers are hemoglobins, lipids and water; values used were 70% oxygenation saturation, 50mM hemoglobin concentration and a composition of 50% water and 15% lipids. The insert shows tissue autofluorescence spectra for excitation wavelengths $\lambda_{exc}$ from 337 to 610nm and emission wavelengths $\lambda_{em}$ from 360 to 750nm. Tissue autofluorescence is much lower at longer wavelengths. Figure adapted from [29].](image)

Figure 2.5: Graph of the absorption coefficient of light in tissue as a function of wavelength. The main absorbers are hemoglobins, lipids and water; values used were 70% oxygenation saturation, 50mM hemoglobin concentration and a composition of 50% water and 15% lipids. The insert shows tissue autofluorescence spectra for excitation wavelengths $\lambda_{exc}$ from 337 to 610nm and emission wavelengths $\lambda_{em}$ from 360 to 750nm. Tissue autofluorescence is much lower at longer wavelengths. Figure adapted from [29].

mammography and visualization of brain function (see [31] for an excellent review). For a full treatise of light propagation in tissue, the reader is referred to [32].

However, the endogenous contrast based on the tissue optical properties is not very high, as signal alterations are of the order of percent or even less. The sensitivity of optical methods can be enhanced significantly by using fluorescence as a contrast mechanism. For example, functional measurements can be performed using the dye indocyanine green (ICG), which binds to blood plasma proteins and stays confined in the vascular system. ICG has been FDA-approved and is used clinically for determining cardiac output, hepatic function and liver blood flow, as well as for ophthalmic angiography [33]. Employing fluorescence in optical imaging has an additional advantage – by administrating fluorescently labeled reporter molecules that are targeted to a specific molecular target, it allows studying molecular processes in the living organism. Targeted fluorescence probes of fluorescent reporter constructs can be designed to visualize specific molecular interactions. A large palette of fluorescent agents for in vivo imaging can also be found commercially. Contrast in fluorescent based imaging is compromized by tissue autofluorescence, which reduces the signal-to-background ratio. Apart from autofluorescence, confounding signals may also arise from non-targeted exogenous sources such as chlorophyll and its breakdown products in the gastrointestinal tract. As shown in Figure 2.5, autofluorescence is lowest in the NIR region.
2.3. Fluorescence-based optical methods

2.3.2 Fluorescence reflectance imaging (FRI)

The simplest way to image fluorescence macroscopically is with the method of fluorescence reflectance imaging (FRI). The light source (typically a white light source with exchangeable excitation filters according to the spectrum of the probe used) illuminates the animal from above. The light intensity distribution at the surface of the animal is recorded with a sensitive camera, also placed above the animal. This basic principle can be expanded with spectral unmixing [34, 35], which records the image at different wavelengths and identifies the contribution of known spectral components to the images, and dynamic contrast imaging [36]. For an excellent review of FRI systems and biomedical applications, the reader is referred to [37]. FRI systems, however, can only provide semi-quantitative information, as shown in Figure 2.6. Due to the nature of light propagation in tissue, the observed signal on the animal surface can appear very diffuse for deeper lying structures, although the source of fluorescence itself is confined. Moreover, as light is heavily absorbed in tissue, the intensity of the fluorescent source cannot be correctly estimated for deeper lying structures. The method of fluorescence molecular tomography, presented in the following section, addresses this problem.

![Figure 2.6: Simulated data showing the impact photon scattering can have on fluorescence imaging in living tissue. (a) shows the illuminated area on the surface of the animal for an 8 mm diameter spherical tumor located in the abdomen for two cases: (b) near the surface, (d) close to the axial center of the animal. (c) and (e) show the outgoing light at the surface for the tumors shown in (b) and (d), respectively. The intensity of the outgoing light in (e) is approximately one-thousandth of that shown in (c). Figure adapted from [37].](image-url)
2. Biomedical imaging modalities

2.3.3 Fluorescence molecular tomography (FMT)

Fluorescence molecular tomography (FMT) was first presented in [8]. FMT models light propagation in tissue to recover the location and the concentration of fluorescent sources, and has been shown [38] to yield more accurate results than FRI. The mathematics behind FMT are quite lengthy and complex. For reasons of clarity, they are described in detail in Appendix A. In this section, the basic principles behind FMT will be discussed.

Method principle

Assume the experiment depicted in Figure 2.7. A tissue contains a fluorophore which is excitable at wavelength $\lambda_{\text{ex}}$ and emits at wavelength $\lambda_{\text{fl}}$. We excite the tissue with a point source of $\lambda_{\text{ex}}$ at position $\mathbf{r}_s$ of its surface – the source can have some coupling losses $\Theta_s$. The light propagates in a diffuse manner (with wavenumber $\kappa_{\lambda_{\text{ex}}}$) inside the tissue, and excites the fluorophore. The fluorophore then acts as a source of light with $\lambda_{\text{ex}}$, which also propagates in a diffuse manner ($\kappa_{\lambda_{\text{fl}}}$) inside the tissue. The intensity of this secondary source depends on the fluorophore concentration $n$ and on its quantum yield. We can measure the flux of light from the surface at wavelengths $\lambda_{\text{ex}}$ and $\lambda_{\text{fl}}$, which, using Fick’s law, we can relate to the intensity of light at the surface. The position of the detector is $\mathbf{r}_d$. Clearly, the flux that we can measure depends on the detector gain and losses $\Theta_d$ and its quantum efficiency $QE_{\lambda_{\text{ex}}}$, $QE_{\lambda_{\text{fl}}}$ at $\lambda_{\text{ex}}$ and $\lambda_{\text{fl}}$ respectively. What we then wish to reconstruct is the position and concentration of the fluorescent source which gives rise to the measured field upon excitation with the laser source. (NB that with time-modulated measurements, the fluorescence lifetime can also be recovered. In this work, a continuous wave (CW) source has been employed).

As described in [39], the measured intensity of the photon field at wavelength $\lambda_{\text{ex}}$ can be described with

$$U_{\text{inc}}(\mathbf{r}_s, \mathbf{r}_d) = QE_{\lambda_{\text{ex}}} \cdot \Theta_s(\mathbf{r}_s) \cdot \Theta_d(\mathbf{r}_d) \cdot U_0(\mathbf{r}_s - \mathbf{r}_d, \kappa_{\lambda_{\text{ex}}}),$$

(2.1)

which is the solution of the nonhomogeneous diffuse wave equation with a point source $\delta(\mathbf{r} - \mathbf{r}_s)$ of wavelength $\lambda_{\text{ex}}$ inside homogeneous tissue.

Similarly, the measured field at wavelength $\lambda_{\text{ex}}$ is
2.3. Fluorescence-based optical methods

Figure 2.7: Schematic of basic FMT experiment. A point source at position \( r_s \) illuminates some tissue (in pink), containing a fluorophore (in green), with one wavelength \( \lambda_{ex} \) (orange). The light is absorbed by the tissue (not depicted here) and is multiply scattered by tissue structures, thus changing its direction. Some of the light excites the fluorophore, which then emits at a longer wavelength \( \lambda_{fl} \) (red). The emitted light is also scattered multiply, and some of it will reach the detector at position \( r_d \) of the surface. Multiple sources and multiple detectors can be used, to increase the available measurements to solve the problem of reconstructing the fluorescent source.

\[
U_{fl}(r_s, r_d) = \int \Theta_s(r_s) \cdot \Theta_f \cdot Q E^{\lambda_{fl}} \cdot \Theta_d(r_d) \\
\cdot U_0(r_s - r_d, \kappa_{ex}) \cdot \frac{n(r)}{1 - i\omega\tau(r)} \cdot \frac{c}{D^{\lambda_{fl}}} \\
\cdot G(r_d - r, \kappa_{fl}) \cdot d^3 r
\]  

(2.2)

which is the solution of the nonhomogeneous diffuse wave equation with a distributed source

\[
U_0(r_s - r_d, \kappa_{ex}) \cdot \frac{n(r)}{1 - i\omega\tau(r)} \cdot \mu_{a,fl}
\]

of wavelength \( \lambda_{fl} \). Equation 2.2 is a direct implication of the Green’s function definition B.1. \( \Theta_f \) is the attenuation of the fluorescent filter at wavelength \( \lambda_{fl} \).

To make those equations more manageable, we can normalize equation 2.2 by 2.1. Therefore, we have:
2. Biomedical imaging modalities

\[
U^{nB}(r_s, r_d) = \frac{1}{\Theta_f} \cdot \frac{U_{fl}(r_s, r_d)}{U_{inc}(r_s, r_d)} \cdot \frac{Q E^\lambda_{ex}}{Q E^\lambda_{fl}}
\]

\[
= \frac{1}{U_0(r_s - r_d, \kappa_{\lambda_{ex}})} \cdot \int U_0(r_s - r, \kappa_{\lambda_{ex}}) \cdot n(r) \cdot \left(1 - i\omega\tau(r) \cdot \frac{c}{D^\lambda_{fl}} \cdot G(r_d - r, \kappa_{\lambda_{fl}}) \cdot d^3r \right)
\]

(2.3)

Note that the measured intensity at \(\lambda_{ex}, U_{inc}\), refers to a homogeneous tissue. However, it can be replaced by a measurement \(U_{inc, meas}\), if \(U_0\) in equation 2.1 is computed for the average optical properties of tissue. This corresponds to a Born approximation, and hence this approach is termed normalized Born approximation. It has been shown [40] that Born normalization renders the reconstruction robust to several errors, such as incorrect boundary modeling and inaccurate tissue optical properties. The reconstruction accuracy was found to be \(\pm 10\%\).

Returning to the experiment, our goal is to reconstruct the position and concentration of the fluorescent source. The functions \(U_0(r_s - r_d, \kappa_{\lambda_{ex}})\) and \(G(r_d - r, \kappa_{\lambda_{fl}})\) have not yet been defined. As with any partial differential equation, an analytical solution can only be given in the case of a regular geometry \(*\). To define these functions in the case of arbitrary geometries, two approaches can be pursued:

- **Analytical approach.** The functions are defined analytically for the case of infinite space and updated upon consideration of boundary conditions. Using the Green’s theorem, the functions are defined using integral of the surface, instead of the volume integral – this approach is therefore a boundary element method [41]. The surface integral is discretized and the surface is approximated by piecewise linear elements (Kirchhoff approximation). By considering the surface instead of the whole volume for the definition of \(U_0(r_s - r_d, \kappa_{\lambda_{ex}})\) and \(G(r_d - r, \kappa_{\lambda_{fl}})\), this approach is less computationally expensive. However, inclusion of varying optical properties (prior information) inside the volume is challenging. In this work, the analytical approach has been used. It is explained in detail in Appendix A.

- **Numerical approach.** With the increase of computational power, the use of finite element methods gained in attractiveness. There, the volume is discretized in elements

\(*\) This is, indeed, what was used for early generation FMT systems – the animal was immersed in index-matching fluid and placed in a cylinder or slab.
and \( U_0(\mathbf{r}_s - \mathbf{r}_d, \kappa^{\lambda_{ex}}) \) and \( G(\mathbf{r}_d - \mathbf{r}, \kappa^{\lambda_{fl}}) \) are determined numerically. An important advantage of this class of methods is that spatially varying optical properties (prior information) can be included more easily.

Methods combining both approaches have also been presented recently [42].

The above considerations, leading to equations for the light propagation in tissue, are called the forward problem. Now that all other terms have been defined, we need to solve the backward problem, i.e. solve the equation for the fluorescent source. This is done numerically – therefore, the tissue is discretized into volume elements and the integral of equation 2.3 can be replaced by a sum, which in turn can be expressed in matrix form.

So far, we have described an FMT experiment for one source-detector pair \((\mathbf{r}_s, \mathbf{r}_d)\). To increase the available information and be able to solve the system, we instead perform measurements of a series of sources \(s_u\) and detectors \(d_v\), thus having \(m = u \times v\) measurements available. Thus, the problem of estimating the distribution of fluorescence concentration \(n\) in matrix form is:

\[
\begin{bmatrix}
\mathcal{P}^n B(\mathbf{r}_{s1}, \mathbf{r}_{d1}) \\
\mathcal{P}^n B(\mathbf{r}_{s1}, \mathbf{r}_{d2}) \\
\vdots \\
\mathcal{P}^n B(\mathbf{r}_{su}, \mathbf{r}_{dv})
\end{bmatrix}
= 
\begin{bmatrix}
W_{11} & W_{12} & \cdots & W_{1n} \\
W_{21} & W_{22} & \cdots & W_{2n} \\
\vdots & \vdots & \ddots & \vdots \\
W_{m1} & W_{m2} & \cdots & W_{mn}
\end{bmatrix}
\times
\begin{bmatrix}
n_t(\mathbf{r}_1) \\
n_t(\mathbf{r}_2) \\
\vdots \\
n_t(\mathbf{r}_n)
\end{bmatrix}
\tag{2.4}
\]

where \(W\) is termed the weight matrix.

The backward problem consists of solving this matrix equation for the fluorescent source. The matrix \(W\) is generally poorly conditioned and not square. Thus, direct inversion is not possible. Instead, an estimate of the fluorescence distribution \(N\) giving rise to the collected data \(P^n B\) is obtained, using least squares. To improve the condition number of the problem, Tikhonov regularization is performed as:

\[
\hat{n} = \arg \min_n \| Q(WN - P^n B) \|^2_2 + \lambda^2 \| L n \|^2_2
\tag{2.5}
\]

where \(L\) is the regularization term and \(Q\) is the inverse square root of the noise covariance. According to the desired properties of the solution, different forms of \(L\) can be utilized, as shown in [14, 15].

21
Existing FMT systems and their applications

Following the advancements of FMT theory, several custom-made systems have been constructed in biomedical research laboratories [43, 44]. Improvements of those systems include spectral unmixing similar to FRI systems [45], full angular coverage [46, 47] and conical mirror design [48]. Commercial FMT systems [49] also exist nowadays. After the first demonstration of protease visualization in tumor in 2002 [8], both custom-made and commercial FMT systems have been employed in molecular imaging studies, most prominently in the field of cancer. Some studies demonstrated proof of principle for visualizing near infrared [50] and green [51] fluorescent proteins in vivo. Commercial imaging probes were utilized to measure tumor vascular volume in mice [52] and examine the effects of anti-VEGF therapy in vasculature [53]. In [54], genetic profiling on lung tumors revealed cathepsin protease overexpression, which were also assessed with FMT with a commercial probe. Probes were also synthesized and fluorescently labeled to access different hallmarks of cancer, including an annexin-based probe labeled with Cy5.5 for assessing apoptosis upon chemotherapy [38] and labeling of tumor-associated macrophages (TAMs) with AMTA860 [55]. Finally, a protease-targeting fluorescent probe was also constructed and used as a photodynamic agent, in order to improve the photodynamic treatment of tumors [56].

These studies underline the advantages of FMT in preclinical research: fluorescently labeled targeted probes that have been used in in vitro experiments can be directly used in in vivo experiments with FMT, and then validated with microscopy ex vivo. This suggests that it is not the depth accuracy per se that renders FMT superior to FRI, but rather its quantitative nature. This is especially evident when assessing therapeutic effects. On the other hand, it must also be noted that most of the aforementioned studies were performed on subcutaneous tumors; the potential of FMT for measurements of deeper-lying structures has thus yet not been fully exploited.

2.4 Hybrid imaging – combining FMT with MRI

As discussed in Chapter 2.1.1, disease phenotypes may display structural, functional, metabolic and molecular alterations. Imaging some of these components can improve the diagnostic accuracy and advance our biological understanding. At the same time, no single imaging modality can provide high spatial resolution, high temporal resolution and high
Hybrid imaging – combining FMT with MRI

sensitivity. Therefore, combinations of modalities (multimodality imaging) are needed. Such combinations can be performed in several ways, with increasing complexity: (a) imaging with different modalities at (roughly) the same timepoint, (b) imaging with a compatible platform, transferred from one modality to the other, or (c) imaging with fully integrated systems – hybrid systems.

Hybrid systems, combining two or more modalities in a single bore, face several constraints [10]; the space is limited, components must be compatible with all modalities and not cause artifacts, and at the same time the performance of each modality must be comparable to the standalone version. Therefore, the costs of development are high. At the same time, hybrid systems are space- and time-efficient. They also ensure identical patient or animal positioning and imaging under identical physiological parameters. Moreover, the readouts can be overlaid – this is of particular advantage when combining high spatial resolution modalities (CT, MRI) with high sensitivity modalities, yielding molecular information (PET, FMT). The advantage of hybrid systems is not only additive; data from one modality (typically the structural imaging modality providing high spatial resolution data) may be used to improve the performance of the other (typically the molecular modality providing low spatial resolution data), yielding better reconstructions. An example of this is attenuation correction in PET in PET/CT systems [57]. Existing data suggest that PET/CT imaging has improved cancer staging in patients [11].

For all these reasons, hybrid systems, with PET/CT [58] as the most prominent example, are increasingly being used into the clinics. Recently, clinical PET/MR systems have also been constructed [59]. Preclinical hybrid systems of course include PET/CT [60] and PET/MR [12], but also FMT/CT systems [13, 61]. In animal research, FMT is an attractive alternative to PET, as provides quantitative molecular information with non-ionizing radiation and stable probes. Recently, several studies have been performed using a platform of a commercial FMT system, compatible with CT, MR and PET scanners of major vendors [62–64]. In view of the additional value that such readouts can provide to existing biomedical applications of FMT imaging as presented in section 2.3.3, it is conceivable that this field will continue to grow.

However, the idea of combining tomographical optical imaging with structural methods, in specific with MRI, is not new. Barbour presented it as early as 1995 [65], suggesting that the soft tissue contrast provided by MRI could provide more accurate DOT reconstruction. In 2000, Ntziachristos combined DOT using ICG with contrast-enhanced MRI, to assess also the functional characteristics of lesions [66]. Additional investigations in this topic were
recently presented by Davis [67,68] and can be found in a review by Niedre and Ntziachristos in 2008 [69].

Why is it, then, that truly hybrid FMT/MRI systems for preclinical applications have not been constructed? The most important reason is that the aforementioned implementations use optical fibers for the laser excitation and fluorescence detection into the sample. Although fibers have the advantage that they do not cause artifacts in MRI, they are bulky. This means that they cannot be integrated in preclinical MRI scanners, but necessitate clinical scanners. Due to the much smaller mouse dimensions compared to humans and the corresponding low fill factor under the MR coil, the spatial resolution of clinical MRI scanners is much lower than achievable in preclinical ones. Moreover, existing MRI mouse protocols cannot be directly transferred. On the FMT side, fibers offer less source-detector pairs than state-of-the-art FMT systems based on cameras, and thus offer an inferior reconstruction quality. Therefore, although those systems have demonstrated the feasibility of the technique and the potential in improving optical reconstructions using MRI information, they involve serious compromises in the quality of both modalities, and thus could not be employed in biomedical applications.

The replacement of optical fibers in FMT/MRI systems requires (a) a theoretical framework for FMT reconstruction using non-contact measurements and (b) the identification of MRI-compatible cameras. The first issue has been addressed by Ripoll in 2003 [70]; furthermore, the field of PET/MRI has identified [71] avalanche photodiodes for use inside the high fields of preclinical MRI scanners. These advances have been utilized in a truly hybrid FMT/MRI system by Stuker et al. [1], which employs non-contact measurements inside a preclinical 9.4T MRI scanner. This thesis investigates further the potential of this method.
Chapter 3

FMT/MRI: Hardware components and development of improved reconstruction tools

This chapter presents tools developed in the course of this PhD thesis, which have been used in the applications presented in the chapters 4, 5 and 6.

3.1 A more compact FMT system

3.1.1 Motivation and goals

A custom-made FMT system existed in the Optical Laboratory of the Animal Imaging Center. It was designed and built by Dr. Florian Stuker based on a prototype existing in the Institute of Electronic Structure and Laser, Forth, Greece, and it was used on first proof-of-concept biological studies with FMT. To enable parallel use of FMT for biological applications and further technological development on the FMT system, we decided to construct a second FMT prototype for biological use.

The specifications for the second system with respect to the existing FMT prototype were (a) more compact size, (b) hardware and software compatibility, (c) equivalent performance and (d) feasibility of reflection and transmission measurements.
3. FMT/MRI: hardware and software developments

Contributors

Dr. Florian Stuker assisted in the system design, Markus Kuepfer from the ETH workshop performed the mechanical construction and Dr. Divya Vats and Dr. Jan Klohs assisted with system validation. Prof. Dr. Jorge Ripoll provided the original system control and reconstruction software and assisted with modifications.

3.1.2 Materials and Methods

System description

The FMT system was designed using Autodesk Inventor (Autodesk Inc., USA) and is shown in Figure 3.1. It was enclosed in a black light-tight box, which measured $745 \times 520 \times 450\text{mm}^3$, compared to $800 \times 600 \times 600\text{mm}^3$ of the first prototype. The basic components of the system were:

1. A continuous wave laser source (solid state laser at 670nm, B&W Tek, Newark, USA), used to excite the fluorophore in the sample. The system is modular and any laser could be connected. The wavelength of 670nm was chosen because of its suitability for in vivo biological applications.

2. A pinhole aperture of 0.5mm diameter, aligned to the fiber output of the laser and used to control the beam spot diameter.

3. A 100mm lens (Thorlabs, Germany) that could be placed on two positions according to the chosen measurement setup (transmission/reflection), so that the beam spot on the sample had a diameter of $\approx 1\text{mm}$.

4. A power meter (PM100, Thorlabs, Germany), used to measure the incident laser power for normalization of the results.

5. A galvanometric scanhead (Scancube 10, ScanLab, Puchheim, Germany) deflecting the laser beam, so that a grid was scanned on the sample surface.

6. Adjustable mirrors (rectangular front surface mirrors, Edmund Optics, NJ, USA for transmission/reflectance measurements, which deflected the beam accordingly. For transmission measurements, the beam was deflected once, by 90°C, so as to penetrate the sample from below. For reflection measurements, the beam was deflected twice by 90°C so as to excite the sample from above. The mirrors were adjusted so that for both
3.1. A more compact FMT system

measurement setups, the laser beam is located at the center of the camera field of view for zero deflection of the galvanometric scanhead.

7. An animal platform connected to a xyz translation stage (Thorlabs, Germany). The platform was heated with water tubing connected to a water bath and equipped with a mask with a tooth bar, which was connected to the gas and isoflurane supply. To accommodate transmission measurements, a rectangular cutout was made in the center of the platform.

8. A lens (Nikon 50mm f/1.4 AF D Nikkor, Tokyo, Japan) to focus the image on the sample plane.

9. A filter wheel (DTA RPF Max 16, Cascina, Italy) with 16 positions for 25.4mm diameter filters. The filters initially included were bandpass filters of 25nm with at 660, 720, 740, 800 and 820nm (Semrock, Rochester, USA), and they were used for detection of the intrinsic and fluorescent field according to the fluorescent probe used in experiments.

10. A CCD camera (iKonM, Andor Corporation, Northern Ireland) with 512 × 512 array size and 16 bits depth, to record and store the photons emitted by the sample. The camera was cooled at −85°C to reduce dark- and readout noise [72].

11. White light sources (electroluminescent plates, Distrelec, Switzerland), used for sample placement and for generation of a reference whitelight image, on which the reconstructed fluorescence was overlaid.

Operation of the FMT system

For detailed instructions on how to operate the FMT system, the reader is referred to a manual for FMT system users in the AIC [73].

Initialization. The FMT acquisition was controlled by software written in LabView v 8.5 (National Instruments, TX, USA). It was originally written by Prof. Dr. Jorge Ripoll and modified for this particular setup. After equipment initialization, the CCD camera was cooled to −85°C. The adjustable parts of the setup (lens and mirrors) were positioned according to the imaging mode (reflection/transmission) and the part of the body imaged.
3. FMT/MRI: hardware and software developments


**Calibration.** A piece of millimeter paper attached to a silicon phantom of the same height as the average minimal height was placed in the center of the camera’s field of view. Two calibration procedures were performed: (a) the stage calibration mapped linearly the deflection of the galvanometric scanhead to the laser beam position in the field of view, as measured by the camera, whereas (b) the pixel size calibration mapped dimensions measured by the camera in pixels to physical dimensions. The generated calibration files could be saved and loaded for different animals, provided that the setup was not moved.

**Source selection.** The anesthetized animal was placed on the heated platform and connected to the isoflurane mask to maintain body temperature and anesthesia. The region of interest (ROI) on the animal was selected interactively based on a white light reference image of the animal. The appropriate laser source grid dimensions \((x \times y)\) and number of sources \((n_x \times n_y)\) were selected to ensure a complete and homogeneous excitation of the ROI. The sources file could also be saved and kept for later use.
3.1. A more compact FMT system

Experiment. The laser and the two filter wavelengths, to record the excitation and emission measurements, were selected according to the used fluorescent probe. Prior to each of the two measurements, the laser power and the camera integration time were adjusted so as to utilize the CCD camera’s 16-bit dynamic range. These settings were kept constant throughout the measurement. For each wavelength, the laser beam was scanned automatically across the defined source grid. For each source position, an image of dimensions $N_x \times N_y$ was recorded, where $N_x$ and $N_y$ are the number of pixels in the x and y directions. The images for all sources $N_s$ were saved in a .tiff stack of size $N_x \times N_y \times N_s$. According to the software version used, a background image file for each wavelength, corresponding to the camera’s dark current at this wavelength, was either saved separately or included in the wavelength’s stack. For each measurement, an automatically generated log file was generated and saved. This file contained relevant information for the reconstruction regarding the setup (laser and filter wavelength, camera type and temperature etc), normalization (laser power and camera exposure time), calibration (two calibration matrices) and sources.

Reconstruction. Reconstruction was performed offline. The reconstruction parameters (use of masking, source exclusion, initial values for the tissue optical properties, boundary mesh dimensions) were entered by the user in a separate .txt file, and they were the same for all experiments within one study.

The reconstruction software, running in Matlab, was originally written by Prof. Dr. Jorge Ripoll and modified in this work. The algorithm is based on the principles explained in Chapter 2. Briefly, reconstruction settings (such as initial assumptions for the optical parameters of tissue, number of sources to use mesh discretization, number of iterations for solution of the backward problem) were input in a .txt file. The whitelight reference image, the excitation and the emission measurements were loaded. Additionally, a binary mask generated by the user could be used to select specific sources. This was especially important for cases where unwanted reflections were recorded. Finally, a .mat geometry file, describing the animal surface, could be input for use in the reconstruction. For details of how this geometry file was generated in this work, the reader is referred to 3.3.3. If no such file was provided, the animal was approximated by a slab.

The fluorescence measurements were first normalized to the excitation measurements according to [39]. The goodness of fit for the absorption parameter of tissue, $\mu_a$, was examined and accordingly updated. The data was masked and the detector positions, i.e. the mesh coordinates for FMT reconstruction according to the discretization given in the reconstruc-
tion settings file, were generated. The animal surface, described by surface patches and vectors, was calculated for these discretization settings. The boundary was removed as described in [74]. The data was median filtered to remove specular variations. The resulting measurement matrix was ready to be used in the inversion. The position of the sources was found according to the reconstruction discretization and the animal surface. This was done so that all the matrices used for the reconstruction have the same dimensions and are in the same coordinate system. The mesh-detector functions, corresponding to the Green’s functions for the differential equations describing diffusion from the source to the mesh and from the mesh to the detector, as described in [75, 76]. The mesh detector functions constituted the weight matrix for the inversion. Finally, inversion of the equation was performed with ART [74]. The resulting matrix values were normalized to the laser power and the exposure time employed in the excitation and emission measurements. The reconstruction yielded a three dimensional map of the fluorescent source distribution within the animal, which could be visualized with Matlab (The Mathworks, Inc., USA) or other programs.

For quantification, regions of interest could be selected on the reconstructed fluorescence source matrix. The value used was the mean value of the reconstructed fluorescence. For visualization and comparison purposes among different animals and timepoints, the data was always scaled to common colormap and transparency values.

### 3.1.3 Results and Discussion

An additional standalone FMT system for biological use in the AIC was successfully designed and constructed. The new system was indeed smaller, but compatible with its predecessor. A series of biological studies was performed on this system; two of these are described in Chapter 4.
3.2 The first FMT/MRI system prototype

3.2.1 Preliminary remarks


The instrumentation is further described in the following patents:

- F. Stuker, K. Dikaiou, C. Baltes and M. Rudin, "MRI Device With Fluorescence Molecular Tomography" System. Pub. No.: EP 2 251 676 [77], and


3.2.2 Summary [1]

The high sensitivity of fluorescence imaging enables the detection of molecular processes in living organisms. However, diffuse light propagation in tissue prevents accurate recovery of tomographic information on fluorophore distribution for structures embedded deeper than 0.5mm. Combining optical with magnetic resonance imaging (MRI) provides an accurate anatomical reference for fluorescence imaging data and thereby enables the correlation of molecular with high quality structural/functional information. We describe an integrated system for small animal imaging incorporating a noncontact fluorescence molecular tomography (FMT) system into an MRI detector. By adopting a free laser beam design geometrical constraints imposed by the use of optical fibers could be avoided allowing for flexible fluorescence excitation schemes. Photon detection based on a single-photon avalanche diode array enabled simultaneous FMT/MRI measurements without interference between modalities. In vitro characterization revealed good spatial accuracy of FMT data and accurate quantification of dye concentrations. Feasibility of FMT/MRI was demonstrated in vivo by simultaneous assessment of protease activity and tumor morphology in murine colon cancer xenografts.
3. FMT/MRI: hardware and software developments

3.2.3 Contributions

**SPAD array control software**

The original control software for the SPAD array (AQUA group, EPFL, Switzerland), written for Linux, was ported to the Windows XP operating system using the Visual Studio 2008 Professional IDE (Microsoft Corporation, USA). Dynamic linked libraries (.dll’s) were made and incorporated into the existing FMT control software, written in LabView v8.5 (National Instruments, TX, USA). To reduce the fixed position noise associated with the SPAD [79], the acquired images were median filtered for use in the FMT reconstruction.

**FMT performance evaluation and assessment of interference from MRI [80]**

Glass tubes of 1.5 mm diameter were filled with 20µl of the fluorescent dye AOI987 [81] in various concentrations and were embedded along the y-axis at z = 1.5mm depth in a silicon-based phantom with tissue-similar optical properties. They were measured using a 660nm CW laser and filters centered at 670nm (intrinsic) and at 720nm (fluorescence). FMT experiments were carried out inside and outside of the magnet and with or without concurrent acquisition of MR images. We could demonstrate that the geometrical accuracy of the reconstruction was not affected by gradient switching, yet the intensity of the reconstructed signal amplitude was slightly reduced (Figure 3.2).

Two 1.5mm tubes at a 2mm center-to-center distance could be clearly resolved (Figure 3.3(a)). The reconstructed fluorescence intensity for a dilution series made from the stock solution depended linearly on the respective concentration (Figure 3.3(b)).

**FMT/MRI overlay**

A first version of software for the registration of the FMT reconstruction with the axial MRI images was written in Matlab (The Mathworks, Inc., USA). Using the whitelight image as a reference, the axial MRI slice corresponding to the center of the laser excitation grid was identified. The FMT reconstruction slice along xy which corresponded to this position was translated and scaled to the dimensions of the MR image. Scaling was performed with cubic interpolation. This software was applied to in vivo data as shown in Figure 3.4.
3.2. The first FMT/MRI system prototype

Figure 3.2: Assessment of the effect of MRI gradient switching on the FMT reconstruction of a fluorophore-containing tube embedded in a phantom. Results of FMT reconstruction with MR gradients (a): not in operation, (b): in operation. No artifacts appeared on the FMT reconstruction. However, the reconstructed fluorescence (in arbitrary units, a.u.) was lower when MRI gradients were in operation. Figures (a) and (b) are scaled by the same factor. Figure from [80].

Figure 3.3: (a): Results of FMT reconstruction along the $xz$ axis for two fluorophore-containing tubes at 2mm distance embedded in a phantom at 1.5mm depth. The two tubes could be clearly separated. (b): Linearity of FMT reconstruction for a series of dilutions of the fluorophore AOI987, contained in a glass tube embedded in a phantom at 1.5mm depth. The mean reconstructed fluorescence depended linearly on the fluorophore concentration. Figure from [80].
3. FMT/MRI: hardware and software developments

**Figure 3.4:** (a): FMT reconstruction results along xy (a) corresponding to the MRI axial slice shown in (b). The overlaid data are shown in (c).
3.3 FMT/MRI coregistration and incorporation of MR surface in the FMT reconstruction

3.3.1 Theory

Coregistration of two modalities means that their two separate coordinate systems have to be mapped onto one [82]. Mathematically, this corresponds to finding a transformation matrix $T$, so that

$$
\begin{pmatrix}
  x_1 \\
  y_1 \\
  z_1
\end{pmatrix}
= T \cdot
\begin{pmatrix}
  x_2 \\
  y_2 \\
  z_2
\end{pmatrix}
$$

(3.1)

The number of unknowns in equation 3.1 depends on the form of the matrix $T$, which is in turn a function of the processes accounted for by the transformation. Examples include translation, rotation, scaling, reflection and shearing, listed in order of increasing complexity. The data for inverting the system of equation 3.1 are provided in pairs, corresponding to the two modalities. A minimal number of pairs is necessary so that the system is (over)determined. In the simplest case, data are pairs of points [39]; in more complicated cases, whole surfaces may be used [83,84]. Correspondingly, equation 3.1 may be solved by direct inversion or iteratively. The practical implementation of the above requires the existence of structures that can be identified with both modalities; these can be fiducial markers or anatomical landmarks. Provided that the geometrical configuration between modalities is constant, the transformation matrix $T$ can be found once, and used to transform subsequent acquisitions.

3.3.2 Considerations for the FMT/MRI hybrid system

On the existing FMT/MRI prototype, one optical reference image (whitelight) was acquired from above the sample. This meant that coregistration could only be performed in two dimensions, although MRI data are three-dimensional. We approached the problem by observing that the whitelight image essentially depicts the animal’s outer surface, as viewed from above. A 2D view of the animal’s surface computed from the MRI data would make the equivalent of the whitelight image. Therefore, pairs of points could be selected to estimate the transformation matrix $T$. This had an additional advantage; the computed MR animal surface, coregistered to the whitelight image, could be included in the FMT reconstruction.
3.3.3 Method

An algorithm for coregistering the FMT and MRI data was developed in Matlab (The Mathworks Inc., MA, USA). The code is presented in detail in Appendix C. Here, the basic steps of the algorithm are explained.

Inputs to the algorithm were one MRI dataset and its corresponding measurement data with FMT. The algorithm had the following steps:

1. **Input and segmentation of the MR data.** The method file, which contained the scanner-native MRI data acquisition parameters, was read. The parameters of interest were the type of sequence (2D/3D), slice orientation (axial/coronal), the spatial resolution along xy, the slice thickness and slice array gap. The data were Gaussian filtered with a $3 \times 3$ kernel. The sample was segmented from background either with the Otsu method [85], or with a user-input value. In the later case, the histogram of the data was provided for guidance. Isosurfaces corresponding to the animal surface $S(x, y, z)$ were extracted from the volume data according to an isovalue provided by the user (Figure 3.5 (b)). For faster computation, the number of isosurface patches could be reduced by a given ratio. Default isosurface visualization was provided for animal and phantom data, for visual inspection.

2. **Computation of height map from the MR data.** The top "sheet" of the animal (i.e. the surface as viewed from above), called height map, was computed by finding the uppermost index for each column along the height axis of the 3D isosurface data, $S(x, y, z_{max})$. The height map was interpolated to the optical camera’s pixel dimensions $dx$, $dy$ with cubic splines. NB that these are not the–much coarser–dimensions of the mesh used for the reconstruction. The data was represented in 3D (Figure 3.5 (c)) and 2D (Figure 3.5 (d)) in color code, where the color corresponded to the height.

3. **Selection of control point pairs on the whitelight reference image and the 2D height map.** Reference points (e.g. fiducial markers or anatomical landmarks) that were required for defining the affine transformation, could be interactively selected on the 2D height map and the reference whitelight image using the Matlab Control Point Selection Tool. As an affine transformation was used, $\geq 3$ control point pairs had to be selected.

4. **Transformation of the MR data to the FMT coordinate system.** The transformation matrix $T$, mapping the MR data to the whitelight data (Figure 3.5 (e)), was computed on the basis of the reference points and stored. The matrix $T_{inv}$, for mapping FMT on
MRI reference data, was also stored. The height map was multiplied by $T$ and thus transformed to the optical system coordinates (Figure 3.5 (f)). The resulting matrix was stored in a `geometry.mat` file.

5. **Use of the transformed MR surface in the FMT reconstruction.** The `geometry` file was read into the FMT reconstruction software, which was modified to compute the surface patches and normals from the file. The surface was interpolated to the reconstruction mesh parameters. (Figure 3.5 (g))

The algorithm described here is not required every time – for example, a stored transformation matrix computed for one dataset can be utilized to coregister others. This requires that the camera and the MR coil have not changed relative positions. This assumption is clearly valid when the same animal is measured at different timepoints, but potentially also among animals. Moreover, the geometry file does not need to be computed with the above procedure; provided that it is registered to the FMT dataset, any geometry file can be used, regardless of how it has been computed.

### 3.3.4 Phantoms

Silicon-based phantoms as described in [87] were not used, as they introduced chemical shift artifacts in MRI. Instead, agar-based phantoms were made as follows: distilled water was mixed with 1% agarose (Sigma-Aldrich, Switzerland) and heated to 90°C under periodic stirring. When the solution temperature reached 60°C, 2% of Intralipid (Sigma-Aldrich, Switzerland) and 0.01% of ink were poured in and stirred. The mix was poured into rectangular ice cube trays and left to solidify in 4°C. Due to shrinkage caused by water loss, agar phantoms were replaced after 1 day. Multicompartment phantoms were made by including Gadolinium (MRI T₁ contrast) and the fluorophore AOI987 in the standard mix. The solidified phantoms were cut in regular shapes and placed in the trays. Still liquid standard mix was poured on them and let solidify. In this way, spatially separated compartments could be included in one phantom. MRI and FMT data from such a phantom are shown in Figure 3.6.

### 3.3.5 Results and discussion

An algorithm was designed to extract the animal surface from MRI data and incorporate it into the FMT reconstruction, after coregistering the two modalities. It was tested and
optimized for both phantom and in vivo data. For example, the optimal MRI slice orientation in order to estimate the height map was found to be the axial one, as shown in Figure 3.7. Ideally, no slice gap should be used in the acquisitions.

Multicompartment phantoms such as the one shown in Figure 3.6 were made. Both with FMT and MRI we found that the compartments were spatially separated. Such phantoms can be used in the future for a next step of MRI information incorporation; namely, consideration of spatially varying optical properties. The anatomical information of MRI can be used to identify individual tissue compartments with potentially different optical parameters (absorption and scattering coefficient). These parameters have to be known (or at least assumed). Simple tissue segmentation algorithms may not be sufficient to adequately determine the spatial extent of individual compartments, however; as shown in Figure 3.8, the MR surface coil of the hybrid FMT/MRI system has a non-uniform sensitivity. Thus, appropriate intensity scaling accounting for the inhomogeneous RF field distribution of the surface coil will have to be applied prior to segmentation.
3.3. FMT/MRI coregistration and use of MR surface

Figure 3.5: Demonstration of MRI and FMT coregistration on a subcutaneous tumor data example. (a): original MRI in axial orientation, (b): segmented MRI images, (c): computed height map from isosurfaces of MRI data, (d) computed height map in 2D. Data are color coded according to each position’s height. (e): reference whitelight image of the same experiment. (f): transformed height map to the FMT data’s coordinate system. (g): reconstructed fluorescence from subcutaneous tumor (mouse injected with the fluorescent agent ProSense (PerkinElmer, USA)), with tumor surface shown in green dots. Data first presented in [86].
Figure 3.6: Volume MRI data from a multicompartment phantom (a) and calculated isosurfaces (b). FMT reconstruction shown in (c). The multicompartment phantom depicted was made as described in section 3.3.4.
Figure 3.7: Computed height maps for MRI measurements in (a) axial and (b) coronal orientation and identical parameters. The axial orientation is much more suitable for the estimation of height using MR.
3. FMT/MRI: hardware and software developments

Figure 3.8: Coronal MR images of the multicompartment phantom shown in Figure 3.6. The coil sensitivity decreases with depth.
Chapter 4

FMT Studies

In this chapter, the results from two in vivo studies performed with the FMT system presented in Chapter 3 are presented. In both studies, novel targeted optical probes were developed in the context of projects running in the group [88,89]. The aim of the FMT measurements were (a) to investigate if these probes could be employed for optical molecular imaging, and (b) to validate the FMT quantification accuracy against a commercial fluorescence reflectance imaging (FRI) system and nuclear imaging methods.

4.1 FMT imaging of pancreatic beta cells in vivo by targeting transmembrane protein 27 (TMEM27)

4.1.1 Preliminary remark

A part of the results in this section are summarized in the paper [88]: "Multimodal imaging of pancreatic beta cells in vivo by targeting transmembrane protein 27 (TMEM27)" by D. Vats, H. Wang, D. Esterhazy, K. Dikaiou, C. Danzer, M. Honer, F. Stuker, H. Matile, C. Migliorini, E. Fischer, J. Ripoll, R. Keist, W. Krek, R. Schibli, M. Stoffel and M. Rudin, published in Diabetologia 2012. The introduction and a part of the discussion in this section are adapted from [88].
4. FMT Studies

4.1.2 Introduction

Pancreatic beta cells produce and secrete insulin in the body. When the demand for insulin is chronically increased, beta cells adapt by altering functionality and mass [90–92]. The gradual loss of beta cells and their function causes hyperglycaemia and eventually type 2 diabetes [91]. Non invasive monitoring of beta cell changes in vivo could serve as an early indicator of diabetes and accelerate therapy development. Beta cells constitute only 1% of the total pancreatic mass and they are scattered in islets of Langherans within the pancreas, next to large organs involved in drug/label metabolism and excretion; all these factors make the development of imaging assays challenging.

In this work, a molecular imaging approach was chosen. The selected target was the transmembrane protein TMEM27, which is selectively expressed on the beta cell surface [93] and in kidney collecting ducts [94]. A monoclonal antibody 8/9-mAb, specific to human TMEM27 (hTMEM27) was developed and labelled for fluorescence and PET imaging in mice.

4.1.3 Materials and methods

Only information relevant to the FMT experiments and their validation with FRI and PET is presented. For details on monoclonal antibody generation, stable cell lines, cell culturing and labeling methods, the reader is referred to [88].

Labeled antibodies

For fluorescence imaging, the generated monoclonal antibody 8/9-mAb was labeled with Alexa 680 (A200000; Molecular Probes, Eugene, OR, USA). In the following, it is referred to as AF 680-8/9-mAb. For control, a non-TMEM27 binding monoclonal antibody labeled with AF680, termed AF 680-anti-haemagglutinin(HA)-mAb, was used. For nuclear imaging, 8/9-mAb and (HA)-mAb were labelled with a chelating ligand containing zirconium (89Zr).

Animal keeping and tumor generation

All procedures were in accordance with the Cantonal Veterinary Office in Zurich. C57BL6 mice and BALB/c nude mice were from Charles River Laboratories (Sulzfeld, Germany). RIP-hTMEM27-tg mice were generated in-house (M. Stoffel). All animals used
in this study were matched for sex and age. Animals were kept under standard housing conditions with free access to water and chlorophyll-free food (Kliba Nafag, Kaiseraugst, Switzerland).

Rat insulinoma INS-1E cells were cultured to carry the pTRE-hTMEM27 construct (INS-1EhTMEM27). Animals were injected subcutaneously in the left thigh with \(3 \cdot 10^6\) of either cultured INS-1E or INS-1E-hTMEM27 cells in 100\(\mu\)l saline (154mmol/1 NaCl). The tumor-bearing mice were administered 200ng/ml doxycycline hydrochloride in drinking water to induce hTMEM27 gene expression [95], with added sucrose (1%) to ameliorate the bitter taste.

**In vivo fluorescence imaging**

**Preparation:** Mice were anaesthetised with 2% isoflurane (Attane; Minrad, Buffalo, NY, USA) in oxygen/air mixture (1:4, vol./vol.). They were first imaged with the FRI system and then transferred to the FMT system, maintaining anesthesia throughout. For both imaging systems, animals were placed on a prewarmed (37\(^\circ\)C) platform contained in all small-animal imaging systems used in this study.

**Protocol for tumor imaging of subcutaneous insulinoma-bearing mice:** *In vivo* fluorescence imaging was performed after the tumors had reached a visible size (\(\geq 3\)mm). 2mg/kg of the targeted labeled antibody AF 680-8/9-mAb were injected on \(n = 3\) INS-1E-hTMEM27 bearing animals and \(n = 3\) INS-1E-bearing animals. 2mg/kg of the control antibody AF680-HA-mAb were injected on \(n = 4\) INS-1E-hTMEM27 bearing animals. All injections were performed i.v. via the tail vein. Imaging was performed longitudinally during the 6 subsequent days.

**Protocol for pancreas imaging of control (C57BL6) and RIP-hTMEM27 transgenic mice:** The animals (\(n = 4\) for both groups) were shaved and epilated in the abdominal region. The targeted labelled antibody AF 680-8/9-mAb was injected i.v. via the tail vein (2mg/kg). Imaging was performed immediately prior to injection and three days post injection.

**FMT imaging:** FMT was performed on a home-made fluorescence molecular tomography (FMT) system described in section 3.1. For each animal, a region of interest (ROI) containing
the tumor was excited by scanning a 5 × 5 continuous-wave laser source at 671 nm across the ROI. Images for the excitation and emission wavelengths were recorded with an Andor iKon CCD camera (LOT-Oriel, Moulin-du-Choc, Switzerland) and filters centered at 660 nm and 720 nm, respectively. The FMT data were reconstructed as described in section 3.1.2. For quantification, the mean reconstructed fluorescence intensity was computed.

**FRI imaging:** This was carried out with a Maestro 500 imaging system (Cambridge Research, Woburn, MA, USA). For AF 680 measurements, a band pass filter from 615 nm to 665 nm and a high-pass filter over 700 nm were used for excitation and emission light, respectively. A series of images were acquired at different wavelengths and then subjected to spectral unmixing as described by Gao et al [34] for unmixing the AF 680 fluorescence pattern from tissue autofluorescence.

**Ex vivo fluorescence imaging**

**Procedure:** Control and RIP hTMEM27-tg mice were injected with 2 mg/kg of AF 680-8/9-mAb or AF 488-8/9-mAb. The animals were sacrificed 72 h (three days) after antibody administration, and abdominal organs (pancreas, liver, kidney and spleen) were excised and imaged.

**FRI imaging:** A band pass filter from 445 nm to 490 nm and a high-pass filter over 515 nm were used for excitation and emission light, respectively, while the filter combination described above was used for AF 680 detection. Spectral unmixing was carried out as previously described [34]. The images were normalized for depth intensity, exposure time and binning differences.

**FMT imaging:** Measurements were performed in transmission mode. A petri dish containing the organs immersed in water was placed on an optical phantom, to protect the camera from direct laser irradiation. Scanning was performed on a region of interest containing all organs.

**PET imaging**

PET scanning of the animals was performed at 24 h and 72 h after tracer injection using small-animal GE VISTA eXplore PET/CT tomograph. Animals were anaesthetised and
scanned for 60 min. After the PET scan on day 3, the mice were sacrificed. This was followed by tissue sampling and gamma counting. Radioactivity accumulated in tissue samples was expressed as percentage of the injected dose (%ID)/g tissue. PET data were reconstructed in a single time frame and analyzed using PMOD software (PMOD Technologies, Zurich, Switzerland). PET data were quantitatively evaluated by ROI analysis, and the standard uptake value was determined by normalizing the activity concentration in the ROI to the injected dose per body weight. Imaged data were visualized by either maximum-intensity projections (MIPs) or horizontal slice series, which were normalized to the injected dose per body weight.

4.1.4 Results

Prior to in vivo imaging studies, the clinical relevance of the protein hTMEM27 and the binding specificity of 8/9-mAb were assessed with human normal tissue microarrays and immunofluorescence, respectively. It was also established that the expression of hTMEM27 in transgenic mice is close enough to expression in humans in vivo (see [88] for details).

The in vivo binding specificity and the kinetics of 8/9-mAb were examined in mice bearing subcutaneous insulinomas. Three groups were included in the experiment; (a) INS-1E-hTMEM27 tumor-bearing animals injected with the targeted antibody AF680-8/9-mAb (positive/positive), with \( n = 3 \), (b) INS-1E-hTMEM27 tumor-bearing animals injected with the control antibody AF680-HA-mAb (positive/negative), with \( n = 4 \), and (c) INS-1E tumor-bearing animals injected with the targeted antibody AF680-8/9-mAb (negative/positive), with \( n = 3 \). In all cases, the mean reconstructed fluorescence calculated with FMT reached its peak one day post-injection and was retained for the subsequent three days, gradually clearing by day six (Figure 4.1). The FMT signal from INS-1E-hTMEM27 tumor-bearing mice injected with AF680-8/9-mAb was clearly distinguishable from the two controls from days one to three. On day three, it was on average seven times larger than signal from the control INS-1E tumors. This effect was also clear from visual inspection of the reconstructed results, shown in Figure 4.2 for one representative animal per group on the third day post injection.

Quantification of FRI results (Figure 4.3) showed a slightly different pattern; the maximum FRI signal for the INS-1E-hTMEM27 tumor injected with AF680-8/9-mAb occurred three days post injection, and did not return to baseline by day six. The behavior of the two control groups was consistent with FMT. On day three, the average FRI signal from INS-
4. FMT Studies

**Figure 4.1:** Mean reconstructed fluorescence with FMT (in arbitrary units) for three groups in the experiment: INS-1E-hTMEM27 tumors upon AF680-8/9-mAb injection (red), INS-1E-hTMEM27 tumors upon injection of control AF680-HA-mAb (green) and control INS-1E tumors upon AF680-8/9-mAb injection (orange). Data are shown for individual tumor-bearing mice. In all tumors, maximum FMT signal occurs at 1 day post injection and is still clear 3 days post injection, until it returns to baseline on day 6. The FMT signal from INS-1E-hTMEM27 tumors with injection of AF680-8/9-mAb on day 3 is on average seven times larger than signal from the control INS-1E tumors.

![Image](image1.png)

1E-hTMEM27 tumor injected with AF680-8/9-mAb was only 2.5 times larger than for the control INS-1E tumors.

The PET results revealed the same kinetics and binding specificity as with FMT; specifically, PET imaging with the 89Zr-labeled antibody showed that at 72 h after injection, the injected dose per gram of tissue %ID/g in INS-1E-hTMEM27 tumors was six to seven times
4.1. FMT imaging of pancreatic $\beta$ cells in vivo by targeting TMEM27

**Figure 4.3:** Quantification of FRI results for three groups in the experiment: INS-1E-hTMEM27 tumors upon AF680-8/9-mAb injection (red), INS-1E-hTMEM27 tumors upon injection of control AF680-HA-mAb (green) and control INS-1E tumors upon AF680-8/9-mAb injection (orange). Figure courtesy of Dr. Vats.

higher than in control tumors (Figure 4.4). These data indicate specific tracer accumulation due to expression of the molecular target and superior quantification of FMT than FRI, as validated with PET measurements.
Figure 4.4: (a) PET of INS-1E-hTMEM27 and control INS-1E tumor (corresponding to groups a and c for FMT experiments) three days after [89Zr]-8/9-mAb injection. (b) Radioactive uptake shown as %ID/g ($p \leq 0.001$). Maximum uptake was seen in tumor (a, middle arrow) compared with liver (a, top arrow) or site of tail injection (a, bottom arrow). Tumor uptake was much higher in INS-1E-hTMEM tumor bearing mice. Figure adapted from [88].
In a next step, the \textit{in vivo} binding of 8/9-mAb to its target, hTMEM27 in the pancreas, was evaluated. For this, RIP-hTMEM27-tg \((n = 4)\) and control mice \((n = 4)\) were injected with the labeled antibody AF680-8/9-mAb and imaged with FMT and FRI three days post injection. In some cases, the FMT signal in RIP-hTMEM27-tg was indeed higher than in control mice – however, this was not consistent, as shown in Figure 4.5. Quantification of the mean reconstructed fluorescence for the two groups showed on average three times higher mean reconstructed fluorescence for the transgenic mice. However, due to the high variability of the data, no significant effect could be found (Figure 4.6(a)). In contrast to this, analysis of the fluorescence intensity measured with FRI for the two groups revealed a significantly higher (3.3-fold) fluorescence intensity in the RIP-hTMEM27-tg mice (Figure 4.6(b); \(p = 0.01\)). It is not known what caused the greater variability in FMT.

![Figure 4.5: FMT results of the abdominal area of RIP-hTMEM27 transgenic and control mice three days post injection of AF680-8/9-mAb, representative animals.](image)

(a) Control mouse injected with AF680-8/9-mAb, (b) and (c): Two different RIP-hTMEM27 transgenic mice injected with AF680-8/9-mAb. Although the FMT signal observed on RIP-hTMEM27 transgenic mice three days post injection was in some cases higher than in controls, this effect was not consistent. Data are scaled with the same scalebar (reconstructed fluorescence in arbitrary units).

To investigate the antibody biodistribution in the abdominal organs and in blood, the animals used for pancreas imaging were euthanized after the \textit{in vivo} session and the abdominal organs (pancreas, liver, kidney and spleen) were excised and imaged. FMT measurements in transmission mode of the organs did not reveal any differences in the pancreatic signal between groups. These were again visible with FRI (post to spectral unmixing with the blood signal) and microscopy. This showed that the targeted probe did bind to the pancreatic islets, but this abdominal signal was not detectable with FMT \textit{in vivo} or \textit{ex vivo}.

51
4. FMT Studies

![Figure 4.6](image)

**Figure 4.6**: Quantitative results from FMT and FRI for the experiments shown in Figure 4.5. Bar graph (mean ± σ) of the (a): normalized mean reconstructed fluorescence calculated with FMT, and (b): mean normalized fluorescence intensity calculated from FRI. Subfigure (b) is adapted from [88].

### 4.1.5 Discussion

A novel application of FMT for imaging of beta cells using an antibody-based fluorescent probe was presented. In a subcutaneous insulinoma model, we could assess both the probe specificity and its pharmacokinetics. Specifically, using FMT we could distinguish the case where the targeted probe was injected on TMEM27-insulinoma bearing animals from the two controls (unspecific fluorescent probe and non-TMEM27 expressing insulinomas). The pharmacokinetics of the probe as assessed with FMT were consistent with PET both qualitatively (form) and quantitatively. This was not the case for FRI results. This confirms the advantage of FMT over FRI for quantitative studies [38, 96].

However, we could not assess the probe’s binding to the pancreas when imaging the abdominal region with FMT. FRI and *ex vivo* analysis confirmed that the probe was indeed binding to its target in the pancreas – thus this problem was FMT specific. A series of reasons may explain this. The abdominal region poses two challenges for FMT imaging. The first is the its average high absorption, caused by the high blood content and organs such as the liver. The second is the presence of autofluorescence from the stomach. Both these aspects render the detection of fluorescence challenging, especially if the specific fluorescence signal is weak. Indeed, the fraction of beta cells in pancreatic tissue is very low (1%), and thus requires specific local enrichment of an imaging probe by a factor of at least 100 compared with the exocrine tissue, blood and adjacent organs, in order to enable reliable assessment of changes in beta cells *in vivo*. This can be achieved with further technological development on the probe as suggested in [88].

The FMT experiments in this study were performed in reflection mode. Reflection mea-
measurements are heavily weighted towards the surface and, while suitable for structures close to it such as subcutaneous tumors (as shown also here), they are potentially not optimal for imaging of deeper lying structures, such as the pancreas. Transmission measurements in vivo need a higher laser power for excitation, and due to the laser used in the experiment, they were not possible at the time of experiments, although the FMT system design accommodated them in principle. Regarding the absorption coefficient, the Born normalization [39] was used, which has been shown with phantoms to yield robust results even in the presence of strong inhomogeneities [40]. It is not known if it is sufficient for the challenging abdominal area. To reduce autofluorescence, chlorophyll-free food was used in this study. However, it is possible that some degree of autofluorescence remained. To remove the stationary effect of autofluorescence, the FMT abdominal measurements on the third day post injection were normalized to the measurements immediately prior to injection. However, it is possible that the non-specific autofluorescence signal could mask the weak pancreatic signal.

The FRI measurements were also performed in reflection mode; however, the commercial FRI system employed in this study accommodated spectral unmixing. By acquiring a series of measurements at different wavelengths and using the known spectrum of AF680, the specific signal could be unmixed from the non-specific background fluorescence. This feature was not available on the FMT system; it is conceivable that spectral unmixing in FMT, as demonstrated in [45], could improve the FMT results. For this, employment of additional excitation wavelengths on the FMT system will also be beneficial.
4. FMT Studies

4.2 FMT imaging of an avidin-GPI reporter system in a mouse tumor model

4.2.1 Preliminary remark

In this section, FMT imaging of a novel avidin-Glycosylphosphatidylinositol (GPI) system monitoring the hypoxia-inducible factors’ (HIFs) activity in a mouse tumor allograft model is presented. The avidin-GPI system and in vivo FRI and SPECT results are described in the paper [89]: “Recording Intracellular Molecular Events from the Outside: Glycosylphosphatidylinositol-Anchored Avidin as a Reporter Protein for In Vivo Imaging” by S. Lehmann, E.G. Garayoa, A. Blanc, R. Keist, R. Schibli and M. Rudin, published in the Journal of Nuclear Medicine 2011(52). Parts of the following introduction and discussion are strongly based on [89].

4.2.2 Introduction

One approach for molecular imaging on animal models is based on reporter gene technology (see Chapter 6.3 of [7] for details). To study the regulation and expression of a target gene, a reporter transgene is put under the control of upstream promoter sequences and inserted into the target cell. Direct reporters are implemented with fluorescent proteins, as known from cell imaging. Applications of direct reporters for in vivo imaging have been presented and also followed up with FMT [51]. To use reporter system with non fluorescence-based imaging modalities, enzymatic reporters can be utilized instead. Enzymatic reporters require a substrate in order to produce a detectable signal. Such substrates carry a signal-generating moiety and can be generated for several modalities [97–99]. Despite additional flexibility, enzymatic reporters depend on reporter accessibility by the substrate, and especially on the substrate’s ability to penetrate cell membranes.

A novel reporter system, avidin-GPI, was implemented by Lehmann et al. [89]. The protein GPI is anchored to the outer cell membrane, making it possible to study intracellular molecular targets without the need for imaging probes to cross the cell membrane. Avidin has a high affinity for biotin [100], and development of biotinylated imaging probes is straightforward and has been described for most of the current imaging modalities [101–104]. As an application, the GPI-avidin reporter system was driven by hypoxia response elements, and thus employed to follow hypoxia response in vivo with FRI and SPECT. The goal of this
4.2. FMT imaging of an avidin-GPI reporter system in a mouse tumor model

FMT study was to perform in vivo monitoring of the HIF transcriptional activity using the GPI-avidin reporter system, and to validate the results against FRI.

4.2.3 Materials and methods

Only information relevant to this chapter is presented here. For details on the reporter construct, stable cell lines generation, immunofluorescence and FACS, the reader is referred to [89]. The following sections have been modified from [89] and expanded for clarity.

In Vivo Allograft Tumor Models

All animal protocols were approved by the Cantonal Veterinary Office in Zurich (license 129/2007 XIMO-Y2) and Aargau. To establish allograft tumors, $10^6$ C51 control or stably transfected pH3SVG cells were subcutaneously injected into the flank of 8– week-old BALB/c nude mice (Charles River Laboratories) for the in vivo fluorescence imaging experiments. Animals were maintained under standard hygienic conditions for the duration of the experiment. Five to seven days before imaging, animals were fed a biotin-free diet (Harlan). Imaging was performed 10d after tumor inoculation when tumors had reached diameters of approximately 510mm. Tumor sizes were monitored by caliper measurements. All animals were sacrificed before tumors reached volumes of $2cm^3$.

Fluorescence reflectance imaging (FRI)

For in vivo fluorescence imaging, animals were gas-anesthetized using 3% isoflurane (MINRAD) and an oxygen-air mixture as carrier gas. Spectral imaging was performed in a fluorescence reflectance imaging system (Maestro 500; Cambridge Research Instruments Inc.) using a bandpass filter for excitation (575 605nm) and a long-pass filter (645nm) for detection. Spectrally resolved detection of the fluorescence signal was achieved by the liquid crystal tunable filter built into the system (bandwidth, 20nm; scanning wavelength range, 400720nm). The tunable filter automatically stepped in 10nm increments from 630 to 850nm. After acquisition of a precontrast image, animals were administered an intravenous injection of 10nmoles of Alexa-594-biocytin or Alexa-594 cadaverine, respectively. Images were then acquired at 60m, 90m and 24h after injection. To use the spectral unmixing software provided by the manufacturer (Maestro 500; Cambridge Research Instruments Inc.), the spectra of autofluorescence were derived from an appropriate region on the origi-
nal, spectral fluorescence images. The pure spectrum of Alexa-594 was obtained by imaging a 0.005mM solution of Alexa-594-cadaverine diluted in saline (0.9% NaCl). The Alexa-594 and the autofluorescence components in the composite images were spectrally unmixed using the spectral unmixing software. Tumor fluorescence was quantified by calculation of tumor-to-background signal ratios on spectrally unmixed images displaying the Alexa-594 component only. Tumor and background regions of interest used for quantification were drawn manually using the Maestro imaging software.

**FMT experiments**

Animals were transferred to the FMT system under anesthesia directly after measurement on the FRI system. They were positioned on the heated animal platform so that the tumor was in the center of the camera’s field of view. A 592nm laser was used for excitation of a 9 × 10 grid on the region of interest. Fluorescence filters at 590nm and at 625nm were used for excitation and emission measurements, respectively. The FMT data were reconstructed as described in 3.1.2. For quantification, the mean reconstructed fluorescence intensity was computed over the tumor volume.

**4.2.4 Results**

**FRI results [89]**

An avidin-GPI reporter pH3SVG construct was designed and used to stably transfect C51 colon carcinoma cells. It drives the expression of avidin-GPI from a minimal SV40 promoter, regulated by three hypoxia response elements. *In vitro* experiments revealed that the reporter was expressed upon treatment with DMOG, which mimicks hypoxia in activating signaling through the hypoxia inducible factor (HIF) pathway, but not under control conditions [89]. Moreover, it was shown that Alexa-594-biocytine interacted specifically with avidin-GPI.

For FMT, we first confirmed that the laser and filters used in the FMT system were suitable for detection of Alexa-594-biocytine (Figure 4.7).

*In vivo* fluorescence experiments (FRI and FMT) were performed to estimate the HIF transcriptional activity by targeting avidin-GPI. Three groups were used for experiments; (a) animals bearing C51 tumor cells with the pH3SVG construct, injected with Alexa-594-biocytine (positive/positive, \(n = 3\)), (b) animals bearing C51 tumor cells with the pH3SVG construct, injected with the control Alexa-594-cadaverine (positive/negative, \(n = 3\)) and
4.2. FMT imaging of an avidin-GPI reporter system in a mouse tumor model

![FMT reconstruction of two Eppendorf tubes filled with saline (top) and Alexa-594-biocytine (bottom).](image)

Fluorescence from the Alexa-594-biocytine could be detected for the combination of laser and filters used in the FMT setup.

(c) animals bearing C51 control tumor cells, injected with Alexa-594-biocytine (negative/positive, \( n = 3 \)).

With FRI, specific targeting of the biotinylated probe to the pH3SVG-positive tumors could already be detected 60min post injection. Alexa-594-biocytin showed accumulation in the liver, kidneys, and urinary bladder, consistent with the role of the liver in biotin storage and urinary excretion of this molecule [105]. Twenty four hours after injection of the tracer, the main signal detected originated from the kidneys and the tumor, implying that most unbound tracer molecules had been cleared from the circulation. There was some residual fluorescence detected in the gastrointestinal tract, potentially due to autofluorescence of food components. The specificity of probe accumulation at the tumor site was analyzed at 24h after tracer infusion in all three study groups. Only for animals carrying pH3SVG C51 tumors injected with Alexa-594-biocytin was a fluorescent signal attributable to the dye molecule detected in the tumor region (outlined in red on the whitelight images, Figure 4.8). Neither C51 tumor animals treated with Alexa-594-biocytin nor pH3SVG C51 animals dosed with Alexa-594-cadaverine displayed a fluorescent signal in the region of interest. The tumor fluorescent signal was clearly increased in pH3SVG animals after injection of the fluorescently labeled biotin (Figure 4.8 C). [89].

The FRI results were consistent with the ex vivo assessment of binding specificities of Alexa-594-biocytine and Alexa-594-cadaverine 24h after injection.
4. FMT Studies

Figure 4.8: (A) In vivo assessment of tumor fluorescence after intravenous injection of either Alexa-594-biocytin or Alexa-594-cadaverine in pH3SVG tumor animals. Images only show the spectrally unmixed Alexa-594 fluorescence component. (B) Original fluorescence, spectrally unmixed, and white light images for pH3SVG animals treated with biocytin or cadaverine and a C51 control animal injected with the biotinylated compound at 24hs after injection. (C) Tumor-to-background ratios calculated on spectrally unmixed fluorescence images. Whereas this ratio did not change for control animals, there was increased tumor fluorescence in pH3SVG tumors 24hs after injection of the dye. The high variability between animals may reflect different degrees of hypoxia in the analyzed tumors. Values are shown as mean ± SD. Figure and caption from [89].

FMT results

Quantification of the FMT results (Figure 4.9) showed a different pattern; whereas the fluorescent signal over time was consistent with FRI (strong enhancement after 2h, return to baseline for 24h) and the signal for the C51 control tumors injected with Alexa-594-biocytine was lowest, the FMT signal for pH3SVG tumors injected with Alexa-594-cadaverine (control) was larger than those injected with Alexa-594-biocytine.

A closer look at these surprising results is shown in Figure 4.10 for one representative animal per group. Due to non-identical positioning for different timepoints, the excited region occasionally contained parts of the stomach region, along with the tumor (Figure 4.10, first row). Overall, the excited region was larger than the actual tumor outline – this was because the tumors on the 10th day after inoculation were not always clearly visible on the reference whitelight images (Figure 4.10, first row).
4.2. FMT imaging of an avidin-GPI reporter system in a mouse tumor model

![Figure 4.9](image)

**Figure 4.9:** Quantification of FMT for avidin-GPI reporter activity. The mean reconstructed fluorescence for each animal and timepoint was expressed as % of the mean reconstructed fluorescence 20min post injection. The results are shown as mean ± std for each of the three groups. The maximum signal enhancement was observed 2hs post injection and slowly decreased afterwards. The C51 control tumors injected with Alexa-594-biocytine showed overall the smallest enhancement. However, both the initial enhancement and the enhancement 24h post injection was larger for the pH3SVG tumors injected with Alexa-594-cadaverine (control) than for the pH3SVG tumors injected with Alexa-594-biocytine.
4. FMT Studies

**Figure 4.10:** FMT reconstruction along the xy axis for one representative animal per group, along time. All data have been scaled with the same factor (colorbar). The corresponding reference whitelight images are in the left column. Notice the fluorescence from non-tumor areas appearing e.g. for the C51 control tumor animal injected with Alexa-594-biocytine.
4.2.5 Discussion

In [89], Lehmann and colleagues presented a new molecular reporter system for tumor hypoxia response, based on GPI-avidin. They showed that biotinylated probes designed for fluorescence and SPECT imaging binded specifically to tumor cells expressing the reporter system. This was not the case for control tumor cells or non-biotinylated probes.

Although we were able to follow some elements of the probe’s pharmacokinetics with FMT, we were not able to detect the specific binding of the biotinylated probe to the pH3SVG tumor cells. This can be attributed to the following reasons: as depicted in Figure 2.5 (see also discussion of corresponding section), both absorption and autofluorescence of tissue are highest around the wavelength of 590nm, which was used to excite Alexa-594. In this study, Alexa-594 was chosen due to its suitability for in vitro validation with microscopy; however, it is not optimal for in vivo applications. This disadvantage affects both FRI and FMT measurements. As shown in Figure 4.8(B), the non-unmixed FRI images (in red) acquired 24h post injection cannot be used to detect the probe’s binding. It is after unmixing with the fluorescent probe’s spectrum (Figure 4.8(B), in blue, and Figure 4.8(A)) that the probe can be separated from the background. On FMT, such a possibility was not offered by the system. An additional correction factor for FRI measurement was introduced by calculating the tumor-to-background ratio for the unmixed images. The background was estimated in a region away from the tumor. In FMT, the measured region was confined to the excitation region; this was done as the measurement time increases linearly with the number of sources used. Care was taken in FMT so that only the tumor region is excited; however, this was not always achieved. One reason for that was that the tumor boundaries were not always visible on the 10th day post inoculation, when experiments were performed.

Several solutions can be employed to overcome the problems outlined here; first, a probe in the NIR region can be used. If this is not possible due to the experimental design, then excitation of two distinct regions, one inside the tumor and one far away from it, will be beneficial, so that tumor-to-background ratio can be estimated. Moreover, multispectral measurements can be employed to separate background autofluorescence from the probe’s fluorescence. Finally, in cases such as the one presented here, where the tumor outline is not visible, MRI measurements enhanced with Gadolinium can be employed to define the tumor.
Chapter 5

Application of a hybrid FMT/MRI system on a transgenic mouse model of amyloid angiopathy

5.1 Preliminary remark

This chapter is adapted from "Compact Hybrid Imaging System Enables Concurrent FMT and MR Imaging in the Mouse Brain" by K. Dikaiou*, F. Stuker*, J. Klohs, A. Elmer, D. Beyeler, J. Ripoll, I. Knuesel and M. Rudin (in preparation).

*The authors contributed equally.

5.2 Introduction

Recently, we presented a first prototype of a small animal FMT/MRI system [1] and showed proof-of-concept on a subcutaneous tumor model. The system eliminated optical fibers by using free-beam laser illumination for excitation and an MR-compatible SPAD array for detection of photons. However, the SPAD array featured a small field-of-view and low sensitivity, which hampered further biological applications. Here, we present a new generation of a hybrid FMT/MRI system, where the SPAD array has been replaced by a low-noise CMOS camera with both enlarged field of view and improved sensitivity. Moreover,
the new system features rails, allowing reproducible positioning and improved animal handling. After assessment of the system performance with phantoms, we have employed the new FMT/MRI system on the APP23 [106] mouse model of Alzheimer’s disease.

Alzheimer’s disease (AD) is a neurodegenerative disease causing dementia, cognitive impairment and memory loss. AD is associated with progressive accumulation of abnormal proteins (amyloid-$\beta$ [A$\beta$] and hyperphosphorylated tau) in the brain [107]. Transgenic animal models overexpressing mutated human APP, leading to enhanced deposition of A$\beta$ plaques in the mouse brain, have been constructed. As amyloid plaques have an average diameter of $\leq 50\mu m$, they can be only be visualized noninvasively with microscopical MRI, which exploits the inherent contrast between the protein aggregates and brain parenchyma. Yet, such high-resolution MRI measurements require very long acquisition times [108]. Alternatively, probes that specifically bind to amyloid plaques can be visualized with molecular imaging techniques to assess A$\beta$ plaque load. The oxazine dye derivative AOI987 has been constructed for NIRF imaging and has been shown to bind to A$\beta$ plaques with high specificity in vivo [81, 84].

AD in patients and transgenic mouse models has also been associated with other MRI findings, such as ventricular enlargement [109], compromised hemodynamic response to vasodilatory stimuli [110], vascular remodeling in the cortex [111] and occurrence of cerebral microbleeds (CMBs) [112], which have been shown to be neighboring amyloid deposits [113]. Combining complementary optical and MRI readouts could thus advance our understanding of AD pathophysiology in the intact organism.

We examined different MRI contrasts to assess the potential of the FMT/MRI system for assessment of structural changes. MRI derived structural information was combined with information on the plaque load as derived from FMT experiments using the dye AOI987. In particular, the surface of the mouse head was extracted from the MRI data, registered to the FMT data set in order to improve the quality of the reconstruction. The Kirchhoff approximation has been used, which locally replaces the curved surface by its tangential plane [114]. To verify that eventual differences between groups detected with FMT were due to the dye’s binding to plaques, ex vivo validation with FRI and confocal microscopy was performed.
5.3 Materials and Methods

5.3.1 Hybrid FMT/MRI system design

This section has been adapted from [87].

The hybrid FMT/MRI system was designed for a Bruker BioSpec 94/30 horizontal small animal MR system operating at 400MHz, equipped with the gradient system B-GA12S2 (Bruker BioSpin MRI, Ettlingen, Germany). It consisted of two modules, one for optical excitation and one for MR transmission/reception and optical detection (Figure 5.1a).

The excitation module (Figure 5.1b) contained MR sensitive parts and was built on a mobile aluminium optical breadboard placed in front of the MR scanner, where the stray field is $\leq 10$ mT. A free-beam 670nm continuous wave laser (Coherent Inc., CA, USA) was aligned to a pinhole of 0.5mm diameter and an antireflectance-coated spherical singlet lens ($f = 1000$ mm, Melles Griot, Bensheim, Germany), so that a $2f$ image of the pinhole would be formed at the scanner’s isocenter. A small portion of the beam corresponding to 10% of its power was deflected to a power meter (Thorlabs, Munich, Germany). The measured laser power was used later in the FMT reconstruction. The main part of the beam entered a galvanometrically driven scan head (Scanlab, Puchheim, Germany) where it was deflected to form the desired laser excitation grid on the sample (animal head) inside the scanner.

The MR transmission/reception and optical detection module (Figure 5.1 (c)) contained only high magnetic field-compatible components, as it was placed in the MR system’s isocenter during measurement. To ensure accurate and reproducible positioning, it was designed to slide on two parallel carbon rods 70mm apart, which were rigidly fixed on the front and the back of the MR scanner prior to experiments. The whole module was integrated in a custom-made animal platform of 114mm diameter, equipped with water tube heating and anesthesia supply via face mask. For RF signal transmission and reception, a home-built rectangular surface transceiver coil (20mm $\times$ 24mm) was fixed at the back flange of the platform, with supplied rods for manual tuning and matching. The coil was made of a flexible PCB substrate with standard copper layer which was curved in a cylindrical shape. It had a rectangular opening of 16mm $\times$ 20mm, revealing the area of interest on the sample for MR and FMT imaging in reflection or transmission mode. For operation in reflection mode, a mirror mounted on the right side of the platform’s back plate deflected the incoming beam by 90° to a second mirror mounted above the sample, which deflected it again by 70° onto the sample surface through the coil cutout. For transmission mode, a third mirror was
mounted under the animal platform and deflected the beam by 90° to illuminate the sample from below. All three mirrors were coated front surface mirrors (Edmund Optics, Karlsruhe, Germany). The diffusive light patterns on the surface of the animal were recorded for each illumination point. An anti-reflection coated fixed focus glass lens (V-4301, FL = 2.1mm, BFL = 5.6mm, Marshall Electronics, El Segundo, CA, USA) was mounted to a custom made light-tight housing comprising a filter wheel with a manual filter switching mechanism (Fig. 7.7b). For measurements at the excitation and fluorescence wavelengths, high-quality bandpass filters (Semrock, Rochester, NY, USA) with peak wavelengths of 660nm and 720nm were used. Filters were characterized by > 90% transmission over a range of 13nm. As their thickness would cause a different focal length for the reference white light image, an antireflection-coated glass slide was placed in one filter wheel slot. The lens-housing assembly was screwed to a custom-made PCB, in front of a 11 × 11µm² CMOS detector array of 256 × 256 pixels (CSEM, Switzerland), yielding a FOV of 55 × 55mm² at a focal distance of 40mm. The data from the camera was stored in an electronic stack placed outside the MR scanner using two 40-pole ribbon cables (Farnell, Switzerland).
Optical acquisition was computer-controlled using an in-house software written in LabView (National Instruments, TX, USA). For MR data acquisition, Paravision 5.0 (Bruker BioSpin MRI, Ettlingen, Germany) was used.

5.3.2 Optical phantom preparation

Tissue-mimicking phantoms for FMT performance evaluation were made out of room temperature vulcanizing (RTV) silicone (Wacker Silicone, Munich, Germany). Tissue optical properties ($\mu_a = 0.2\text{cm}^{-1}$ and $\mu_s' = 10\text{cm}^{-1}$) were simulated by adding TiO$_2$ particles (Alfa Aesar, Karlsruhe, Germany) and carbon black powder (Alfa Aesar, Karlsruhe, Germany) as scattering and absorption agents respectively. Tissue equivalence was confirmed with a near infrared spectrometer (OxiplexTS, Illinois, USA) operating at 690nm. The phantoms were poured in a cuboid metal mold of 13mm depth. Cylindrical metal rods with 1.5mm diameter were inserted into the silicon mass when liquid to create holes at various spatial coordinates. When the phantoms were dry, the metal rods were removed and glass capillaries of 1.5mm outer diameter, 0.6mm inner diameter) were inserted. The capillaries were filled with $10\mu$l of the dye Cy5.5 or the dye AOI987 used in the in vivo study. Both dyes have similar spectral properties.

5.3.3 Animals

All experimental procedures conformed to the national guidelines of the Swiss Federal Act on Animal Protection and were approved by an official committee (Cantonal Veterinary Office, Zurich, Switzerland). The APP23 mice, which overexpress the human amyloid precursor protein (APP) and develop amyloid plaque deposits from six months of age [106], were generated at the Novartis Institute for Biomedical Research, Basel. Two transgenic (APP23) mice and three wild-type (wt) littermates (Novartis) at 24 months of age and of both sexes were used. Animals were kept at standard housing conditions with a 12-hour dark/light cycle and free access to water and chlorophyll-free food (Kliba Nafag, Kaiseraugst, Switzerland).
5. Application of FMT/MRI on a transgenic mouse model of amyloid angiopathy

5.3.4 NIRF dye

The oxazine derivative AOI987, a NIRF ligand binding to amyloid plaques \textit{in vivo} [81] was used in this study. AOI987 was synthesized according to published procedures [81] (laboratory of Prof. K.-H. Altmann, Institute of Pharmaceutical Sciences, ETH Zurich, Switzerland). The dosage for each animal was calculated according to [81] (0.1mg/kg, 0.9% saline vehicle).

5.3.5 \textit{In vivo} FMT/MRI imaging

\textit{Animal preparation}

Anesthesia was induced with 3\% isoflurane (Abbott, Cham, Switzerland) in a 4:1 air/oxygen mixture and maintained with 1.2\% isoflurane through a face mask. The head fur was removed with an electric shaver and epilating cream and the tail was cannulated with a 30G needle (0.3mmx13mm, BD Microlance, Drogheda, Ireland). The animal was placed on the support of the FMT/MRI system so that the head was in the center of both the MR coil FOV and the camera FOV. The animal’s body temperature was monitored with a rectal temperature probe (MLT415, ADInstruments, Spechbach, Germany). Temperature was kept at 36°C using a warm-water circuit integrated into the animal support.

\textit{In vivo imaging protocol}

The MR coil was tuned and matched and scout scans were acquired to adjust positioning. FMT calibration was performed once at the beginning of each experiment as described in section 3.1.2. An FMT dataset was acquired prior to AOI987 injection. The light emitted upon laser excitation in a $8 \times 12$ grid on the mouse head was collected using the 660nm and 720nm filters. Additionally, a white light reference image without laser excitation was acquired. The complete FMT measurement lasted 8 minutes. A volume of AOI987, calculated separately for each animal as described above, was administered intravenously (i.v.) into the tail vein as a bolus, followed by saline flush of equal volume. FMT datasets with identical parameters to the first one were acquired every 20 minutes after dye administration over a period of 3 hours. MRI measurements were acquired between FMT acquisitions over the course of three hours, with the following parameters:
5.3. Materials and Methods

**T2-weighted spin echo sequences** Nine axial slices comprising the brain were acquired using a T2-weighted spin-echo (RARE) sequence with the following parameters: RARE factor 8, FOV = 20.45 × 20.45mm², matrix size 256 × 256, slice thickness 0.5mm, interslice distance 1mm, T_E/T_R = 11/2500ms, number of averages NA = 2, total imaging time 2min40s. Alternatively, ten coronal slices comprising the brain were acquired with the same parameters apart from NA = 3, which translated into a total imaging time of 4min.

**T1-weighted spin echo sequences** Nine axial slices comprising the brain were acquired with a T1-weighted spin-echo (RARE) sequence with the same geometry parameters as above and the following changes: RARE factor 4, T_E/T_R = 8.2/1500ms, number of averages NA = 4, total imaging time 4min48s.

**T2*-weighted gradient echo sequences** Nine axial slices comprising the brain were acquired with a T2*-weighted gradient-echo (FLASH) sequence with the same geometry parameters as above and the following changes: T_E/T_R = 5/383ms, pulse angle α = 35°, number of averages NA = 1, total imaging time 3min49s.

**3D MR angiograms** 3D MR angiograms were acquired with a gradient-echo sequence (FLASH) with the following parameters: FOV = 20 × 16 × 8mm³, matrix size 248 × 200 × 100, T_E/T_R = 2.5/15ms, pulse angle α = 30°, number of averages NA = 2, total imaging time 7min27s. Angiograms were generated with maximum intensity projections (MIPs) using Volview (Kitware, USA).

**5.3.6 Ex vivo optical imaging**

The animals employed in the FMT/MRI in vivo experiments were decapitated 3 hours following the AOI987 administration under deep anesthesia and the brains were surgically removed without prior animal perfusion.

**Ex vivo FRI**

Axial brain slices of 1mm thickness were cut in a brain matrix using a razor blade. Planar fluorescence imaging was performed using the Maestro 500 epi fluorescence imaging system (previously Cambridge Research, Woburn, MA, USA, now PerkinElmer, USA). A
5. Application of FMT/MRI on a transgenic mouse model of amyloid angiopathy

615 – 665 nm band pass filter and a 650 nm long pass filter were used for excitation and emission light, respectively. Images from 650 to 850 nm were acquired in 10 nm steps and then subjected to spectral unmixing according to [34], to separate fluorescence from background. The spectral library of the uninjected dye was used for unmixing. Acquisition and processing were performed with the Maestro 3.0.1 software.

**Ex vivo confocal Microscopy**

Brains were snap-frozen on dry ice immediately after extraction and were cryosectioned in slices of variable thickness 10 – 80 µm. Qualitative evaluation of the dye labeling was done with a confocal microscope (LSM-710, Zeiss) using the Plan-Apochromat 20× (NA = 0.8) objective. Autofluorescence and AOI987 were visualized using sequential acquisition of each channel. The pinhole aperture was set to 1.0 Airy unit for each channel. Single plane images (512 x 512 pixels, zoom 1) or tile scans (15 x 6) were acquired. For visual display, both channels were merged using the image analysis software Imaris (Bitplane, Zurich, Switzerland). Cropping of images and adjustments of brightness and contrast were identical for each labeling and done using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

**5.3.7 Data processing and analysis**

**MR surface extraction and FMT/MR co-registration**

The surface of the animal head was extracted and the resulting 2D height map was coregistered with the optical reference whitelight image as described in section 3.3. The procedure for one representative animal is illustrated in Figure 5.2. Animal movement, assessed by the difference of the whitelight reference images acquired every twenty minutes, was found minimal. Therefore, the transform matrix for the coregistration was computed only once for each animal and used to coregister all FMT measurements acquired during the 3 hs of the experiment.

**FMT reconstruction**

FMT reconstruction was performed using the normalized Born approximation of the diffusion equation [39], which normalizes the fluorescence images by their corresponding excitation images to correct for optical inhomogeneities within the mouse head [40]. The ratios were fed into the forward model [115] which predicts the fluorescence signal on the sample.
5.3. Materials and Methods

Figure 5.2: Surface extraction from MR and optical/MR coregistration. (a) Slices of the reference T2 spin echo MR sequence prior (left) and post (right) smoothing and segmentation. (b) Calculated isosurface displaying the surface of the mouse head. The same isovalue was used for all animals. (c) Calculated height map from (b), interpolated to the pixel dimensions of the camera (top) and whitelight optical image of the same animal. (d) Selection of one control point on the two dimensional projection of the height map and the whitelight optical image. Three control point pairs were used per animal. The points were selected based on anatomical landmarks of the animal (beginning of the two ears and eye curvature). Dark spots visible on the reference whitelight image in (c) were fiducial markers (fish roe) experimented with for the co-registration. Although these markers could be used on phantom experiments, the resolution of MRI in the in vivo experiments was not sufficient to resolve them.

surface by solving the diffusion equation analytically. For the sample’s boundaries in the equation we used the stored animal boundary described above, represented by its tangent plane and its normal vector at each point according to the Kirchhoff approximation [114]. The forward model was inverted with the algebraic reconstruction technique (ART), where the initial assumption for the fluorophore distribution is iteratively improved using a least square minimization procedure. The reconstruction yielded a three dimensional map of the fluorescent source distribution within the subject. The same procedure for FMT reconstruction was performed for all animals and timepoints.

For FMT quantification, the mean reconstructed fluorescence (MRF) value was used. To remove inter-animal variability, as shown in different pre-injection MRF values, each animal’s MRF values were normalized to the animal’s maximum MRF value, observed at the first timepoint upon dye administration, and expressed it in %. These percentages were plotted for all animals versus time. Exponential curves were fitted (Matlab Curve Fitting Toolbox) to the transgenic and wildtype group measurements separately, excluding the pre-
injection points so as to have single-mode curves.

5.4 Results

5.4.1 FMT and MR performance assessment

The experiments and analysis of the FMT/MRI system’s performance using phantoms have been performed mainly by Dr. Florian Stuker and are described in detail in his PhD thesis [87]. A modified version of this work is included in the manuscript in preparation. For reasons of copyright and clarity, only an abridged version of the main findings is included here.

Crosstalk

Cross-talk between FMT and MRI was assessed with the SNR of successive and concurrent imaging of a standard water phantom with both modalities. No SNR change was observed for optical measurements performed (a) outside the MRI scanner, (b) inside the MRI scanner and (c) inside the MRI scanner during MRI acquisition. However, the SNR of the MR images was reduced by a factor of ten when the optical system was in operation. We discovered that this was due to the camera’s power supply and was not related to actual acquisition of optical data. This problem can generally be addressed with shielding the camera, its supply and the connecting cables (A. Elmer, M. Augath, unpublished results). In this study, FMT and MRI were performed in an interleaved fashion instead, as done on other hybrid systems (e.g. FMT/XCT [61]).

Spatial resolution

To assess the spatial resolution provided by FMT, phantoms containing two fluorescent tubes at varying distances and depths were measured. The minimal experimentally realizable distance was 2mm. The two tubes were considered resolved if two distinguishable peaks were visible on the Gaussian fitted reconstruction results along the plane perpendicular to the tubes. Tubes 2mm deep and 2mm apart could be resolved. Simulations revealed that tubes separated by 1mm should be readily resolvable using this setup [1].
5.4. Results

Depth estimation

The accuracy of depth estimation by FMT was assessed with measurements of a single tube embedded in a phantom in depths of $1−5\text{mm}$. Fluorescent sources $1−3\text{mm}$ deep inside the phantom could be attributed to their correct height. We found that sources embedded at $\geq 4\text{mm}$ depth were attributed closer to the surface than their real distance. This effect is due to the reflection geometry utilized in this setup, which is known to weigh surface sources more [43].

Quantification analysis

The proportionality of the reconstructed fluorescence to the local dye amount was assessed with phantom measurements of a tube containing linear dissolutions of the dye Cy5.5. The mean reconstructed fluorescence varied linearly ($R^2 = 0.978$) with the dye amount (range of concentration $2.14−15\text{pmol}$, dissolved in $2.5\mu\text{l}$ saline). Based on the linear relation between fluorescence intensity and dye concentration and knowing the background signal intensity, we estimated the detection limit of the hybrid FMT/MRI systeme for AOI987 to be $2\text{pmoles}$.

5.4.2 In vivo FMT and MR imaging

MRI and MRA

We examined if ventricular enlargement was present on APP23 mice compared to wild type mice, as stated in [109]. For this, we manually segmented the ventricles from brain tissue using the $T_2$—weighted MR coronal images (two representative sets are shown in Figure 5.3). No differences were found in the ventricle volume between the two mouse strains.

On the $T_2^*$—weighted MR axial images of one transgenic animal, we observed focal hypointensities of round shape at areas within the brain parenchyma of both cortical and subcortical structures in APP23, but not in age-matched control mice(Figure 5.4(a)). On the corresponding $T_1$— and $T_2$—weighted MR images of the same slice, these abnormalities were less apparent. Nevertheless, we observed decreased intensity of the $T_1$—weighted signal at some of these positions (Figure 5.4(b)), albeit with a lower contrast and a smaller radius, and sometimes surrounded by a slightly brighter halo (ring enhancement). These findings are compatible with the MRI characteristics of cerebral microbleeds (CMBs) [116]. CMBs are
5. Application of FMT/MRI on a transgenic mouse model of amyloid angiopathy

![Figure 5.3: $T_2$-weighted MR images in coronal orientation for a representative pair of a wild type mouse (a-d) and an APP23 mouse (e-h). No obvious morphological differences between the two groups are present. The ventricles were manually segmented and their volume was calculated for comparison between groups.](image)

associated with several neurological disorders, including AD, and are indicative of an underlying vascular pathology [112]. The decreased $T_2^*$ signal from CMBs is caused by the contained hemosiderin, which creates a local inhomogeneity in the magnetic field surrounding the CMB. This leads to the so-called "blooming effect" in susceptibility-weighted gradient-echo images, i.e. the pathology appears larger than it actually is. In contrast, this effect is refocused when using spin-echo pulse sequences, which leads to more confined lesions. We did not observe such focal hypointensities on $T_1$-weighted MR images of wild type mice (Figure 5.4(c)).

To demonstrate the versatility of the FMT/MRI combination, we also recorded MR angiograms of the mouse cerebral vasculature, as AD has been associated with vascular remodeling in the cortex [111]. We examined if TOF angiograms of the mouse brain could be acquired with the FMT/MRI setup. We performed 3D time of flight (TOF) MR angiography (MRA) with an isotropic resolution of $80\mu m$. We found that major vessels and structures in the mouse brain could be identified.
Figure 5.4: (a): Axial slice of $T_2^*$-weighted MRI for one APP23 mouse. Red arrows point at focal hypointensities which are postulated to be CMBs (see text). (b): The same slice, acquired with a $T_1$-weighted MRI sequence. Red arrows indicate these CMBs regions that also appear hypointense with a $T_1$ contrast. As $T_1$ contrast is not optimal for CMB detection, and thus the number of hypointensities detected is much smaller. (c): Same sequence as in (b) for a wildtype mouse. No focal hypointensities were found.
5. Application of FMT/MRI on a transgenic mouse model of amyloid angiopathy

Figure 5.5: MIP of MRAs for one APP23 mouse. Figures (a)-(d) represent different views of the MIP, visualized every 27°. Visible structures are annotated in (a) as follows. 1: vertebrate artery, 2: basilar artery, 3: internal carotid artery, 4: posterior cerebral artery (PCA), 5: pterygopalatine artery (extracranial), 6: circle of Willis, 7: middle cerebral artery (MCA), 8: anterior cerebral artery.
5.4. Results

**FMT reconstruction**

**Use of the co-registered MRI animal surface in the FMT reconstruction.** We compared the FMT reconstruction results with and without the use of the co-registered MR surface. In the second case, the mouse shape was approximated by a slab. Representative results are shown for one animal in Figure 5.6. The slab reconstruction yielded more artifacts (apparent as a bright spot close to the surface) and less accurate assessment of fluorescence distribution, present both in reconstructed sources outside the excited area (Figure 5.6 (d), reconstruction results over and on the left ear) and reconstructed sources only on the edge of the excited area (torus shape shown in Figure 5.6 (c)). These slab reconstruction artifacts are caused by the curvature of the mouse head; in order to calculate the position of the laser grid points of the sample, the FMT reconstruction algorithm projects the coordinates of the galvanometric mirror onto the sample surface. Obviously, curved surfaces cause a diversion of the excitation grid from the uniformly distributed rectangular pattern produced by the galvanometric mirror. When the true sample surface is not taken into account, the excitation grid points are erroneously assigned. In the ill-posed FMT problem, where small deviations in the measured data can cause large deviations in the reconstructed data, this can cause severe artifacts.

![Image](image.png)

**Figure 5.6:** FMT reconstruction results for the same animal (APP23 mouse 3hs post AOI987 injection) and reconstruction parameters, apart from the use of surface. (a) Reconstruction with the slab approximation for the mouse head, shown in detail in (c) and (d). (b) Reconstruction incorporating the coregistered MR surface, shown in detail in (e) and (f). All data are shown in the same scale.
Assessment of tracer kinetics. For all mice in the study, an intense fluorescence signal was observed in the brain immediately after AOI987 administration, which decreased over time 5.7. The quantitative FMT results from the measurements performed every 30min over a period of 3hs are presented in Figure 5.8. The temporal evolution of the fluorescence signal for both groups (wild type and APP23) could be approximated with exponential decay. The decay rate for APP23 mice was faster than for wildtype mice, indicating specific dye retention in APP23 mice.

Figure 5.7: FMT reconstruction results for the same animal, measured with FMT (a) immediately after and (b) 3hs post AOI987 administration. Data are shown in the same scale. The co-registered MR surface, utilized in the reconstruction, is shown in dots. The initially spread fluorescence signal decreased in intensity over time, but was also more spatially confined.
5.4. Results

Figure 5.8: Time course of FMT signal upon AOI987 injection. Markers on graph represent the mean reconstructed fluorescence (MRF) for each animal and timepoint as a % of the MRF value immediately upon injection. Fluorescence decay over time was observed for both groups (APP23, purple; wildtype, turquoise), and could be approximated with a fitted exponential curve ($R^2 = 0.8$ and $R^2 = 0.5$, respectively). The decay constant was slower for APP23 ($b = -0.0014\text{min}^{-1}$) than for wild type mice ($b = -0.0043\text{min}^{-1}$), indicating a slower dye washout in APP23 due to dye binding to A\textbeta plaques.
**Ex vivo validation**

To verify that the increased signal observed with FMT was caused by binding of AOI987 to cerebral structures, we sacrificed all animals following the FMT/MRI *in vivo* study and extracted the brains 3h post AOI987 injection. FRI imaging was performed on brain slices of 1mm thickness for one APP23-wildtype pair. Spectral unmixing was applied to separate the contributions of AOI987 and intrinsic background to the overall fluorescence intensity using the known spectral features of AOI987. As shown in Figure 5.9(a), the wildtype mouse (top) could be clearly distinguished from the APP23 mouse (bottom). In brains of APP23 mice, AOI987 was found to have accumulated in the cortex, but also in subcortical brain regions. The AOI987 signal was quantified by averaging the spectrally unmixed AOI987 signal, normalized to the exposure time, over all slices shown in Figure 5.9(a). As shown in Figure 5.9(b), the total AOI987 signal in the APP23 brain was seven times higher than in the wildtype brain.

The remaining animals used in the *in vivo* study have been analyzed using confocal microscopy (Figure 5.10), to demonstrate that the accumulation of AOI987 was found predominantly at the sites of the amyloid plaques rather than being unspecifically distributed throughout the brain parenchyma. Therefore, we can conclude that the spatial distribution of AOI987 (top row) reflects the spatial distribution of plaques.
5.4. Results

(a) Spectrally unmixed FRI images of 1mm thick brain slices from one APP23 (top) and one wildtype mouse (bottom), extracted 3hs post AOI987 injection. Both animals were 24 month-old littermates. The spectrum of the uninjected dye AOI987 was used to unmix fluorescence (turquoise) from background (pink). (b): Quantification of measurements shown in (a). Average AOI987 accumulation in the APP23 brain was seven times higher than in the wildtype brain.

Figure 5.9: (a): Spectrally unmixed FRI images of 1mm thick brain slices from one APP23 (top) and one wildtype mouse (bottom), extracted 3hs post AOI987 injection. Both animals were 24 month-old littermates. The spectrum of the uninjected dye AOI987 was used to unmix fluorescence (turquoise) from background (pink). (b): Quantification of measurements shown in (a). Average AOI987 accumulation in the APP23 brain was seven times higher than in the wildtype brain.
5. Application of FMT/MRI on a transgenic mouse model of amyloid angiopathy

**Figure 5.10:** Representative confocal images of 40µm thick brain slices from one wildtype (left) and one APP23 mouse (right panel). Animals were 24-month old littermates. The brains were extracted and snap-frozen immediately after the FMT/MRI *in vivo* imaging session, 3h post AOI987 injection. The imaged slice was selected so that the cortex was visible. Autofluorescence, caused by the protein lipofuscin which accumulates in brain tissue with increasing age [117], was measured with the confocal microscope’s green filter and is depicted in green. AOI987 signal, measured with the Cy5 filter of the confocal microscope, is depicted in magenta. Although lipofuscin, characterized by dots on the autofluorescent channel, was present in both animals (middle panel), the characteristic plaque shape (middle right) was predominantly detected on the APP23 animal. AOI987 signal for the wildtype animal colocalized with autofluorescence, whereas on the APP23 animal it surrounded the plaque, as expected. Tile scans of both animals (top panel) show that AOI987 fluorescence is indeed much higher on the APP23 animal than on the wildtype one. AOI987 is highly present on the brain cortex of the APP23 mouse, but not exclusively.
5.5 Discussion

Trangenic mouse technology has enabled the study of human diseases preclinically, by genetically engineering animals which display certain aspects of the human pathology. *In vivo* imaging can unveil associated aberrations in structure, function or metabolism in a longitudinal and noninvasive manner, with the goal of translating these findings for diagnostic or therapeutic applications in the clinics. However, visualization of minute pathological findings *in vivo* is challenging, even with the increased resolution and sensitivity of structural imaging techniques (CT, MRI) designed for small animal imaging. Molecular imaging offers an alternative approach, by detecting the distribution of targeted probes with sensitive imaging modalities such as PET or FMT. The construction of hybrid imaging systems, combining structural/functional (CT, MR) with complementary molecular (PET, FMT) imaging techniques in one bore, is therefore attractive for *in vivo* imaging of transgenic mouse models.

Recently, we presented the first prototype of an FMT/MRI system designed for the limited available space of a preclinical MRI scanner [1]. We chose MRI due to its superior resolution and soft-tissue contrast, but also due to its capabilities for structural and functional measurements. FMT, on the other hand, provides quantitative molecular readouts with stable probes and non-ionizing radiation, and additionally lends itself to *ex vivo* microscopical validation. Proof of concept of the first FMT/MRI prototype was demonstrated using a subcutaneous tumor model. However, the SPAD detector array used involved several limitations for FMT imaging; it had a small field of view, a small array size compared to CCD cameras used on standalone FMT systems, and high fixed-position noise [79], which compromised the achievable sensitivity. All these issues hampered the further use of this system for biomedical applications.

In this work, we have presented a new generation FMT/MRI system featuring two major improvements: (a) replacement of the SPAD array with a low-noise CMOS camera [118] with both enlarged field of view and improved sensitivity, and (b) technical modification allowing for reproducible positioning and improved animal handling. We verified the performance of this new system with phantom measurements. We found that FMT could clearly resolve sources placed 2mm apart in 1mm depth; analysis of the line profile was necessary to verify this separation distance in a depth of 2mm. The depth localization of fluorescent sources deeper than 3mm was found to be systematically smaller than the theoretical depth, even though the detector could resolve deeper located sources. Inaccurate visualization of
deep lying structures is an inherent problem of optical imaging, which is caused by the strong absorption and scattering of light in tissue; with increasing depth of fluorescent sources, the number of photons exiting the sample surface is also reduced. The increase of the sensitivity and the FOV of the optical detector as well as the increase of source–detector pairs in FMT experiments, as performed here, can improve the detection of fluorescence originating from deeper lying structures. One additional reason for the depth underestimation of deeper-lying sources is the reflection geometry employed in these FMT experiments. Reflection measurements, in contrast to measurements in transmission mode, have been shown to weigh surface fluorescence more than deeper structures. The modification of the system for measurements in transmission mode can possibly improve this behavior.

A strength of FMT compared to FRI systems is the quantitative, molecular information it provides. To verify that this was the case in our setup, phantom experiments revealed that the reconstructed fluorescence depended linearly on the amount of dye used, and that the minimum detectable concentration of AOI987 was 2 pmoles.

Optical measurements in the brain using fluorescent targeted probes have been reported in the past [81, 84, 119–121]. In general, however, the brain is a challenging region for FMT measurements, due to high absorption from the skull and the head’s curvature, which renders a slab approximation for FMT reconstruction inaccurate. Information on the surface of the animal’s head had to be included in the FMT reconstruction. The quality of the improved procedure was demonstrated by the overlay of the FMT with the MRI anatomical scan, which shows massive dye accumulation in the cerebral cortex. Even considering that fact that the FMT results for data recorded in reflection mode are surface weighted, the high cortical and the low subcortical dye concentration are compatible with the ex vivo results. A second aspect confirming the quality of the quantitative data derived from the FMT reconstruction is the analysis of the temporal behavior of the signal. In line with the findings of Hintersteiner et al. [81], we found the fluorescence signal to decay exponentially, indicative of clearance of the non specifically-bound dye. The decay was slower in the transgenic animals reflecting specific retention of the dye; three hours after the administration of AOI987 we measured a specific dye retention of 50% estimated according to $\frac{I_{(\text{APP23};3h)} - I_{(\text{wild-type};3h)}}{I_{(\text{APP23};3h)}}$. This value is in good agreement with the one reported by Hintersteiner et al. [81], who reported a specific binding of 40 to 50% for 16 months old APP23 mice, suggesting that FMT can be used to quantitatively assess processes at a molecular level with a time resolution of currently a few minutes.

We confirmed the in vivo FMT findings with FRI on brain slices extracted immediately
after the *in vivo* imaging session. We found that the residual fluorescence in the APP23 brain was significantly higher than in the wildtype, although the difference was much larger than the one observed with FMT *in vivo* 3hs post injection. This can be attributed to two effects. First, the skin autofluorescence and the photon absorption by the skull and blood *in vivo*, which are common between groups, reduce the contrast-to-background ratio. Second, Hintersteiner and colleagues showed [81] that the residual plasma levels of AOI987 two hours after injection were 10% of the initial concentration and that the elimination from the brain was only slightly lower. It is conceivable that the plasma fluorescence, which is common for the two groups and potentially higher for the wildtype animals (as no specific binding of AOI987 to Aβ occurs), contributes to the total detected signal and hampers its quantification. In contrast, ex *vivo* FRI was performed on excised brain slices in the absence of blood, and AOI987 was spectrally unmixed [34] from autofluorescence, thus eliminating the sources of *in vivo* background mentioned here.

One of the advantages of FMT is the straightforward validation of the *in vivo* findings with *ex vivo* microscopy, especially when using targeted probes which remain bound after histological protocols. We could verify histologically that the APP23 brain presented considerably more Aβ plaques than the age-matched wildtype, and that AOI987 colocalized with these plaques. The AOI987 distribution pattern was concordant with the FRI findings.

One obvious issue is that FMT could not unveil the AOI987’s distribution pattern on the brain evident on the *ex vivo* data. This is partly caused by the diffuse propagation of light in tissue, limiting the spatial resolution, and by measuring in reflection mode, which prevents the accurate reconstruction of subcortical signal sources as discussed previously. However, it is also caused by the ill-posedness of the FMT inverse problem. Several solutions for the fluorescent source distribution can give rise to the measured data, as shown in [15]; the FMT results here represent one (the simplest) of these solutions. As demonstrated by the *ex vivo* findings, this solution does not fully correspond to the actual spatial distribution of fluorescence, although the quantification (accessed by the mean reconstructed fluorescence) is correct.

In order to constrain the inverse problem, prior information needs to be included in FMT. This can be done in several ways; one data-driven approach for a priori information for the backward problem is reported in [84], addressing the same problem of reconstructing AOI987 signal on APP23 mice. There, the authors segmented the mouse head in three tissue types (brain, cortex and soft tissue) and utilized three different regularization matrices for the inversion. However, the selection of the three areas is empirical, as AOI987 penetrates
5. Application of FMT/MRI on a transgenic mouse model of amyloid angiopathy

the blood brain barrier (BBB) non-preferentially [81]. Although plaques on APP23 mice are indeed predominantly found in the cortex (as also shown by the ex vivo data in [84]), with increasing age they can also occupy regions of the hippocampus and thalamus as well [81]. For a given mouse and timepoint, the actual plaque distribution is unknown and can only be determined ex vivo. In longitudinal studies of animal models developing a phenotype over large periods of time, such as in AD, ex vivo data from different timepoints are difficult to acquire. For such studies, the use of FMT for estimating the total plaque load is attractive as global quantification results have been shown to be reasonably accurate.

An alternative approach which does not make assumptions on dye distribution is the incorporation of a priori information in the forward problem. There, the high resolution anatomical images obtained with MR can be segmented in regions containing approximately homogeneous optical properties, which can be assigned according to published values. As this labeled segmented data is coregistered to the FMT component, it can be used in the FMT reconstruction, instead of assuming that the whole sample has homogeneous optical properties. This approach has been presented in [122] for a hybrid FMT/XCT system. We expect that the superior MR soft tissue contrast compared to CT can yield even better FMT reconstruction results in the future.

In conclusion, we have described the design of a hybrid FMT/MRI system and characterized its performance using tissue phantoms of known composition and geometry. Reconstructions have demonstrated accurate geometric reconstruction of point sources with some limitations regarding depth information and accurate quantification. In vivo it has been demonstrated that fluorescence signals can be recorded with high sensitivity from brains of APP23 and age-matched wildtype mice. Dynamic FMT imaging enabled monitoring of the specific enrichment of the plaque specific dye AOI987 in the brains of APP23 mice. FMT reconstruction using information on the mouse head surface yielded information on the amount of tracer retained and on its spatial distribution. Accurate reconstruction of the tracer distribution, in particular capturing of subcortical sources, was not possible due to the surface weighting of data acquired in reflection mode and due to approximations made in the reconstruction (internal anatomical features of the mouse head were not considered). Future developments will include transmission mode imaging and inclusion of prior anatomical information in the reconstruction process.
Chapter 6

Application of a hybrid FMT/MRI system to study tumor vascularity

6.1 Preliminary remark

This chapter is adapted from "Tumor vascularity measurements using a novel FMT/MRI hybrid system" by K. Dikaiou, C. Germanier, M. Desai, M. Augath, F. Stuker, G. Batsios, M. Migueis, R. Keist, J. Ripoll and M. Rudin (in preparation).

6.2 Introduction

Imaging has become an indispensable tool to study tumor progression both in clinics and preclinical research, as it enables noninvasive disease followup. Several aspects of cancer such as tumor size, hypoxia, oxygen metabolism and neovascularity can be probed in the clinics with CT, MRI, SPECT and PET [123]. These clinically established imaging modalities are complemented by optics-based methods such as microscopy, fluorescence reflectance imaging and fluorescence molecular tomography (FMT), which are employed in tumor research using animal models. In such studies, exogenous fluorescently labeled agents targeting tumor physiological and molecular processes such as angiogenesis [52], protease activity [8, 124], ECM remodeling [125] and apoptosis [38] are imaged. Alternatively, the
expression levels of specific molecules can be probed using reporter genes such as fluorescent proteins that are expressed under the control of a promoter of interest.

As each technology has its strengths and limitations, multimodal systems (FMT/XCT [13], FMT/MRI [64], FMT/PET [62, 126], PET/MR [59]) are increasingly being used in cancer research to convey complementary information. A straightforward solution for multimodal imaging is the use of a sample stage that is compatible with different modalities [62, 64, 126]. This concept allows for proper co-registration of imaging data sets, assuming that the sample is not moved during the transfer, but obviously does not allow for simultaneous measurements, and is typically used to obtain an anatomical reference data set.

True hybrid systems [13, 59] combine two modalities in a single bore, thereby enabling simultaneous measurements with both modalities. Hybrid systems can reduce imaging times and ensure identical positioning and physiological parameters of the subject during data acquisition.

Recently, we presented [1] a hybrid FMT-MRI system fitting into the limited space of a small animal MR system. FMT has the advantage of providing quantitative molecular information [8] without ionizing radiation, in contrast to PET; moreover, a palette of fluorescent molecular probes is currently available commercially. MRI, on the other hand, can provide high resolution and soft tissue contrast, but can also be employed for functional measurements [127]. In [1], we have demonstrated proof-of-principle on a subcutaneous tumor model, with simultaneous acquisition of structural information using MRI and assessment of protease activity using FMT. In that study, we did not utilize the potential of MRI for both structural and functional measurements in tumors, which renders simultaneous data acquisition particularly attractive.

Dynamic contrast-enhanced MRI (DCE-MRI) [128–130] is an established tool to assess tumor vasculature. The signal enhancement in T$_1$-weighted MRI data upon injection of small-sized contrast agents extravasating into the extravascular/extracellular space (EES), such as Gd-DOTA, is a combined function of microvascular density, tumor blood flow and vessel permeability. Using pharmacokinetic models, estimates of the vascular transfer constant (K$_{trans}$) and the EES volume ($v_e$) can be made. Additionally, with dynamic susceptibility contrast MRI (DSC-MRI), the signal decrease in T$_2$ or T$_2^*$ weighted MRI images upon injection of magnetite nanoparticles, which stay within the vasculature, can be used to assess tumor vascular volume ($rTBV$) and tumor blood flow ($TBF$) [127].

In this work, we investigated the feasibility of concurrent FMT and DCE-/DSC-MRI measurements in two mouse tumor models using a hybrid FMT/MRI setup derived from the one...
6.3 Methods

6.3.1 Hybrid system setup

The hybrid FMT/MRI system used in this study is described in Chapter 5.3.1.

6.3.2 Animals

All experimental procedures conformed to the national guidelines of the Swiss Federal Act on Animal Protection and were approved by an official committee (license 168-2010, Cantonal Veterinary Office, Zurich, Switzerland). Female nude Balb/C mice (Janvier, France) of 8-10 weeks of age were used. Animals were kept at standard housing conditions with a 12-hour dark/light cycle and free access to water and chlorophyll-free food (Kliba Nafag, Kaiseraugst, Switzerland). One group of mice \( (n = 5) \) was injected with \( 10^6 \) C51 colon carcinoma cells and one group \((n = 4)\) with \( 10^5 \) 4T1 mammary carcinoma cells subcutaneously on the right thigh flank. Animals were identified by ear markings and were scored every second day once the tumors became visible. Tumor length and width were assessed with caliper measurements. In vivo imaging was performed for all animals at 7-10 days post tumor inoculation.

6.3.3 Hybrid in vivo imaging

After preliminary experiments described in Appendix D, we chose the experimental protocol depicted in Figure 6.1.

Previously described in [1]. To probe tumor vascularity with FMT, we chose a commercially available vascular fluorescent probe, AngioSense®680EX. This macromolecular probe has been used to assess both tumor blood volume and extracellular space [52,53] upon measurement at two distinct timepoints. We compared the DCE- and DSC-MR and FMT readouts, to evaluate whether FMT measurements with AngioSense®680EX could indeed be used as an equivalent of respective MRI procedures. Finally, we compared all in vivo findings to ex vivo measurements of vascularity and perfusion using confocal microscopy.
6. Application of FMT/MRI to study tumor vascularity

Figure 6.1: Experimental protocol of the study. The top part of the graph indicates procedures in the preparation room and the bottom part procedures in the hybrid FMT/MRI system. The acquisition parameters for MRI and FMT are described in detail in the text of section 6.3.3.

Animal preparation

Animals were anesthetized with an initial dose of 3% isoflurane (Abbott, Cham, Switzerland) in a 4:1 air/oxygen mixture and were maintained at 37°C body temperature. The temperature was monitored with a rectal temperature probe (MLT415, ADInstruments, Spechbach, Germany). The tail vein was cannulated with a 30G needle (0.3mmx13mm, BD Microlance, Drogheda, Ireland). 100µl of AngioSense®680EX (PerkinElmer, MA, USA) were administered intravenously, followed by saline flush of the same volume. The mice were positioned on the animal support of the hybrid FMT/MRI system on their left side so that the tumor was in the center of both the MR coil FOV and the camera FOV. Anesthesia was gradually reduced to 1.5% isoflurane. The animal temperature was kept at 37°C using a warm-water circuit integrated into the animal support. The total anesthesia duration was approximately 2 hours on the first experimental day and 30 minutes on the second day. All experiments were carried out on the hybrid FMT/MRI setup described in section 5.3.1.

MRI

For MRI, high resolution images of ten axial slices comprising the tumor were acquired with a $T_1$-weighted gradient-echo (FLASH) sequence with the following parameters: field of view FOV = 22 × 22mm$^2$, matrix size 256 × 256, slice thickness 0.5mm, $T_E/T_R = 2.526/200$ms, pulse angle $\alpha = 20^\circ$, number of averages NA = 6, total imaging time 3m40s. Subsequently, DCE-MRI was performed on a single slice at the middle of the tumor with a $T_1$-weighted FLASH sequence with the following parameters: FOV = 22 × 22mm$^2$, matrix size 256 × 90, slice thickness 1mm, $T_E/T_R = 3.16/15$ms, pulse angle $\alpha = 20^\circ$, number of averages NA = 1, number of repetitions $N_R = 500$, total imaging time 11m50s. A bolus
of 100µl of the contrast agent Dotarem (Guerbet, France) was injected on the 70th scan repetition with a spectrometer-triggered infusion pump (infusion rate 3ml/m). Immediately after the dynamic scan, a high resolution post-contrast T₁-weighted image with identical parameters to the first one was acquired.

The T₂ protocol comprised a high resolution T₂-weighted spin-echo (RARE) sequence with identical geometry to the T₁-weighted high resolution images and following parameters: RARE factor = 8, field of view FOV = 22 × 22mm², matrix size 256 × 256, slice thickness 0.5mm, Tₑ/Tᵣ = 11/2500ms, number of averages NA = 4, total imaging time 5m20s. Subsequently, DSC-MRI was performed on the same slice as DCE-MRI with a T₂-weighted FLASH sequence as follows: FOV = 22 × 22mm², matrix size 256 × 90, slice thickness 1mm, Tₑ/Tᵣ = 3.16/15ms, pulse angle α = 20°, number of averages NA = 1, number of repetitions Nᵣ = 60, total imaging time 1m21s. Bolus injection of 50µl of the contrast agent Endorem (Guerbet, France) was triggered automatically on the 20th scan repetition. Immediately after the dynamic scan, a high resolution post-contrast T₂-weighted image with identical parameters to the first one was acquired.

FMT imaging was performed twice; 2h post AngioSense®680EX injection (first experiment day) and 24h post AngioSense®680EX injection (second experiment day). Depending on experimental planning, the FMT measurement on the 2h timepoint was performed either before or immediately after the series of T₁-weighted scans. In order to position the laser grid on the tumor and map the camera coordinates to physical coordinates, calibration of the galvanometric head and the camera was performed on millimeter paper fixed to a plastic block with the same height as the tumor. Due to the reproducible positioning of the hybrid system, the calibration parameters were loaded and controlled prior to each FMT scan. For FMT measurements, a white light reference image (without laser excitation) was acquired. Subsequently, the light emitted upon laser excitation in a 8 × 8 grid comprising the tumor was collected twice, using the 660nm and the 720nm filters, corresponding to excitation and emission respectively. The images and FMT hardware parameters (laser power, camera exposure) were saved for off-line reconstruction. Acquisition of each full FMT measurement lasted approximately 5 minutes.
6. Application of FMT/MRI to study tumor vascularity

6.3.4 Immunofluorescence and immunohistochemistry

Animals were deeply anesthetized with 5% isoflurane in a 4:1 air/oxygen mixture and were tail vein cannulated. To access functional vasculature and thus perfusion, a dose of 20mg/kg of a 10mg/ml solution of the DNA-staining dye Hoechst 33342 (Sigma-Aldrich, Switzerland) was administered intravenously one minute prior to animal sacrifice via cervical dislocation. The tumors were harvested immediately, embedded in OCT medium (Tissue-Tek* O.C.T. Compound, Sakura* Finetek, The Netherlands) and snap-frozen in liquid nitrogen. Cryoslices with a thickness of 14µm were cut at approximately the middle of the tumor and were fixed in PFA. The slices were stained for the endothelial marker CD31 (primary antibody rabbit anti-mouse CD31, Abcam, UK, secondary antibody goat anti-rabbit Alexa 488, Invitrogen, USA) and the macrophage marker f4/80 (primary antibody rat anti-mouse f4/80, 1:500, AbD Serotec, UK, secondary antibody goat anti-rat Alexa 594, Invitrogen, USA), according to standard procedures. One slice per animal was additionally H&E stained.

6.3.5 Microscopy

The stained slices were imaged with the Axio Imager Z1 microscope with ApoTome and AxioCam MRm (Zeiss, Germany) using a 10x objective and DAPI/Alexa594 filters. Images were acquired and contrast-corrected with the software ZEN 2011, Blue Edition.

6.3.6 Analysis of MR data

Dynamic MRI scans

Regions of interest (ROIs) comprising the tumors and control ROIs of the same size containing the gastrocnemius muscle were selected on all dynamic MR datasets with the program BioMAP6 (Martin Rausch, Novartis Institute for Biomedical Research, Basel, Switzerland). The mean intensity and standard deviation over the ROIs were extracted for each animal. Further processing was performed in Matlab R2012b (The Mathworks Inc., MA, USA) as described below.

T1-weighted MRI scans For the contrast-enhanced $T_1$-weighted dynamic images, the GdDOTA concentration in each voxel of tissue, $C_{t,GdDOTA}$, was estimated from the signal enhancement curves as described in [127]:

92
6.3. Methods

\[ E[R_1(t)] = \frac{S[R_1(t)] - 1}{S[R_0]} \approx \frac{r_{1,GdDOTA}}{R_{10}} \cdot C_{t,GdDOTA}. \] (6.1)

where \( S[R_0] \) and \( S[R_1](t) \) indicate the signal intensity prior and post GdDOTA administration, respectively, and \( R_{10} \) is the relaxation rate without contrast agent. \( S[R_0] \) was estimated by the mean of all 70 timepoints prior to GdDOTA injection. Using a two-compartment model comprising the plasma and extracellular/extravascular space (EES) [128], the GdDOTA concentration in tissue is:

\[ C_{t,GdDOTA}(t) = k \int_0^t e^{(-K_{\text{trans}}/v_e)(t-t')} \cdot C_{p,GdDOTA}(t) \, dt' + v_p \cdot C_{p,GdDOTA}(t), \] (6.2)

where \( K_{\text{trans}} \) is the transfer constant of GdDOTA from the plasma into the EES, \( v_p \) and \( v_e \) the plasma and EES volume, respectively, and \( C_{p,GdDOTA} \) the tracer plasma concentration. As our protocol involved bolus injection of the tracer and by assuming instant tracer/blood mixing, we approximated the GdDOTA concentration in plasma \( C_{p,GdDOTA} \) by a step function \( (C_p(t) = 0, \quad t < t_0 \text{ and } C_p(t) = C_p, \quad t > t_0) \), where \( t_0 \) was the point of GdDOTA administration. Moreover, we assumed the tracer concentration in circulation to be negligible compared to the concentration in tumor. Therefore, equation 6.2 is simplified into

\[ C_{t,GdDOTA}(t) = v_e \cdot C_{p,GdDOTA}(1 - e^{(-K_{\text{trans}}/v_e)t}) \] (6.3)

Its initial slope of equation 6.3 is then proportional to \( K_{\text{trans}} \), as

\[ \frac{d}{dt} C_t(0) = K_{\text{trans}} \cdot C_{p,GdDOTA} \] (6.4)

and the tracer final uptake in tissue is proportional to \( v_e \), as:

\[ \lim_{t \to \infty} C_{t,GdDOTA}(t) = v_e \cdot C_{p,GdDOTA} \] (6.5)

We fitted the GdDOTA concentration in tissue to a biexponential function

\[ f(t) = ae^{bt} + ce^{dt} \] (6.6)

using non-linear least squares, as shown in Figure 6.2(a). The goodness of fit was de-
6. Application of FMT/MRI to study tumor vascularity

scribed with the coefficient of determination $R^2$. The initial slope and the final uptake were calculated from the fitted curve. In cases where the GdDOTA concentration curve could not be approximated with a biexponential function due to its shape (Figure 6.2(b)), it was first smoothed with cubic splines and the resulting piecewise polynomial form was differentiated once. The segment corresponding to GdDOTA’s first pass was estimated automatically from the first zero crossings of the differentiated curve, as shown in Figure 6.2(c). A linear function was fitted to the segment and its slope was assigned to $K_{trans}$. The extravasated volume $v_e$ was estimated from the area under the GdDOTA tissue concentration curve.

![Figure 6.2](image)

Figure 6.2: Fitting procedures for the GdDOTA enhancement curves, illustrated for two representative animals. The enhancement curves have been computed according to 6.1 and are shown as a function of time, setting the injection timepoint as 0. (a) Enhancement curve (red), which can be fitted with a biexponential function shown in brown. $K_{trans}$ and $v_e$ have been computed from equations 6.4 and 6.5. (b) Enhancement curve which cannot be fitted by a biexponential function. To estimate $K_{trans}$, the initial slope of the curve has been estimated using a linear function fitted to the increasing segment of the enhancement curve, shown in gray. The increasing segment of the enhancement curve in (b) has been computed by the zero-crossing points of the derivative of the spline-approximated enhancement curve, as shown in (c).

T2-weighted MRI scans  From the contrast-enhanced T2-weighted dynamic images, the relative tumor blood volume was estimated from the steady-state in signal decrease curves as described in [127]:

$$rTBV = - \frac{1}{r_{2,Endorem} C_{p,E}(t)} \ln \left[ \frac{S_E(t)}{S_0} \right],$$  \hspace{1cm} (6.7)

where $r_{2,Endorem}$ is the Endorem’s $R_2$ relaxivity. As we know neither endorem’s $R_2$ in tumor tissue nor the actual plasma concentration, we can only estimate relative blood volumes according to $rTBV \propto - \ln \left[ \sum_i S_E(i) / \sum_j S_E(j) \right]$ with $15 < j < 20$ and $65 < i < 70$. Moreover, the blood flow index ($BFI$) was estimated by the slope of a linear equation fitted to the segment of the Endorem uptake curve immediately upon injection ($24 < k < $
6.3. Methods

29). The fitting was performed with non-linear least squares. Also here, the goodness of fit was described with the coefficient of determination $R^2$.

Pre- and post- high resolution MRI scans

For the high resolution pre-and post-injection $T_1$ and $T_2$-weighted images, maps of the final uptake of contrast agent were calculated for all slices according to equations 6.1 and 6.7, respectively, where $S_0$ corresponded to the pre-injection images. These maps reflected the leakage space $v_e$ and tumor blood volume $rTBV$ for $T_1$- and $T_2$-weighted pairs respectively. An example of such maps is shown in Figure 6.3. Regions of interest corresponding to the tumor and the gastrocnemius muscle were selected on the $v_e$ and $rTBV$ maps, using the ROIs of the dynamic scans as a guide.

![Figure 6.3: Illustration of $v_e$ maps from high resolution $T_1$ pre- and post-images of a mouse bearing a C51 tumor. (a) shows the difference image between the post-and the pre-$T_1$ scan for one animal in the study. The enhancement in the tumor (top) is visible. (b) shows the corresponding $v_e$ map.](image)

6.3.7 Reconstruction and analysis of FMT data

FMT reconstruction was performed using the Kirchhoff approximation of the normalized Born ratio as described in [39, 114]. Briefly, the emission measurements were divided by the excitation measurements and normalized to the camera exposure time and the laser power, in order to correct for optical inhomogeneities within the animal and camera factors. Sources that presented reflections by the metal of the MR surface coil were excluded from the reconstruction. The mean reconstructed fluorescence in arbitrary units was calculated for each animal. As only the tumor region was excited, ROIs outside the tumor could not be selected. Incorporation of the animal surface, as computed by the MRI images, into the
6. Application of FMT/MRI to study tumor vascularity

FMT reconstruction, is in progress.

6.3.8 Statistical analysis

For MRI, the extracted parameters $K_{\text{trans}}$, $v_e$, $BFI$ and $rTBV$ for tumors were normalized for each animal to the corresponding values for muscle. For FMT, the mean reconstructed fluorescence from the tumor for each animal at 2h and 24h were used instead. All values were compared between the two tumor type groups with one-way ANOVA. Statistical significance ($p \leq 0.05$) was derived from a two-tailed $t$-test analysis.

6.3.9 Analysis of immunofluorescence data

Composite immunofluorescence images were imported in ImageJ (NIH, USA) and split into the RGB channels corresponding to Hoechst 33342 and CD31. Each channel was automatically thresholded and segmented. The mean value and standard deviation for each staining was saved for statistical analysis.

6.4 Results

6.4.1 DCE-MRI results

The GdDOTA tumor and muscle uptake curves for all animals in the study are depicted in Figure 6.4(a). The 4T1 tumors showed steady uptake of GdDOTA (Figure 6.4(b)), whereas C51 tumors (Figure 6.4(c)) presented both an initial signal drop immediately upon GdDOTA injection and a signal decrease 42s to 2m post GdDOTA injection, after which they slowly increased towards steady state. This temporal profile was not observed in the control areas (muscle) of both groups. However, the steady state GdDOTA muscle uptake was lower for the C51 animals compared to the 4T1 animals (mean value 0.45 versus 0.3, respectively).

Statistical analysis of the derived parameters from the GdDOTA uptake curves revealed that the normalized $K_{\text{trans}}$ in C51 animals was smaller than in 4T1 animals (Figure 6.5(a)), however not significantly so ($p = 0.69$). Normalized $v_e$ (6.5(b)) was found to be significantly smaller in C51 animals than in 4T1 animals ($p = 0.046$).
6.4. Results

Figure 6.4: GdDOTA uptake curves for tumor (red) and muscle (green) as a function of time. (a): GdDOTA uptake profiles for all animals in the study ($n = 7$). 4T1 animals are depicted in dashed line, C51 animals in solid line. (b) GdDOTA uptake profiles for 4T1 animals, depicted as mean±sem ($n = 4$) (c) GdDOTA uptake profiles for C51 animals, depicted as mean±sem ($n = 3$).

Figure 6.5: Normalized dynamic parameters from GdDOTA uptake curves, calculated and shown separately for the two groups as mean ± sem, where $n = 4$ for the 4T1 group and $n = 3$ for the C51 group. (a): Normalized $K_{trans}$, (b) normalized $v_e$.

The Endorem uptake curves are shown in Figure 6.6 (mean ± sem) for all animals in the study. No qualitative differences in the tracer uptake curve in tumor or muscle were observed.
6. Application of FMT/MRI to study tumor vascularity

**Figure 6.6**: Endorem uptake curves for tumor (red) and muscle (green) as a function of time, shown for all animals in the study.
6.4. Results

6.4.2 High resolution MRI results

To probe further into possible reasons for the differences in GdDOTA uptake in the two tumor types, we analyzed the high resolution pre- and post-images. The $v_e$ and rTBV maps for four representative animals (two from each group) are shown in Figures 6.7 and 6.8, respectively. The spatial distribution of $v_e$ is markedly different the two tumor types; in the 4T1 tumors it appears uniformly distributed across the tumor, whereas in the C51 tumors regions of different behavior are visible. The rTBV maps show that perfusion of even tumors of the same type can also differ markedly (compare (a) with (b) or (c) with (d) in Figure 6.8).

![Figure 6.7: High resolution $v_e$ maps for four representative animals of the study. (a), (b): 4T1 tumors. (c), (d): C51 tumors.](image)

![Figure 6.8: rTBV maps for the animals of Figure 6.7.](image)

Not surprisingly, quantification of the mean $v_e$ and rTBV values among the two groups (Figure 6.9) revealed high variability. The differences between the two tumor types were found non-significant for both $v_e (p = 0.75)$ and $rTBV (p = 0.63)$. 
6. Application of FMT/MRI to study tumor vascularity

Figure 6.9: Normalized parameters from high resolution images uptake curves, shown separately for the two groups as mean ± sem, where $n = 3$ for the 4T1 group and $n = 3$ for the C51 group. (a): $v_e$, (b) $rTBV$. 
6.4. Results

6.4.3 FMT results

The hypothesis underlying the FMT study was that the initial readout (2h) constitutes a measure of the average $rTBV$, while the difference between the intensities at 24h and 2h reflected $\nu_e$. Quantification of FMT results revealed that the initial AngioSense®680EX signal (2hs p.i.) was higher on C51 animals than on 4T1 animals, compared to the MRI-derived $rTBV$ assessment for the same animals (Figure 6.10(a)). The results obtained for the two tumor types are shown in Figure 6.10 (b). Again, the variability within groups, and especially within the C51 group, was high, which may be attributed to the heterogeneity typically observed in large tumors.

![Graph](image)

Figure 6.10: Quantification of FMT reconstruction results. (a) Mean reconstructed fluorescence according to timepoint and tumor type. (b) AngioSense®680EX enhancement according to tumor type. All values represent mean ± standard deviation.

6.4.4 Histology results

Histological analysis of the central slices in all tumors in this study revealed that the tumor vascularity pattern was different for the two tumor types in this study. Representative tile images yielding an overview on the whole tumor are shown in Figure 6.11. In 4T1 tumors, positive areas for both CD31 (endothelial marker) and Hoechst 33342 (perfusion) stainings were highly concordant. In C51 tumors, however, we observed extended areas for which the two stainings were non-overlapping. This indicates that in these regions vessels have been formed (positive for CD31 staining) but are not functional (negative for the perfusion marker Hoechst 33342). The disconnect between perfused tumor tissue and the presence of vascular endothelial cells is illustrated in Figure 6.12.
6. Application of FMT/MRI to study tumor vascularity

Figure 6.11: Tile confocal images of tumor slices corresponding to the animals shown in Figures 6.7 and 6.8. The endothelial marker CD31 staining is shown in red, perfusion as accessed with Hoechst 33342 is shown in blue. (a),(b): 4T1 tumors; (c),(d): C51 tumors.

Figure 6.12: Representative slice showing difference of perfusion, as assessed with Hoechst 33342, and existence of vessels, as assessed with CD31.
6.5 Discussion

Truly hybrid systems for preclinical research, combining more modalities in one design, have been presented recently (PET/MRI [59], FMT/XCT [13], FMT/MRI [1]). They have demonstrated equal [59] or superior [13] imaging quality compared to standalone systems, due to the fact that prior information can be used for solving the inverse problem. Specifically in cancer research, the benefit of multimodality to identify biomarkers for disease progression and therapy response has been shown [64] using sequential measurements. One advantage of hybrid systems utilizing MRI as a structural modality is the ability to combine anatomical information with functional and metabolic measurements, adding to the classical structural/molecular combination.

We have employed a custom-made hybrid FMT/MRI system for simultaneous assessment of tumor vascularity on two mouse tumor models. DCE- and DSC-MRI was performed with both Gd- and iron-based contrast agents according to standardized procedures, and a vascular fluorescent probe was used for analogous measurements using FMT. In vivo measurements were validated by ex vivo analysis of excised tumors by assessing the structure and functionality of the tumor neovasculatures using the perfusion marker Hoechst 33342 and the endothelial marker CD31 respectively. Preclinical and clinical experience with tumors has shown that tumors are highly heterogeneous; even under the controlled conditions of the present study, this effect was clear in all the quantitative MRI and FMT measures. The high resolution with which microscopy can discern the highly inhomogeneous areas [131] in tumors is not achievable with either MRI or FMT. Still, ensemble descriptions from those in vivo modalities have the potential to detect differences that are associated to what is observed ex vivo.

The GdDOTA uptake curves from the DCE-MRI experiments revealed differences between the two groups in the study. These differences could be further clarified in high resolution contrast uptake maps. We found that the C51 tumors in this study had highly inhomogeneous vascularity, in contrast to the 4T1 tumors. Moreover, the variability within the C51 group was higher for all quantitative measures employed in this study. Microscopical tumor examination confirmed that on C51 tumors vessels were indeed formed, but were not functional. On 4T1 tumors, however, regions containing vessels and perfused regions were highly concordant. This difference may be attributed to two reasons. First, as visible on the high resolution images, the C51 tumors were much smaller than 4T1 tumors. It is possible that at the time of the experiment, vessel formation on C51 tumors was at an earlier
stage of progress. Second, it has been shown in [132] that 4T1 tumors are highly aggressive and establish lethal metastases as early as two weeks post inoculation. As formation of new vessels is related to tumor malignancy, the more developed vascularity pattern observed on 4T1 tumors may reflect an inherent difference between the two tumor types. To clarify between these two hypotheses, longitudinal studies on different days post tumor inoculation will be necessary.

In this study, we did not find differences in AngioSense®680EX enhancement as assessed with FMT. The reduced sensitivity of FMT compared to MRI in this application appears to be in contrast to the known higher sensitivity of FMT compared to MR [10]. A possible reason behind this surprising result is that AngioSense®680EX is not a molecular agent, but a vascular agent without target selectivity; therefore, it is likely that the contrast-to-background ratio in this experiment was not high. Moreover, although the chosen timepoints of 2h/24h are stated to correspond to mostly vascular/whole tumor signal respectively, it is almost certain that the agent’s behavior is not as clear cut in biological tissue, and is putatively different between tumor types. Indeed, FMT with various molecular probes has demonstrated significantly differences in their distribution patterns [62].

A common finding for both MRI and FMT in this study was that lumped parameters averaged within groups revealed common tendencies (lower \( v_e \) but higher \( rTBV \) on C51 tumors) in a consistent manner. However, with the estimation of \( v_e \) from dynamic measurements being the single exception, the variability within groups was so high that no statistical significance was found. As already stated, this variability is a common finding in tumors and renders the evaluation of effects difficult. Novel methods, addressing the issue of tumor heterogeneity specifically might be of interest in this context [133]. The MRI results suggest that measurements with high spatial resolution and/or high temporal resolution can unveil these differences better. In FMT, such a high spatial resolution depiction is beyond reach; the diffuse propagation of light in tissue [27] and the linearization of the inverse problem for the reconstruction of the fluorescent sources [134] limit the achievable resolution to \( > 1 \text{mm} \), which implies that patchy structures within the ROI cannot be reconstructed. Nevertheless, the volume average FMT analysis should yield comparable results to the volume-average MRI readouts that are typically used to assess the efficacy of therapeutic interventions [127]. Our data indicate that apart from the variability in the data this is in fact the case. Additionally, FMT measurements with high temporal resolution with this setup are currently not feasible. We expect that both the incorporation of structural prior information from MRI in the reconstruction of FMT data and the development of and the use of dynamic contrast-
enhanced FMT measurements with improved temporal resolution will enhance the potential of the hybrid approach.
Chapter 7

Conclusions and Outlook

The objective of this work was to (a) to improve the performance of a hybrid FMT/MRI system recently developed at the Animal Imaging Center, Institute for Biomedical Engineering, University and ETH Zurich [1] by utilizing MRI-derived information to improve the quality of the FMT reconstruction, and (b) to demonstrate the potential of the FMT/MRI combination on selected biomedical applications.

7.1 Conclusions

7.1.1 FMT applications

We constructed a more compact FMT system based on an existing prototype and used it in two in vivo imaging studies. The first study aimed at assessing the expression of the transmembrane protein TMEM27 on pancreatic beta cells with a fluorescently labeled antibody in the NIRF region. Quantitative FMT results from subcutaneous insulinomas were in complete agreement with PET results and superior to FRI. However, the variance of FMT results from the pancreas was larger than both FRI and PET. We believe that the compromised performance of FMT when imaging the abdomen is due to the high background in this region, caused by high absorption and autofluorescence. Spectral unmixing, which can separate the probe signal from background using their different spectral signatures, can potentially
7. Conclusions and Outlook

alleviate this problem.

The second application aimed to monitor the activity of hypoxia-inducible factors (HIFs) in a mouse tumor allograft model using a novel GPI-avidin molecular reporter system and a fluorescently labeled biotinylated probe. Imaging was performed in the visible domain of the spectrum ($\lambda = 594\text{nm}$), to facilitate in vitro and ex vivo microscopy. With FMT we could follow the temporal evolution of the fluorescent signal in a consistent way with FRI, but we could not distinguish between the specific and the unspecific probe on tumors bearing the molecular reporter system. We postulate that this was due to (a) the wavelength domain (594nm) not being suitable for in vivo imaging, as tissue displays high absorption and autofluorescence, and (b) the high background signal related to the probe’s biodistribution. Here, spectral unmixing and parallel measurement of control regions outside the tumor could improve results.

Put together, the results of both studies indicate that FMT can provide quantitative information with comparable quality to PET and superior to FRI systems. This holds when measuring with FMT within the NIRF region and under conditions of sufficient signal to background ratio, which might be compromised by high absorption or high autofluorescence. This is especially relevant when using targeted probes whose targets occur in low abundance. These confounding factors of course apply to any fluorescence-based optical in vivo method; as shown here, existing commercial FRI systems alleviate them to some extent by utilizing spectral unmixing, which takes into account the spectral properties of the injected probe. We therefore believe that incorporation of such tools in our FMT system could improve their performance significantly.

On a more general note, both these studies clearly demonstrated one advantage of FMT imaging in preclinical research; both targeted probes used here were fluorescently labeled and assessed in vitro for specificity, in vivo for sensitivity and pharmacokinetics and ex vivo to confirm specific binding to the tissues of interest. Fluorescence imaging thus enabled the seamless integration of in vivo studies with microscopy techniques, using the same probe.

7.1.2 FMT/MRI: system prototypes, coregistration and use of the sample surface in the FMT reconstruction

This work contributed in the development and characterization of a first hybrid FMT/MRI prototype for small animal imaging developed in the Animal Imaging Center, Institute for Biomedical Engineering, University and ETH Zurich. This system was the first of its kind to
replace optical fibers for sample excitation and signal detection by free laser beam illumination and an MR-compatible SPAD array camera. Contributions included the adaptation of the FMT hardware control software for this system, performance characterization of the FMT component with respect to spatial & depth resolution, quantification linearity and crosstalk between components and a first version of overlaying the FMT and MRI datasets. Although proof of principle of this system was demonstrated on a subcutaneous tumor model, both the limited SPAD array size (and corresponding FOV) and its non-uniform sensitivity hampered further biological applications.

These problems were addressed in a second hybrid FMT/MRI prototype, where the SPAD array was replaced with a sensitive CMOS MR-compatible camera with a higher array size and sensitivity. Additionally, the new system featured an improved design for the FMT/MRI insert based on rails, which enabled accurate repositioning and easier animal handling. Contributions to this system were related to design, choice of the CMOS camera over other available solutions and assessment of FMT/MRI crosstalk.

The new CMOS camera provided superior optical images which could be used for FMT/MRI coregistration. This was combined with detection of the sample surface by MRI and its inclusion in the FMT reconstruction as follows; isosurfaces corresponding to the sample were found and used to compute its top surface, which can be thought of as the sample view from above, similarly to the view provided by the reference whitelight image of the FMT dataset. Control points from these two images could be selected interactively for the computation of the affine transformation between the two images. The sample surface, registered to the whitelight image, was then used in the FMT reconstruction by virtue of the Kirchhoff approximation [114]. This provided a more realistic model of the sample compared to the previously used slab representation. Optimal MRI acquisition parameters for this procedure were identified with phantom and in vivo experiments. It must be noted that this procedure has to be carried out only once during the experiment; provided that the distance between the camera and the coil of the FMT/MRI system remains constant, the registration transform matrix can then be employed to co-register all subsequent FMT and MRI datasets.

### 7.1.3 Biological applications of the FMT/MRI system

Two biological studies were performed to assess the potential of the hybrid FMT/MRI system.
7. Conclusions and Outlook

In a first step, we aimed to combine structural readouts provided by MRI with molecular readouts provided by FMT, and it was performed on a transgenic mouse model of Alzheimer’s disease (AD). This mouse model, APP23 [106], overexpresses the mutated human amyloid precursor protein (APP), which leads to enhanced deposition of amyloid $\beta$ plaques in the mouse brain after 6 months of age. We employed 24–month old APP23 mice and age-matched wildtype littermates. We used structural MRI measurements with different contrasts to assess morphological changes that have been associated with APP23 mice. No ventricular enlargement on transgenic animals was found [109], but in one transgenic animal focal hypointensities, attributed to cerebral microbleeds [112, 116], were detected. MR angiography revealed that major vessel structures in the brain could be identified, suggesting that this system could be used to assess vascular remodeling, a process shown to occur on AD mouse models [111].

The axial MR data was used to estimate the mouse head’s surface, which was registered to the FMT reference whitelight image and used in the FMT reconstruction. Using dynamic FMT measurements over a timecourse of 3hs, we were able to follow the kinetics of AOI987, a NIRF Cy5.5 conjugate which has been shown to bind to A$\beta$ plaques in vivo with high specificity. The exponential decay of the reconstructed fluorescent signal over time was slower in transgenic mice, suggesting specific dye retention. Ex vivo FRI and confocal microscopy measurements from the excised brains revealed that residual AOI987 signal was indeed higher on transgenic mice, that transgenic mice had plaques and that AOI987 had binded on them.

These results show that MRI is a valuable structural modality for hybrid systems, as it offers not only high spatial resolution, but also adjustable high soft-tissue contrast which can be used to assess several aspects of tissue morphology. Prior information from MRI, employed here in the form of the animal surface, can improve the FMT reconstruction by providing a more realistic model of the imaged animal. Dynamic FMT measurements can be employed to quantitatively follow molecular processes with a temporal resolution of currently some minutes. Finally, in vivo validation of FMT in vivo readouts with FRI and microscopy can be performed seamlessly when using targeted fluorescence probes.

In a next step, we combined structural and functional MRI readouts with FMT information. For this, we studied tumor vascularization on two subcutaneous tumor models (breast carcinoma 4T1 and color carcinoma C51). During their growth, tumors induce the formation of new vessels in order to cover their increasing oxygen needs, a process called angiogenesis. The extent to which these vessels are functional and perfuse the tumor is variable. The
study of tumor vascularity is biomedically relevant, as it has been associated with tumor malignancy and response to antiangiogenic drugs. We performed dynamic contrast-enhanced MRI with two commercially available contrast agents based on gadolinium (Dotarem) and super paramagnetic iron oxide nanoparticles (Endorem), which influence the local $T_1$ and $T_2$ tissue relaxation rates, respectively. Endorem remains in the vasculature, and thus reflects the tumor blood volume (TBV), whereas Dotarem leaks out rapidly, reflecting the extravascular-extracellular space volume ($v_e$). Kinetic modeling of $T_1 -$ and $T_2 -$ weighted dynamic sequence allows estimates of these two different aspects of tumor vascularity. As an equivalent to these readouts, we chose a NIRF dye, AngioSenseEX®680, which has been used to assess these aspects when measured at two distinct timepoints [52, 53].

Using the FMT/MRI system, we were able to perform DCE-MRI and high resolution measurements prior and post contrast agent administration. As observed in many tumor studies, the biological variability was high. However, differences between groups were observed on the Dotarem uptake curves and the high resolution contrast uptake maps. They suggested that the distribution of leaky vessels in C51 tumors was highly inhomogeneous, in contrast to 4T1 tumors. We could verify these results ex vivo using a perfusion marker and endothelial cell staining reflecting vessels. We did not find significant differences in the uptake and enhancement of AngioSenseEX®680 between the two tumors in the study, although the observed tendencies were consistent with all MRI results. This may be attributed to the low spatial resolution of FMT and the absence of fast dynamic measurements equivalent to MR.

These results show the potential of the FMT/MRI hybrid system for functional measurements. Functional protocols assessing the vascular response upon contrast agent administration are used in tumor imaging [127], but also in neuroimaging applications [135]. Combination of such protocols with molecular information provided by FMT can thus be used to access structural, functional and metabolic changes in animal models of disease.

The method of FMT, treated in this work, is used predominantly in preclinical applications. The high absorption of light by tissue limits its applications in the clinical setting to soft tissues with relatively low absorption coefficient, such as the breast. On the other hand, MRI is established both preclinically and clinically. In this work, we have shown the added value of a hybrid FMT/MRI system for imaging small animals. We believe that the multiplexed information that this system provides has the potential for clinically translatable findings in the future.
7. Conclusions and Outlook

7.2 Outlook

7.2.1 Exploiting the potential of MRI for metabolic information

In this work, the advantages of the high soft tissue contrast provided by MRI and its potential for functional measurements have been demonstrated. However, the potential of MRI as a component of the hybrid FMT/MRI system has been anything but exhausted. Further work can be pursued along several lines, some of which are stated here.

As discussed in section 2.1.1, several diseases are known to be associated with altered metabolic processes. Metabolic information can be obtained with magnetic resonance spectroscopy (MRS) and spatially resolved with chemical shift imaging (CSI). Therefore, it is intuitive to include this type of information on the existing FMT/MRI system. However, CSI has high demands on excitation field homogeneity and SNR which cannot be accommodated with the current FMT/MRI setup. A system redesign would thus be necessary, involving a volume resonator for homogeneous MR excitation and multiple MR detection coils, potentially cryogenic [136], to increase the SNR without the inefficient increase of acquisition time by the power of two. Such a redesign would have to be compatible with the spatial limitations of the FMT/MRI system, related to the small MRI bore diameter and the requirement of a coil window for optical excitation and detection, and it would be highly challenging.

A highly interesting alternative with less technological demands would be the employment of dynamic nuclear polarization (DNP). DNP achieves polarization of NMR-sensitive nuclei such as $^{13}$C up to $10^6$ times more than their polarization at thermal equilibrium, and therefore the DNP signal can be detected with surface coils at room temperature. The approach is limited by the relaxation of the polarized state, which in biological tissues is typically less than one minute. However, DNP has been successfully employed recently to assess enzymatic processes in tumors associated with glycolytic metabolism (pyruvate-lactate conversion [137]) and necrosis (malate-fumarate conversion [138]), as well as tumor response to antiangiogenic therapy [139]. DNP measurements have recently been performed in our lab using a room temperature surface coil (G. Batsios, unpublished results). Replacement of the existing MR coil in this setup with a double-tunable 1H/$^{13}$C coil is feasible. The combination of FMT/MRI with DNP is not only technologically applicable, but potentially highly attractive for tumor biology studies. Potential applications could study the interplay of metabolism (DNP) and vascularization (MRI) with hypoxia, angiogenesis or tumor microenvironment (FMT).
7.2.2 Improving the FMT component

In this work, almost all FMT experiments have been performed in reflection mode. As discussed in Chapter 4, reflection measurements are strongly weighted towards the surface. This was shown also in Chapter 5, where the fluorescent source depth was correctly estimated for sources to a depth of 5 mm. Reflection measurements are sufficient when the fluorescent sources are indeed close to the surface, as is the case for subcutaneous tumors. For other biologically relevant applications such as deep-seated orthotopic tumors and brain imaging, transmission measurements can provide improved depth reconstruction. In the current FMT/MRI setup, this can be accommodated with a mirror underneath the sample. Phantom experiments to estimate the depth resolution of the FMT system in transmission mode will have to be carried out, so that the performance is evaluated prior to in vivo studies.

In the current FMT/MRI system, one camera is placed above the sample. This compromises the sensitivity, as not all photons exiting the sample are detected. Moreover, as shown by Ripoll et al. in [74], the method of boundary removal requires that the total flux exiting the sample is recorded by the camera. Therefore, detection of photons exiting at several angles will improve the reconstruction accuracy. The addition of multiple cameras in the current design is challenging, due to spatial limitations – cameras with a more compact design will have to be employed. Detection from multiple angles can be performed either by placing several cameras around the sample or by rotating a single camera around the sample. The latter solution is cheaper, but requires an MR-compatible motor system. Such systems have indeed been constructed in the field of MR-compatible robot technology, as discussed in [140]. It must be noted, however, that they have been designed for clinical MR scanners, where the spatial requirements are less strict and the static magnetic field lower. It remains to be examined if they could be utilized in the present setup. Provided that optical detection from multiple angles can be performed, the animal surface can also be recorded by the camera, as shown in [84]. This would provide the coregistration problem discussed in Chapter 3.3 with many more control point pairs, and would thus make its solution more stable.

An additional improvement in the FMT system is the visualization of the whole mouse body. As shown in section 4.2, this would allow the estimation of probe pharmacokinetics and can provide control ROIs outside the tissue of interest, to estimate target-to-background ratios. Currently, FRI systems offer this possibility by moving the animal stage further away from the camera. Again, spatial limitations of the current FMT/MRI setup do not allow for such stage movement. Full body imaging with FMT can be implemented with either multiple cameras over different positions of the sample along the z axis or a motor system to translate
the animal stage along the \( z \) axis, while the camera and coil remain at their positions. In any case, the coil arrangement around the animal will have to be modified to accommodate for sample illumination and signal detection in this updated geometry.

Regardless of the choice of implementation, the effective increase of the FMT’s FOV will be associated with larger laser excitation grids and therefore linearly increased acquisition time. Considering that the acquisition of a full FMT dataset of a \( 8 \times 8 \text{mm}^2 \) tumor was 5min with the current setup (Chapter 6), it is evident that the acquisition time for the whole animal in one angular orientation would exceed one hour, which would be prohibitive for \textit{in vivo} studies. An alternative could be the excitation of the whole animal with a laser sheet instead of points on a grid, implemented for example with cylindrical lenses. Measurements at both excitation and emission wavelengths could provide a quantitative readout (Born ratio), as shown in [38, 141]. This approach could be used for a fast full scan, used to choose the region of interest for the actual FMT measurements following. Moreover, it could be used to follow dynamic procedures with a higher temporal resolution.

### 7.2.3 Improving the FMT reconstruction with MRI prior information

One of the main problems in FMT is that the actual distribution of optical properties in tissue is not known. Currently, tissue inhomogeneity is addressed in the FMT reconstruction by use of the Born ratio [39], where the fluorescence measurements are divided by the excitation measurements. Born normalization has been shown to improve the FMT reconstruction [40]; however, it essentially provides an ”average” estimate for the optical inhomogeneities. On FMT standalone systems, no information on the distribution inside the tissue can be provided.

In this work, we have employed a hybrid FMT/MRI system. A first step in utilizing information from MRI into the FMT reconstruction has been performed, in acquiring the sample surface. Given the high spatial resolution and soft tissue contrast of MRI, the obvious next step is the inclusion of tissue types visible on MRI, as discussed in section 3.3.5. This could be achieved with segmentation of tissue from the MR images and lookup of its optical properties from published tables. The latter step must be approached with caution, as data in these tables generally concern excised tissue, which has always undergone some degree of dehydration. Segmentation from MR images could be performed semi-automatically, if a suitable soft tissue MR contrast is used. In the case where an MRI surface coil is used (as is the case here), prior correction of the MRI intensity values will have to be performed.
to compensate for the non-uniform sensitivity of the coil. After these steps, incorporation of the tissue information in the FMT reconstruction could be done by updating the weight matrix. In this work, a boundary element method has been used for FMT reconstruction. It is possible that the use of finite elements instead could make this incorporation easier.

It must be noted here that interfaces between tissue types of different optical properties will also introduce reflection, as discussed in Appendix A. Therefore, appropriate boundary conditions would have to be defined for every such interface. This procedure would complicate the FMT reconstruction considerably. Whether boundary conditions have to be defined for all interfaces or if a simplified version of them will be sufficient for \textit{in vivo} imaging remains to be seen.
Appendix A

Mathematics of light propagation in tissue

In this chapter, the mathematics behind the forward problem of FMT, first presented in Chapter 2.3.3, are presented in more detail. This appendix treats in specific the diffuse propagation of light in tissue and the resulting wave equation describing its propagation when using a single-wavelength source for tissue excitation.

A.1 The diffusion equation for light propagation in tissue

Light propagation in tissue can be described by the radiative transfer equation (RTE). A detailed study of the RTE is outside the scope of this work; briefly, the RTE is essentially an energy conservation equation. It states that a beam of light loses energy through divergence and extinction (including both absorption and scattering away from the beam) and gains energy from light sources in the medium and scattering directed towards the beam.

In highly scattering media, the RTE can be approximated by a much simpler time-dependent diffusion equation [142] which describes light propagation upon excitation with a source term:

\[
\frac{1}{c} \frac{\partial}{\partial t} U(r, t) - \nabla \cdot (D(r) \cdot \nabla (U(r, t))) + \mu_a \cdot U(r, t) = S_0(r, t) \tag{A.1}
\]
A. Mathematics of light propagation in tissue

where the optical diffusion coefficient is given as

\[ D(r) = \frac{c}{3 \cdot (\mu_a(r) + \mu_s'(r))} \]  \hspace{1cm} (A.2)

and \( \mu_a \) is the medium’s absorption coefficient and \( \mu_s' \) is the reduced scattering coefficient of the medium, which is related to the medium’s scattering coefficient via an anisotropy factor:

\[ \mu_s' = (1 - g) \cdot \mu_s \]  \hspace{1cm} (A.3)

The reduced scattering coefficient is of special importance in the diffusion equation we are examining; the transport mean free path, \( l_{tr} \), is defined as:

\[ l_{tr} = \frac{1}{\mu_s'} \]

i.e. the transport mean free path describes the distance which light travels before the propagation direction is completely randomized due to several scattering events. In other words, the transport mean free path defines the distance where the scattering can be regarded as isotropic [143].

The photon current then, according to Fick’s law, is

\[ J(r, t) = -D(r) \cdot \nabla(U(r, t)) \]  \hspace{1cm} (A.4)

Validity of the diffusion approximation  The diffusion approximation is valid for a variety of optical tomography schemes of tissues. Essentially, it describes light propagation accurately when scattering is much stronger than absorption and it is almost isotropic. Moreover, the scattering events much occur much faster than temporal variations of the total photon flux. Under these premises, solutions of the diffusion equation are excellent approximations to solutions of the RTE [65]. These assumptions can be violated when imaging weakly scattering regions, such as the cerebrospinal fluid in the human head [144], when imaging in the blue region of the spectrum, where tissue fluoresces strongly [145], or when geometries with short source-detector separations are employed [146]. Such situations are not considered in this work.
A.2 From the diffusion equation to the Helmholtz equation – frequency-modulated source and homogeneous medium

In almost all the experimental setups, the light source with amplitude $S_0$ is modulated at a single frequency $\omega$. The case where $\omega = 0$, which corresponds to excitation with a continuous wave (CW) source, is included in this case. The diffusion equation becomes

$$\frac{i \cdot \omega}{c} \cdot U(r, \omega) - \nabla \cdot (D(r) \cdot \nabla(U(r, \omega))) + \mu_a \cdot U(r, \omega) = S_0(r).$$  \hspace{1cm} (A.5)

If we additionally assume a homogeneous medium, where $D(r) = D$, then equation A.5 takes the form

$$\nabla^2(U(r, \omega)) + \kappa^2(U(r, \omega)) = S_0(r),$$  \hspace{1cm} (A.6)

which we recognize as a Helmholtz (wave) equation with wave number

$$\kappa = \sqrt{-c \cdot \mu_a + i \cdot \omega} \hspace{1cm} (D)$$  \hspace{1cm} (A.7)

or, for the CW case,

$$\kappa = i \sqrt{\frac{\mu_a}{D}}$$  \hspace{1cm} (A.8)

The solutions $U(r)$ of the Helmholtz equation are called diffuse photon density waves (DPDW’s) and are strongly damped [147].

The nonhomogeneous Helmholtz equation is solved with the Green’s function method, presented in detail in Appendix B. For simplicity, the equations (B.1) and (B.2) are repeated here. The solution to equation (A.6) for any source $S_0(r)$ is

$$y(r) = \int_a^b G(r - r')S_0(r')dr',$$

where $G(r)$ is

$$G(r - r') = -\frac{1}{4\pi} \frac{e^{ik|r-r'|}}{|r - r'|}.$$  \hspace{1cm} (A.10)
A. Mathematics of light propagation in tissue

A.3 Solution of the diffusion equation for an inhomogeneous medium – the Born approximation [30]

So far, we have solved the diffusion equation for a homogeneous, infinite medium. Here we treat the case where inhomogeneities, i.e. objects with a different refractive index than the rest of the medium, are present.

Let’s assume that the wavenumber is a scalar function of position, i.e.

\[ \kappa(r) = \kappa_0 \cdot [1 + n_\delta(r)] , \]

where \( n_\delta(r) \) are refractive index deviations. The homogeneous wave equation can be rewritten as

\[ (\nabla^2 + \kappa_0^2) u(r) = -\kappa_0^2 [n^2(r) - 1] u(r) = -\mathbf{o}(r) \cdot u(r) \quad (A.11) \]

The right term of the equation (A.11) is called forcing function. We consider the field \( u(r) \) to be the sum of two components, \( u_0(r) \), termed incident field, and \( u_s(r) \), termed scattered field. The incident field is the field that would be present without any inhomogeneities, and it is a solution to the homogeneous Helmholtz equation. The scattered field is the field attributed solely to inhomogeneities. Then, equation (A.11) becomes

\[ (\nabla^2 + \kappa_0^2) u_s(r) = -\mathbf{o}(r) \cdot u(r), \quad (A.12) \]

We can represent the forcing function of the inhomogeneous wave equation as a summation of weighted shifted pulses, i.e.

\[ \mathbf{o}(r) \cdot u(r) = \int \mathbf{o}(r') u(r') \delta(r - r') dr' \]

and therefore the solution to A.12 will be given with help of the Green’s function:

\[ u_s(r) = \int G(r - r') \mathbf{o}(r') u(r') dr' \]

or, by substituting \( u(r) \),
A.4. The non-homogeneous Helmholtz equation for a non-infinite medium

\[
  u_s(r) = \int G(r - r')o(r')u_0(r')dr' + \int G(r - r')o(r')u_s(r')dr'.
\]

By assuming that \( u_s(r') \ll u_0(r') \) (we will define later what this means), then

\[
  u_s(r) \approx \int G(r - r')o(r')u_0(r')dr' = u_b(r')
\]

which is known as the first Born approximation. For the second Born approximation, we would use \( u(r) = u_0(r) + u_b(r') \) so that \( u^{(2)}_b(r') = \int G(r - r')o(r')u_B(r')dr' \). The \( i \)-th approximation would then be

\[
  u^{(i+1)}_b(r') = \int G(r - r')o(r')u^{(i)}_B(r')dr'.
\]

The total field inside the tissue equals to

\[
  u(r) \approx u_0(r) + u_B(r) + u^{(2)}_B(r) + \cdots
\]

Equation (A.14) is known as the Born series [134] and each of its terms corresponds to successively higher orders of scattering of the incident field. If only the first term in the series is retained, this is known as the first Born approximation – typically simply referred to as Born approximation.

**Validity of the first Born approximation** For the Born approximation to be valid, the phase change between the incident wave and the wave inside the object should be less than \( \pi \) [30].

A.4 Solution of the non-homogeneous Helmholtz equation in a non-infinite medium

So far, we have only considered infinite volumes, i.e. we haven’t formulated boundary conditions for the diffusion equation yet. An obvious issue with boundaries is that the diffusion approximation is valid deep in the interior of the medium but not within a boundary region of the order of two or three transport mean free paths [148]. Therefore, we need to choose boundary conditions which yield solutions that are identical to the ones of RTE deep inside the medium interior and vanishing at a certain distance outside the physical boundary.
A. Mathematics of light propagation in tissue

A.4.1 Formulation of boundary conditions for photon diffusion

The transition between two media is not only related to different diffusion constants, but also to a change in the refraction index, causing reflection. The conditions that we impose on the boundary are (a) continuity of flux and (b) change of intensity according to the material discontinuity. For details on the derivation of those boundary conditions, the reader is referred to [148]. The resulting boundary conditions at the interface $S$ between two media $0$ and $1$ with corresponding indices of refraction $n_0$, $n_1$ and diffusion coefficients $D_0$, $D_1$ are [75]:

$$
U_1(r)|_s - \left(\frac{n_1}{n_0}\right)^2 \cdot U_0(r)|_s = CD_0 \frac{\partial U_0(r)}{\partial n}|_s
$$

$$
D_0 \frac{\partial U_0(r)}{\partial n}|_s = D_1 \frac{\partial U_1(r)}{\partial n}|_s
$$

(A.15)

where $C$ is given by

$$
C = \frac{2 - R_{j \rightarrow 0} - R_{j \rightarrow 1}^{0 \rightarrow 1}}{R_{j \rightarrow 0}^{1 \rightarrow 0}}
$$

(A.16)

and $R_{j \rightarrow k}$ represents the power reflectivity on passing from medium $j$ to medium $k$ as described in [148].

The boundary conditions of A.15 can be replaced by simpler, approximate boundary conditions as formulated in [75]:

$$
U_1(r)|_s \approx \left(\frac{n_1}{n_0}\right)^2 \cdot U_0(r)|_s
$$

$$
D_0 \frac{\partial U_0(r)}{\partial n}|_s = D_1 \frac{\partial U_1(r)}{\partial n}|_s
$$

(A.17)

It has been shown in [75] that the error introduced by this approximation is in the order of 3%.
A.4. The non-homogeneous Helmholtz equation for a non-infinite medium

A.4.2 Solution of equation

We assume a point source of frequency $\omega$ at the position $r_s$ in medium 1. Then, for each of the two media 0 and 1, we formulate the Helmholtz equations stated in (A.6) both for $U_0$, $U_1$ and for $G_0$, $G_1$, as the Green’s functions are solutions to the Helmholtz equation. The $U_0$ and $U_1$ must satisfy the boundary conditions of A.17. We multiply the equations and apply the Green’s theorem, which states that the sum of fluid outflows at any point inside the volume is equal to the total outflow summed about an enclosing area. NB that in this way we are moving from the considering the whole volume to considering only the boundary. This approach, which is less expensive computationally, is the core of any boundary element method [41].

In this way, we obtain inside the volume:

$$U_0 = U_{\text{inc}}(r) +$$

$$+ \frac{1}{4\pi} \int_S \left[ U_0(r') \frac{\partial G(\kappa_0 |r - r'|)}{\partial n'} - G(\kappa_0 |r - r'|) \frac{\partial U_0(r')}{\partial n'} \right] dS'$$

(A.18)

where

$$U_{\text{inc}} = \frac{1}{4\pi} \int_V G(\kappa_0 |r - r'|) \frac{S_0(r')}{D_0} d^3r'$$

(A.19)

is the average intensity obtained in the absense of the surface. Equation A.19 is actually a direct implementation of equation A.9 – since we know the Green’s function for the Helmholtz equation in a homogeneous, infinite space, we can calculate the intensity in this space for any source distribution.

Equation A.18 states that, in the presence of boundaries, the intensity inside the volume will be equal to what would be generated in an infinite space and additionally contributions from the surface due to reflections. Solving the integral of A.18 will provide the intensity at any part of the volume. However, this surface integral can be solved analytically only for regular surfaces – this is the reason why the first FMT systems placed the sample inside a cylinder or slab filled with index-matching fluid. The integral of A.18 can be solved numerically for arbitrary geometries.
A. Mathematics of light propagation in tissue

A.5 The Kirchhoff approximation for arbitrary geometries

The Kirchhoff approximation assumes that the surface is replaced at each point by its tangent plane [114], and is therefore a first-order approximation of the surface. The integral in the equation A.18 can thus be replaced by a sum. In terms of the Green’s function, this can be written as

\[
G^{KA}(r_s, r_p) = g(\kappa |r_s - r_p|) * [1 + R_{ND}(r_p)]
\]

(A.20)

where \(g(\kappa |r_s - r_p|)\) is the Green’s function for infinite space, * denotes convolution and \(R_{ND}\) is the reflection coefficient for diffusive waves in real space. The vectors \(r_s, r_p\) correspond to the vectors \(r, r'\) previously. The expression for \(R_{ND}\) can be found in [114]. The equation A.18 can be thus rewritten for the Green’s function as:

\[
G^{KA}(r_s, r_p) = g(\kappa |r_s - r_p|) + \frac{\Delta S}{4\pi} \sum_{p=1}^{N} \left[ C_{nd} D \frac{\partial g(\kappa |r_s - r_p|)}{\partial n_p} + g(\kappa |r_s - r_p|) \right] \frac{\partial G^{KA}(r_s - r_p)}{\partial n_p}.
\]

(A.21)

The error of the Kirchhoff approximation was shown [76] to be less than 5%, whereas the computation time for the solution of A.21 will increase only linearly with the system size. Comparing with calculation using the Green’s function for infinite space, the Kirchhoff approximation was found superior by one order of magnitude.

A.6 Measurements in free space

So far, we have considered the detector points \(r_d\) to be inside the volume. In this section, the calculations for estimating the intensity distribution from measurements outside the volume as shown in [70] will be presented.

Equation A.18 can be written using the net outward flux \(J_n\) and the inward flux due to a source \(J_{src}^-\) by substituting the boundary conditions of A.17 as follows:
A.6. Measurements in free space

\[ U(r) = \frac{1}{4\pi} \int_S \left[ C_{nd} D \left( \frac{\partial g(\kappa |r_s - r_p|)}{\partial n_p} + g(\kappa |r_s - r_p|) \right) \right] \left\{ J_n(r') + J_{src}(r') \right\} dS'; \quad r \in S. \] (A.22)

After the flux at the boundary has been determined, the free-space propagation of the diffuse intensity radiated from surface \( S \) must be modeled. Assuming that each differential \( dS \) area acts as a Lambertian source, and accounting for the effect of refraction index mismatch, the total flux \( J_{det} \) detected at \( r_d \) is

\[ J_{det}(r_d) = \frac{1}{\pi} \int_S J_n(r') \Gamma(r', r_d) dS' \] (A.23)

where the function \( \Gamma(r', r_d) \) depends on the surface–detector angle and the NA of the detectors and accounts for light propagation in free space from the surface to the detector [70, 115, 149]. Obviously, the integral of equation A.23 can be replaced with a sum by using the Kirchhoff approximation as previously discussed.

If we can measure experimentally the light that emerges from all points of the surface \( S \), then we can measure the total distribution of the emerging flux \( J_n \) and substitute it directly into A.22. This is the principle of the boundary removal method presented in [74].

Then, the measured infinite-case average intensity \( U_{inc} \) at each detector position \( r_i \) for a total of \( N \) detectors can be found as

\[
U^{(inc)}_{meas}(r_i) = C_{nd} J_{n_{meas}}(r_i) + \frac{1}{4\pi D} \sum_{j=1}^{N} \left[ C_{nd} D \left( \frac{\partial g(\kappa |r_j - r_i|)}{\partial n_j} + g(\kappa |r_j - r_i|) \right) \right] J_{n_{meas}}(r_j) dA_j; \quad r_i \in S. \] (A.24)

**Accuracy of the boundary removal method [74]** In order for the obtained values for \( U^{(inc)}_{meas} \) to be accurate, the following conditions need to be met: (a) The (average) optical properties of volume \( V \) need to be known a priori, (b) the surface geometry must be known accurately and (c) we must have all values of the surface outward flux measured in order for the series in A.24 to be equivalent to a surface integral.

The authors in [74] examined the effect of reduced number of measurements and reduced
A. Mathematics of light propagation in tissue

angular coverage of the object. They found that the error increased linearly with the detector width and was less than 1%. The angular coverage was more critical; whereas 10% error was found when the detectors were close to the sources, the error reached 30% when measuring on the other side of the object. As one in principle does not know where the sources are a priori, this suggests that full-angular measurements are needed in order to obtain accurate images.

**Excitation of fluorescent sources**

In a fluorescence molecular tomography experiment, sources are introduced at an excitation wavelength $\lambda_{ex}$, giving rise to an excitation field $U_0$. Fluorescence is regarded as a function $h(\tau)$, which governs the absorption of radiation at wavelength $\lambda_{ex}$ and (partial) re-emission as a source at the longer wavelength (lower energy) $\lambda_{em}$. The quantity $h$ is a product of the fluorescent material concentration and its conversion efficiency. As re-emission is a Poisson process, it is characterized by lifetime $\tau$. In the frequency domain $h(\mathbf{r}, \omega)$, can be written as

$$h(\mathbf{r}, \omega) = h_0 \frac{1}{1 + i\omega\tau(\mathbf{r})} = \frac{1 - i\omega\tau(\mathbf{r})}{1 + (\omega\tau(\mathbf{r}))^2} \quad (A.25)$$
Appendix B

Green’s functions

This chapter is based on [150], an excellent book on partial differential equations in Greek.

B.1 Definition and usage of Green’s functions

Non-homogeneous differential equations can be solved with the Green’s function method. Essentially, the Green’s function is an expansion of the method of matrix inversion for problems of partial differential equations. Let us assume the non-homogeneous differential equation

$$Ly = f$$

where $L$ is a second order derivative operator. Solving B.1 is equivalent to inverting the derivative operator $L (y = L^{-1} f)$. Logically, this procedure leads to an integral operator. The solution of the differential equation can thus be written as:
B. Green’s functions

\[ y(x) = \int_a^b G(x, x') f(x') dx' \]

\[ \Rightarrow Ly = L \int_a^b G(x, x') f(x') dx' = \int_a^b LG(x, x') f(x') dx' \]  \hspace{1cm} (B.1)

but as \( Ly = f \),

\[ \int_a^b LG(x, x') f(x') dx' = f. \]

However,

\[ \int_a^b \delta(x' - x) f(x') dx' = f(x), \quad a \leq x \leq b \]

and thus

\[ LG(x, x') = \delta(x - x') \equiv \delta(x' - x) \]  \hspace{1cm} (B.2)

This means that the Green’s function is a solution for the non-homogeneous differential equation of the problem, with a point source in its right part. This result is a consequence of the superposition principle for linear differential equations. Any source term \( f(x) \) can be considered as a continuous superposition of point sources \( \delta(x - x') \), with coefficients \( f(x') \).

To construct the Green’s function for a differential equation, we need to solve the homogeneous equation for \( x \neq x' \) (i.e. treat the space \((a, b)\) in two parts, \((a, x')\) and \((x', b)\)) and then assemble the solution at \( x = x' \). For a differential operator \( L = A(x)D^2 + B(x)D + C(x) \), the Green’s function at \( x = x' \) is continuous but its derivative is not – there is a jump of \( 1/A(0) \). The Green’s function also satisfies all the homogeneous boundary conditions of the problem.

The advantage of solving a differential equation with the Green’s function exists only when the Green’s function can be expressed in a closed form.
B.2 The Green’s function of the operator $\nabla^2 + k^2$ in infinite space. Solution of the non-homogeneous Helmholtz equation

The equation

\[(\nabla^2 + k^2)u(r) = f(r)\]  

appears in problems of wave scattering, where $f(r)$ plays the role of the source and $u(r)$ is the resulting wave. The natural boundary condition to be applied for this equation is that in infinity, i.e. for large $r$, the solution will have the form of a wave which moves away from the source $f(r)$.

The equivalent equation to B.3 for the Green’s function is

\[ (\nabla^2 + k^2)G(r, r') = \delta(r - r'), \]  

which, due to the rotational and translational symmetry of the problem, takes the much simpler form

\[ (\nabla^2 + k^2)G(r) = \delta(r). \]  

The corresponding homogeneous equation to B.4 is

\[ (\nabla^2 + k^2)G(r) = 0, \]  

for which possible solutions are

\[ G(r) = \left( \frac{\sin kr}{r}, \frac{\cos kr}{r} \right) \text{ or } G(r) = \left( \frac{e^{ikr}}{r}, \frac{e^{-ikr}}{r} \right) \]

Out of those solutions, the one representing a wave moving away from the source is

\[ G(r) = A \frac{e^{ikr}}{r} \]

$A$ is found by considering that for $k = 0$, equation B.5 becomes the Poisson equation, and thus shall coincide with the Green’s function of the Laplacian operator, which is $-1/4\pi r$. Therefore, the Green’s function for this problem will be
B. Green’s functions

\[ G(r) = - \frac{1}{4\pi} \frac{e^{ikr}}{r} = - \frac{1}{4\pi} \frac{e^{ik|r|}}{|r|} \]  \hspace{1cm} (B.6)

or more generally

\[ G(r - r') = - \frac{1}{4\pi} \frac{e^{ik|r - r'|}}{|r - r'|}. \]

Thus, the solution of the equation B.3 is

\[ u(r) = - \frac{1}{4\pi} \int \frac{e^{ik|r - r'|}}{|r - r'|} f(r') dV' \]  \hspace{1cm} (B.7)
Appendix C

Developed software

C.1 Computation of sample surface from MRI data and co-registration with the whitelight reference image of the corresponding FMT dataset
C. Developed software

C.1.1 Main script

mr_main.m

%input and visualization

[mrDataSet, mrDataPars] = i_readMR();

%select slices if too many

if (size(mrDataSet,3)>10)
    prompt = {'Lower value:', 'Higher value:'};
    dlg_title = ...
    strcat('There are: ', num2str (size(mrDataSet,3)),' slices. ... Enter the slices to use:');
    num_lines = 1;
    def = {'1', num2str(size(mrDataSet,3))};
    answer = inputdlg(prompt,dlg_title,num_lines,def);
    lowestslicetouse = str2double (answer{1});
    highestslicetouse = str2double (answer{2});
    mrDataPars.lowestslice = lowestslicetouse;
    mrDataPars.highestslice = highestslicetouse;
    mrDataSet = mrDataSet (:,:,lowestslicetouse:highestslicetouse);
end

v_montage(mrDataSet, 'Montage of original MR data');

%denoise if camera artifacts are present on the MR images.

button_denoise = questdlg('Select denoising','Denoising',...
    'yes','no','no');
mrDataPars.denoised = button_denoise;
if (strcmp (button_denoise, 'yes'))
    [mrDataSet_denoised] = d_denoise(mrDataSet);

else
    mrDataSet_denoised = mrDataSet;
end
v_montage(mrDataSet_denoised,'Montage of MR denoised and Wiener filtered');

%flip data

button_flip = questdlg('Select flipping','flipping',...
    'ud','no','no');
mrDataPars.flipped_for_orientation = 'no';
[mrDataSet_denoised] = ...
d_flip(mrDataSet_denoised, mrDataPars, button_flip);

[mrDataSet_flipped_read_orientation] = ...
d_flip_for_read_direction(mrDataSet, mrDataPars);

%smooth data

mrDataSet_s = d_smooth(mrDataSet_flipped_read_orientation);
v_montage(mrDataSet_s, 'Montage of smoothed MR data');

%segmentation and visualization

button_seg = questdlg...
    ('Select segmentation method','Segmentation method',...
        'otsu','alzheimer value', 'value', 'otsu');
[mrDataSet_seg, BW] = d_segment(mrDataSet_s, button_seg, mrDataPars);
v_montage(mrDataSet_seg, 'Montage of segmented MR data');

mrDataPars = v_iso(mrDataSet_seg, mrDataPars);

%compute height map, scale

height_map = d_find_height(mrDataSet_seg, mrDataPars);
C. Developed software

%read optical data

[mFMT, optiWhite, mrDataPars] = i_readOpt(mrDataPars);

%interpolate height map

[height_map_interp, mrDataPars] = d_interp_height...
(height_map, mrDataPars, mFMT);
v_height(height_map_interp, mrDataPars, ’surf’, ’yes’);

%smooth height map

height_map_s = medfilt2(height_map_interp, [3 3]);
v_height(height_map_s, mrDataPars, ’surf’, ’yes’);

%scale – necessary for coregistration

[height_map_scaled] = d_scale(height_map_s);

%Interactive selection of control points.
%Alternatively, the control points can be loaded from a .mat file.

[xyinput_out, xybase_out] = ...
cpselect(height_map_scaled, optiWhite, ’Wait’, true);

%coregister

[xyinput_out, xybase_out, height_registered, mrDataPars] = ...
d_coregister(height_map_scaled, optiWhite, ...
mrDataPars, mFMT, height_map_interp);

%save the mr data parameters

mrDataPars_directory = uigetdir (mrDataPars.mrDataPathName);
mrDataPars_file = strcat (mrDataPars_directory, ‘\mrDataPars.mat’);
save mrDataPars_file mrDataPars;
C.1.2 Important functions

function [mrDataSet, mrDataPars] = i_readMR()

%Reads the MR dataset and its corresponding parameters
%from the method file.
%They include acquisition parameters (Te, Tr, flip angle),
%the dataset dimension (2D, 3D), the spatial resolution,
%slice distance etc.

%NOTE: the MR dimensions are stated in mm,
%whereas the FMT dimensions in cm.
%This is why we multiply everything by 0.1
%when we read the values here.

[mrDataFileName,mrDataPathName,filterIndex] = uigetfile...
    ({{’.hdr’, ’Analyze files (*.hdr)’},’Select the hdr dataset’});

mrDataInfo = analyze75info([mrDataPathName,mrDataFileName]);
mrDataSet = analyze75read(mrDataInfo);

%parameters of interest

mrDataPars.spatial_resolution = zeros (1,2);
mrDataPars.slice_thickness = 0;
mrDataPars.slice_gap = 0;
mrDataPars.slice_distance = 0;
mrDataPars.mrDataPathName = mrDataPathName;

mrDataPars.slice_offset = 0;
mrDataPars.read_offset = 0;
mrDataPars.phase_offset = 0;

%The method file is the corresponding
%parameter file of the fid. Thus:
%fid-method
%2dseq-reco
C. Developed software

[methodFilename, methodPathname, filterIndex] = uigetfile( ... 
    strcat (mrDataPathName,'/*.*'),... 
    'Select the corresponding METHOD file');

fid = fopen([methodPathname,methodFilename]);

while 1
    tline = fgetl(fid);
    if ~ischar(tline), break, end
    %% general parameters
    if (~isempty (findstr(tline, '##OWNER'))) 
        mrDataPars.date = fgetl(fid);
    end
    if (~isempty (findstr(tline, '##$Method='))) 
        equality_index = findstr (tline, '=');
        mrDataPars.method = tline (equality_index+1:end);
    end
    if (~isempty (findstr(tline, '##$PVM_EchoTime='))) 
        equality_index = findstr (tline, '=');
        mrDataPars.TE = str2num(tline (equality_index+1:end));
    end
    if (~isempty (findstr(tline, '##$PVM_RepetitionTime='))) 
        equality_index = findstr (tline, '=');
        mrDataPars.TR = str2num(tline (equality_index+1:end));
    end
    if (~isempty (findstr(tline, '##$PVM_NAverages='))) 
        equality_index = findstr (tline, '=');
        mrDataPars.Navg = str2num(tline (equality_index+1:end));
    end
    if (~isempty (findstr(tline, '##$PVM_ScanTimeStr=')))
        mrDataPars.ScanTime = fgetl(fid);
    end
    if (~isempty (findstr(tline, '##$PVM_SpatDimEnum=')))
        mrDataPars.dimensions = str2num (tline(end-1));
    end
    if (~isempty (findstr(tline, '##$PVM_SPackArrSliceOrient=')))
        mrDataPars.orientation = fgetl(fid);
    end
end
C.1. MR Surface and registration to whitelight image of FMT

end
if (~isempty(findstr(tline, '#$PVM_SpatResol=')))
tline = fgetl(fid);
index_space = findstr(tline, ' ');
if (mrDataPars.dimensions == 2)
    mrDataPars.spatial_resolution = 0.1*...
        [str2num(tline(1:index_space(1)-1)), ...
        str2num(tline(index_space(1)+1:end))];
elseif (mrDataPars.dimensions == 3)
    mrDataPars.spatial_resolution = 0.1*...
        [str2num(tline(1:index_space(1)-1)), ...
        str2num(tline(index_space(1)+1:index_space(2)-1)), ...
        str2num(tline(index_space(2)+1:end))];
end
end
if (~isempty(findstr(tline, '#$PVM_SliceThick')))  
equality_index = findstr(tline, '=');
mrDataPars.slice_thickness = ...
    0.1*(str2num(tline(equality_index+1:end))); 
end
if (~isempty(findstr(tline, '#$PVM_SPackArrSliceGap=')))
mrDataPars.slice_gap = 0.1*(str2num(fgetl(fid)));
end
if (~isempty(findstr(tline, '#$PVM_SPackArrSliceDistance=')))
mrDataPars.slice_distance = 0.1*(str2num(fgetl(fid)));
end
if (~isempty(findstr(tline, '#$PVM_SPackArrReadOrient')))  
tline = fgetl(fid);
mrDataPars.read_orientation = tline();
end
if (~isempty(findstr(tline, '#$PVM_SPackArrReadOffset')))  
tline = fgetl(fid);
mrDataPars.read_offset = str2num(tline);
end
if (~isempty(findstr(tline, '#$PVM_SPackArrPhase1Offset')))  
tline = fgetl(fid);
C. Developed software

    mrDataPars.phase_offset = str2num (tline);
    end
    if (~isempty (findstr(tline, '##$PVM_SPackArrSliceOffset')))
        tline = fgetl(fid);
        mrDataPars.slice_offset = str2num (tline);
    end
end
fclose(fid);

%the 3rd dimension of the matrix is the slice thickness,
, for a 2D dataset.
%This allows us consistent visualization.

if mrDataPars.dimensions == 2
    mrDataPars.spatial_resolution (3) = mrDataPars.slice_distance;
end
end
function [mrDataSet_seg, BW] = ...
d_segment(mrDataSet, method, mrDataPars)

BW = zeros(size(mrDataSet,1),size(mrDataSet,2),size(mrDataSet,3));
BW = logical(BW);
mrDataPars.segmentationMethod = method;

%% scale dataset so that visible for manual segmentation

[mrDataSet_scaled] = d_scale(mrDataSet);

figure;imhist(mrDataSet_scaled(:,:,floor(mean(size(mrDataSet,3)))));
set(gcf, 'Name', 'Histogram of scaled MR image')

% fill out BW

switch method
    case 'manual'
        for i=1:size(mrDataSet_scaled,3)
            BW(:,:,i) = roipoly(mrDataSet_scaled(:,:,i));
        end
    case 'otsu'
        for i=1:size(mrDataSet_scaled,3)
            level = graythresh (mrDataSet_scaled (:,:,i));
            BW(:,:,i) = im2bw(mrDataSet_scaled (:,:,i), level);
        end
    case 'value'
        prompt = {'Enter gray value:'};
        dlg_title = 'Enter thresholding value';
        num_lines = 1;
        def = {'0.5'};
        answer = inputdlg(prompt,dlg_title,num_lines,def);
        answer = str2double (answer{1});
        mrDataPars.manualsegmentationvalue = answer;
end
C. Developed software

for i=1:size(mrDataSet_scaled,3)
    BW(:,:,i) = im2bw(mrDataSet_scaled (:,:,i), answer);
end

case 'alzheimer value'
    for i=1:size(mrDataSet_scaled,3)
        BW(:,:,i) = im2bw(mrDataSet_scaled (:,:,i), 0.08);
    end
end

%% masking of the input dataset

mrDataSet_seg = double(mrDataSet).*BW;
end
C.1. MR Surface and registration to whitelight image of FMT

```matlab
function [mrDataPars] = ...
v_iso(mrDataSet, mrDataPars)

%% make maps

if (~isfield(mrDataPars, 'xMRMap'))
    mrDataPars.xMRMap = [0:size(mrDataSet,1)-1]*...
                        mrDataPars.spatial_resolution(1);
    mrDataPars.yMRMap = [0:size(mrDataSet,2)-1]*...
                        mrDataPars.spatial_resolution(2);
    mrDataPars.zMRMap = [0:size(mrDataSet,3)-1]*...
                        mrDataPars.spatial_resolution(3);

    [mrDataPars.XMRMap, mrDataPars.YMRMap, mrDataPars.ZMRMap] = ...
                meshgrid (mrDataPars.xMRMap, mrDataPars.yMRMap, ... 
                        mrDataPars.zMRMap);
end

button_v_iso = questdlg('Select object type:',...
'Object type','in vivo', 'phantom','phantom');

%% isovalues

if strcmp (button_v_iso, 'in vivo')
    pl_face_color = [1, .75, .65];
    pl_face_alpha = 0.9;
    isovalue = 3000;
    mrDataPars.isovalue = isovalue;

elseif strcmp (button_v_iso, 'phantom')
    pl_face_color = [0.85, 0.85, 0.88];
    pl_face_alpha = 0.9;
    isovalue = 1800;
end

r_value = 0.5;
```
C. Developed software

mrDataPars.r_value = r_value;
figure;
[faces,verts,colors] = isosurface(mrDataPars.yMRMap, ...
mrDataPars.xMRMap, mrDataPars.zMRMap, ...
    mrDataSet, isovalue, mrDataSet)
    pl = patch ('Vertices', verts, 'Faces', faces, ...
        'FaceColor', pl_face_color, ...
        'edgecolor', 'none', 'FaceAlpha', pl_face_alpha);
reducepatch (pl, r_value);
colormap jet;
p2 = patch(isocaps(mrDataPars.yMRMap, ...
mrDataPars.xMRMap, mrDataPars.zMRMap, ...
    mrDataSet, isovalue), 'FaceColor','interp',...
    'EdgeColor','none');
reducepatch (p2, r_value);

view(3);
colormap(gray)
axis equal;
set(p2,'AmbientStrength',.4)
set (pl, 'SpecularColorReflectance', 1, 'SpecularExponent', 50);
set(gcf, 'Name', 'Isosurfaces')
function [height_map] = ...
    d_find_height(mrDataSet_seg, mrDataPars)

% we assume a right-hand system inside the MR scanner where:
% z is feet-to-head
% y is left-to-right and
% x is down-to-up.

% AXIAL
% We assign the indices i,j,k to x,y,z respectively.
% To compute the sample % height, we need to find
% the uppermost x for a column given by each (j,k).
% However: in matlab, the axis origin is in
% the upper left corner and not % the lower left one.
% The height map will be a 2D matrix with dimensions (y,z),
% and values the height of each point.
% This is because we have eliminated x.
% For example, if the mrDataSet is axial (133,200,30),
% its height map will be(200,30).
% The reason why the end result does not seem so extremely
% sheared is that in an axial 2D measurement, the z resolution
% is much coarser than the x,y ones.

if strcmp (mrDataPars.orientation, 'axial')
    height_map = zeros(size(mrDataSet_seg,3), size(mrDataSet_seg,2));
    for k=1:size(mrDataSet_seg,3)
        for j=1:size(mrDataSet_seg,2)
            index = find(mrDataSet_seg(:,j,k),1,'first');
            if isempty(index)
                height_map(k,j)= 0;
            else
                height_map(k,j)= abs(index - size(mrDataSet_seg,2));
            end
        end
    end
    height_map = height_map*mrDataPars.spatial_resolution(1);
C. Developed software

```matlab
height_map = fliplr(height_map);

%CORONAL
% here, the xyz are different.
% x and y are the dimensions of each coronal image
% z is the height.
% Therefore, we need a xy projection and to vary the z.

elseif strcmp(mrDataPars.orientation, 'coronal')
    height_map = zeros(size(mrDataSet_seg,1), size(mrDataSet_seg,2));
    for i=1:size(mrDataSet_seg,1)
        for j=1:size(mrDataSet_seg,2)
            index = find(mrDataSet_seg(i,j,:),1,'last');
            if isempty(index)
                height_map(i,j) = 0;
            else
                height_map(i,j) = index;
            end
        end
    end
    height_map = height_map * mrDataPars.spatial_resolution(3);
    height_map = rot90(height_map,2);
end
end
```
function [height_map_interp, mrDataPars] = ...
  d_interp_height(height_map, mrDataPars, mFMT)

%AXIAL (zy)
%get the same resolution as dx and dy from the mFMT structure.
%Will make coregistration and visualization easier.

%z in MR is the rows, thus their distance equals to dy from FMT.  
%y in MR are the columns, thus their distance equals to dx from FMT
%those could also be the other way round, although here dx = dy

if strcmp (mrDataPars.orientation, 'axial')
  mrDataPars.zMRMap_FMT = min(min(mrDataPars.zMRMap)):...
    max(max(mrDataPars.zMRMap));

  mrDataPars.yMRMap_FMT = min(min(mrDataPars.yMRMap)):...
    max(max(mrDataPars.yMRMap));

  [X,Y] = meshgrid (mrDataPars.yMRMap, mrDataPars.zMRMap);
  [XI,YI] = meshgrid (mrDataPars.yMRMap_FMT,mrDataPars.zMRMap_FMT);

  height_map_for_interp = NaN(size(height_map));
  height_map_for_interp(height_map>0) = height_map(height_map>0);
  height_map_interp = interp2(X,Y,height_map_for_interp,XI,YI);

%CORONAL (xy)

elseif strcmp (mrDataPars.orientation, 'coronal')
  mrDataPars.xMRMap_FMT = min(min(mrDataPars.xMRMap)):...
    max(max(mrDataPars.xMRMap));

  mrDataPars.yMRMap_FMT = min(min(mrDataPars.yMRMap)):...
    max(max(mrDataPars.yMRMap));
C. Developed software

```
[X,Y] = meshgrid (mrDataPars.yMRMap, mrDataPars.xMRMap);
[XI,YI] = meshgrid (mrDataPars.yMRMap_FMT,mrDataPars.xMRMap_FMT);

height_map_for_interp = NaN(size(height_map));
height_map_for_interp(height_map>0) = height_map(height_map>0);

height_map_interp = interp2(X,Y,height_map_for_interp,XI,YI);
end
```
C.1. MR Surface and registration to whitelight image of FMT

function [height_filled_numbers, mrDataPars, t_opti_mr] = ...
    d_coregister(xyinput_out, xybase_out, transform_type, ...
    height_map_scaled, optiWhite, mrDataPars, mFMT)

    % computation of transform. Types can be ‘similarity’ or ‘affine’.
    
    t_opti_mr = cp2tform(xyinput_out, xybase_out, transform_type);
    mrDataPars.transformmatrix = t_opti_mr;

    % scaling for the different dx and dy from the camera.
    
    scaling_factor = mrDataPars.dxFMT/mrDataPars.dyFMT;

    [xyinput_out, xybase_out] = ...
        cpselect(height_map_scaled, optiWhite, ‘Wait’, true);

    % registration.
    
    height_filled_numbers = imtransform...
        (height_map_scaled, t_opti_mr, ‘bicubic’, ‘XYScale’, ... 
        [scaling_factor 1],’FillValues’, min(min(height_map_scaled))), ... 
        ’XData’,[1 size(optiWhite,1)], ’YData’,[1 size(optiWhite,2)], ... 
        ’Size’, size(optiWhite));
function [] = v_coregistered_height...
    (mrDataPars, height_filled_numbers)

% visualization

figure ('Name', '2D view of coregistered height');
imagesc(mrDataPars.xFMTMap, mrDataPars....
yFMTMap, height_filled_numbers); axis equal;

figure('Name', '3D view of coregistered height');
surf(mrDataPars.xFMTMap, mrDataPars.yFMTMap, ...
flipud(height_filled_numbers), 'EdgeColor', 'interp'); axis equal
C.2 Use of coregistered surface in FMT reconstruction

The original FMT reconstruction code, written by Prof. Jorge Ripoll, has been modified to incorporate the registered sample surface in the reconstruction. Only the corresponding modification is presented here, along with the original version, which uses the slab approximation, for comparison.

```matlab
function [mFMT,dUbnd_fluo,dUbnd_intr,Ustd_fluo,Ustd_intr] = ...
    mfmt_remove_boundary(mFMT)

... %ORIGINAL CODE.
%Developed and kindly provided by Prof. Jorge Ripoll.
%Slab approximation, to be used if no geometry file is loaded.

if ~isfield(mFMT,'geom_file') || isempty(mFMT.geom_file)
    disp('mini-FMT :: No geom_file found. Assuming Flat interface');

    mFMT.Rsrf        = reshape(Xdet,1,Num)';
    mFMT.Rsrf(:,2)   = reshape(Ydet,1,Num);
    mFMT.Rsrf(:,3)   = mFMT.L;
    mFMT.dS          = ones(Num,1)*(Navg*mFMT.dx)*(Navg*mFMT.dy);
    mFMT.Nsrf(:,3)   = 1; % pointing outward
    % --
    mFMT.Rsrf        = mFMT.Rsrf(mindx,:);
    mFMT.Nsrf        = mFMT.Nsrf(mindx,:);
    mFMT.dS          = mFMT.dS(mindx);

%MODIFIED CODE.
%Developed in this work.
%Use of sample surface, to be used if a geometry file is loaded.
```
C. Developed software

else
    disp(‘Using input geometry file’);
    load (mFMT.geom_file);
    mFMT.Rsrf = reshape(Xdet,1,Num’);
    mFMT.Rsrf(:,2) = reshape(Ydet,1,Num);

%Interpolation of surface
to the number of elements used for the reconstruction.

    test = mex_detectors(height_filled_numbers,xd,yd,Navg);
    mFMT.Rsrf(:,3) = reshape(test,1,Num);
    mFMT.dS = ones(Num,1)*(Navg*mFMT.dx)*(Navg*mFMT.dy);

    mFMT.Nsrf = zeros(size(mFMT.Rsrf));

%Generation of surface normal vectors,
pointing out from each surface patch.

    [Nx,Ny,Nz] = surfnorm(height_filled_numbers);

    test = mex_detectors(Nx,xd,yd,Navg);%Nx
    mFMT.Nsrf(:,1) = reshape(test,1,Num);
    test = mex_detectors(Ny,xd,yd,Navg);%Ny
    mFMT.Nsrf(:,2) = reshape(test,1,Num);
    test = mex_detectors(Nz,xd,yd,Navg);%Nz
    mFMT.Nsrf(:,3) = reshape(test,1,Num);
    % --
    mFMT.Rsrf = mFMT.Rsrf(mindx,:);
    mFMT.Nsrf = mFMT.Nsrf(mindx,:);
    mFMT.dS = mFMT.dS(mindx);
end;

...
C.3 Simultaneous visualization of coregistered FMT and MRI datasets

Simultaneous display of the FMT reconstruction results and the coregistered MRI dataset is performed with software. The code written here has been based on the default visualization functions of the FMT reconstruction results, written by Prof. Jorge Ripoll.

%Load the reconstructed FMT data, where the surface has been used.

sel = {'*.mat','matfiles (*.mat)'};
[file,folder] = uigetfile(sel, 'mini-fmt :: SELECT RECONSTRUCTED DATA');
matfile = fullfile(folder,file);
load(matfile);

if isfield(matfile,'Xrec'),
    mFMT = matfile;
end

%reshaping of Xrec data in 3D

nx = mFMT.xmesh(3);
ny = mFMT.ymesh(3);
nz = mFMT.zmesh(3);
data = reshape(mFMT.Xrec, nx, ny, nz);

%original reconstruction mesh.

[X, Y, Z] = ndgrid(linspace((mFMT.xmesh(1)),(mFMT.xmesh(2)),nx), ...
    linspace((mFMT.ymesh(1)),(mFMT.ymesh(2)),ny), ...) ...
    linspace((mFMT.zmesh(1)),(mFMT.zmesh(2)),nz));

%higher density mesh to be used for visualization.
C. Developed software

variable_for_interp = 3;
[XI, YI, ZI] = ndgrid(linspace((mFMT.xmesh(1)),...
(mFMT.xmesh(2)),variable_for_interp*nx), ... 
    linspace((mFMT.ymesh(1)),(mFMT.ymesh(2)),variable_for_interp*ny), ... 
    linspace((mFMT.zmesh(1)),(mFMT.zmesh(2)),variable_for_interp*nz));

xdet_interpolated = linspace((mFMT.xdet(1)),...
(mFMT.xdet(2)),variable_for_interp*nx);
ydet_interpolated = linspace((mFMT.ydet(1)),...
(mFMT.ydet(2)),variable_for_interp*ny);
zdet_interpolated = linspace((mFMT.mesh_z(1)),...
(mFMT.mesh_z(end)),variable_for_interp*nz);

%preparation for interpolation

P = [2 1 3];
X = permute(X, P);
Y = permute(Y, P);
Z = permute(Z, P);
XI = permute(XI, P);
YI = permute(YI, P);
ZI = permute(ZI, P);
data = permute(data, P);

%interpolation of the FMT reconstructed data.

interpolated_xrec = interp3(X,Y,Z, data,XI,YI, ZI);

%loading of default visualization options.

options = fmt_render_opt();

%3D visualization of FMT reconstruction.
jump = 1;
figure('Name', '3D');
for z = 1:jump:size(interpolated_xrec,3)
    set (surf(XI(:,:,z), YI(:,:,z), ZI(:,:,z), ...
    interpolated_xrec(:,:,z), 'Tag', 'fmt_rec'),...
    options.volume);
    hold on
end
for y = 1:jump:size(interpolated_xrec,2)
    set(surf(squeeze(XI(:,y,:)), squeeze(YI(:,y,:)),...
    squeeze(ZI(:,y,:)), squeeze(interpolated_xrec(:,y,:)),...
    'Tag', 'fmt_rec'), ... 
    options.volume);
    hold on
end
for x = 1:jump:size(interpolated_xrec,1)
    set(surf(squeeze(XI(x,:,:)), squeeze(YI(x,:,:)),...
    squeeze(ZI(x,:,:)), squeeze(interpolated_xrec(x,:,:)),...
    'Tag', 'fmt_rec'), ... 
    options.volume);
    hold on
end
alpha(findobj(gca,'Tag','fmt_rec'),'color');
set(findobj(gca,'Tag','fmt_rec'),'FaceAlpha','interp');

%summation - "coronal" view.

test = squeeze(sum(data,3));

optiWhite = (mFMT.Uflat);

[X2d,Y2d] = meshgrid ...
(linspace((mFMT.xmesh(1)),(mFMT.xmesh(2)),nx), ... 
    linspace((mFMT.ymesh(1)),(mFMT.ymesh(2)),ny));

[XI2d,YI2d] = meshgrid ...
C. Developed software

(linspace((mFMT.xmesh(1)),(mFMT.xmesh(2)),size(optiWhite,1)), ...  
linspace((mFMT.ymesh(1)),(mFMT.ymesh(2)),size(optiWhite,2)));

ZI2d = interp2(X2d,Y2d,test,XI2d,YI2d);
B = flipud(rot90 (ZI2d, 1));
figure(’Name’, ’Rotated fmt recon view’);
imagesc(B);axis equal; colormap gray;
figure;imagesc(rot90(optiWhite));axis equal; colormap gray;

%read the axial MRI dataset that has been used
to generate the sample height and
coregister with the FMT whitelight image.

[mrDataSet, mrDataPars] = i_readMR();

%make the ’coronal’ view of the axial dataset.

mrDataSet_mean = squeeze(sum(mrDataSet,1));

[X2d,Y2d] = meshgrid (linspace((mFMT.xmesh(1)),(mFMT.xmesh(2)),nx), ...  
linspace((mFMT.ymesh(1)),(mFMT.ymesh(2)),ny));

[XI2d,YI2d] = meshgrid ...
(linspace((mFMT.xmesh(1)),(mFMT.xmesh(2)),size(optiWhite,1)), ...  
linspace((mFMT.ymesh(1)),(mFMT.ymesh(2)),size(optiWhite,2)));

ZI2d = interp2(X2d,Y2d,test,XI2d,YI2d);
B = flipud(rot90 (ZI2d, 1));
figure(’Name’, ’Rotated fmt recon view’);
imagesc(B);axis equal; colormap gray;%ok
figure;imagesc(rot90(optiWhite));axis equal; colormap gray;

%interpolate to a denser grid.

[XMR_original, ZMR_original] = meshgrid...
C.3. Display of coregistered FMT and MRI datasets

\[
\text{XMR, ZMR} = \text{meshgrid}(\text{linspace(1, 256,256), linspace(1, 20,20)});
\]
\[
\text{XMR}_\text{original}, \text{ZMR}_\text{original} = \text{meshgrid}(\text{linspace(1, 256,256), linspace(1, 20,100)});
\]
\[
\text{ZI} = \text{interp2}(\text{XMR}_\text{original}, \text{ZMR}_\text{original},...\text{mrDataSet\_mean'}, \text{XMR}, \text{ZMR});
\]

% visualize the result

\[
\text{figure; imagesc(mrDataPars.spatial\_resolution(1)*... linspace(1, 256,256),... mrDataPars.spatial\_resolution(3)* ZMR(:,1),ZI);}
\]
\[
\text{colormap gray; axis equal;}
\]

% rotate the coronal view like the height map, so that we can apply the same transform.

\[
\text{coronal\_rotated} = \text{flipud(d\_rotate\_heightmap(ZI, ... mrDataPars, 'rot90\_clockwise'))};
\]
\[
\text{figure('Name', 'Rotated coronal view');}
\]
\[
\text{imagesc(coronal\_rotated); colormap gray;}
\]

% load the transform directory.

\[
\text{sel = {'*\_mat','matfiles (*.mat)'}};
\]
\[
[\text{tfile, tfolder}] = \text{uigetfile(sel, 'select transform directory');}
\]
\[
\text{transform = fullfile(tfolder,tfile);}
\]
\[
\text{load(transform);}
\]
\[
\text{initial\_directory = 'D:\experiments.data\... ALZ\_STUDY\analysis\coregistrations\946';}
\]
\[
\text{load (strcat(initial\_directory, '\optiWhite.mat'))};
\]

% scaling for the different dx and dy from the camera.
C. Developed software

```
scaling_factor = mFMT.dx/mFMT.dy;
scaling_factor = mrDataPars.spatial_resolution(3)/mrDataPars.spatial_resolution(2)

%registration.

coronal_coregistered = imtransform(coronal_rotated, t_opti_mr,...
    'bicubic', 'XYScale', [1 1], 'FillValues', NaN,...
    'XData',[1 size(optiWhite,1)], 'YData',[1 size(optiWhite,2)],...
    'Size', size(optiWhite));

figure ('Name', '2D view of coregistered coronal');
imagesc(mFMT.mesh_x, mFMT.mesh_y,coronal_coregistered);axis equal;
imagesc(coronal_coregistered);axis equal;

%# show overlayed images

figure, imshow(coronal_coregistered,[]), hold on
hImg = imshow(B,[]); set(hImg, 'AlphaData', 0.1);
min(get(hImg, 'CData'))

%# also we can specify a colormap

colormap hsv
```
Appendix D

Preliminary studies for the tumor vascularity study

D.1 Study design

In order to design the study protocol, we compared reported methods to study tumor vascularity with FMT and MRI (Figure D.1).

For MRI, we chose the combination of Endorem and Dotarem to assess tumor blood volume and tumor leakage space due to the availability of these contrast agents and the existence of established tools for kinetic modeling. For FMT, we decided against ICG (which would be the closest equivalent to Dotarem), due to the low temporal resolution of the FMT component. Instead, we concentrated on two commercially available NIRF dyes, AngioSense®680EX and AngioSpark®680, which have been used to measure tumor vascularity. Due to the pegylation of both those molecules, their pharmacokinetics are much slower than MR agents; it has been reported that they remain in the vasculature for the first 4 hours post injection and they leak out to the interstitial space 24 hours post injection. Therefore, each agent could be used as an equivalent to both the Endorem and the Dotarem readout, if it was measured at different time points post injection.
D. Preliminary tumor vascularity studies

<table>
<thead>
<tr>
<th>Low molecular weight agents</th>
<th>Large molecular agents (blood pool agents)</th>
<th>Agents accumulating at sites of angiogenesis</th>
<th>No agents – TOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-DOTA: T1+, –</td>
<td>Vasovist: T1+, –</td>
<td>RGD-USPIOs: alpha-beta integrins</td>
<td>Not suitable for tumor: small vessels and slow flow</td>
</tr>
<tr>
<td>First pass, then very fast to interstitial space (no membrane penetration)</td>
<td><em>Binds reversibly to albumin</em></td>
<td><em>Sensitivity? Competition with IntegriSense?</em></td>
<td></td>
</tr>
<tr>
<td>(half-life: 90’ in humans)</td>
<td><em>Pre-post imaging</em></td>
<td><em>Pre-post imaging, 6h in between</em></td>
<td></td>
</tr>
<tr>
<td>DCE-MRI</td>
<td>Half-life 22’ in rat</td>
<td>Maximum loss @ 1h, 30’T=6h possible</td>
<td></td>
</tr>
<tr>
<td>Bolus injection</td>
<td>Bolus or infusion for tc80”</td>
<td>Infusion, bolus possible</td>
<td></td>
</tr>
<tr>
<td>MW</td>
<td>Endorem (SPIOs): T2/T2*+, –</td>
<td>SPI/0o-120, ~100nm diameter</td>
<td></td>
</tr>
</tbody>
</table>

**Figure D.1:** Comparison of MRI (top row) and optical (bottom row) readouts for tumor vascularity measurements.

D.2 Choice of NIRF agent

Preliminary experiments were performed to choose between AngioSense®680EX and AngioSpark®680 for this study.

D.2.1 Materials and methods

**Animals**

Female nude Balb/C mice (Janvier, France) of 8-10 weeks of age were used for all experiments. For determination of the optimal NIRF probe, \( n = 10 \) animals were injected subcutaneously with \( 10^6 \) C51 colon carcinoma cells on the left thigh. On day 8 post tumor inoculation, either AngioSense®680EX (\( n = 5 \)) or AngioSpark®680 (\( n = 5 \)) was administered intravenously at the recommended dosage. All animals were measured 2hs and 24hs post injection. For determination of *in vivo* NIRF timepoints, \( n = 5 \) animals were injected with \( 10^6 \) C51 colon carcinoma cells and \( n = 5 \) animals were injected subcutaneously with
10^4 4T1 breast carcinoma cells on the left thigh. All animals were measured 90 ± 30 min and 24 ± 1 h post injection.

**In vivo FRI experiments**

All animals were measured using a commercial FRI system (Maestro 500, previously Cambridge Research, Woburn, MA, USA, now PerkinElmer, USA). A band pass filter from 615 nm to 665 nm and a high-pass filter over 700 nm were used for excitation and emission light, respectively. A series of images were acquired at different wavelengths and then subjected to spectral unmixing as described by Gao et al [34] for unmixing the AF 680 fluorescence pattern from tissue autofluorescence.

**D.2.2 Results**

The results of the FRI studies are shown in Figure D.2 for four representative animals. Both tested agents showed a signal increase 24 h post injection compared to 2 h post injection. This is consistent with the leakage of the agents into the interstitial space over time. The detected signal with AngioSpark®680 2 h post injection was higher than the AngioSense®680EX signal on the same timepoint – however, the biodistribution of AngioSpark®680 24 h post injection was much more spread. This is unfavorable, as it is a source of background which can affect the quantification of the specific tumor signal. Therefore, we decided to employ AngioSense®680EX in this study.
D. Preliminary tumor vascularity studies

Figure D.2: FRI measurements 2hs (top row) and 24hs (bottom row) post AngioSense®680EX and AngioSpark®680 injection for four representative animals. The AngioSpark®680 signal was higher than AngioSense®680EX 2hs p.i. The AngioSpark®680 biodistribution 24hs p.i. was much more diffuse than the biodistribution of AngioSense®680EX at the same timepoint.
D.3 Choice of *in vivo* MRI protocol

In section D.1, we hypothesize that both Endorem and AngioSense®680EX 2hs p.i. can be used to assess vasculature whereas Dotarem and AngioSense®680EX 2hs p.i. can be used to assess EES. Therefore, the first two measurements should be performed concurrently on the first experimental day and the second two on the second experimental day. However, it is known that Endorem is uptaken by macrophages, where it can be retained up to three days. The magnetite nanoparticles that constitute Endorem cause local field inhomogeneities which can interfere with $T_1$ MRI measurements upon Dotarem injection on the next day.

The degree of Endorem uptake depends on the presence of macrophages, which varies according to tumor type and timepoint. To assess this, we performed microscopy analysis of immunofluorescently labeled tumor sections. Representative results, shown in Figure D.3, confirmed that macrophages were indeed present on both tumor types. Therefore, we decided to perform both Dotarem and Endorem measurements on the same day, with the Dotarem measurements preceding. In this, we assumed that the changes occurring in tumor perfusion within one day were insignificant.

![Figure D.3: Confocal microscopy of immunofluorescently labeled tumor sections. (a) DAPI staining for DNA, (b) CD31 staining for endothelial cells, (c) f4/80 staining for mouse macrophages, (d) overlay of all three channels.](image)
D. Preliminary tumor vascularity studies

D.4 Choice of *ex vivo* validation approach

One advantage of using optical *in vivo* imaging methods is the easy *ex vivo* validation of results, using microscopy. We assessed if we could detect AngioSense®680EX on tumor slices of animals sacrificed 24hs post AngioSense®680EX injection without any additional staining targeting the vasculature. We found that the AngioSense®680EX signal on unfixed slices was very weak for both tumor types. Upon PFA fixation, the free AngioSense®680EX signal was washed out, as AngioSense®680EX does not contain fixable groups. This excluded the use of AngioSense®680EX for *ex vivo* validation.

This *ex vivo* analysis had a surprising additional finding; contrary to what is currently known about AngioSense®680EX, we found that 24hs p.i. it was bound by macrophages. This was seen on both unfixed and fixed slices. It is not known at what point this uptake occurs.

![Confocal microscopy](image)

**Figure D.4:** Confocal microscopy of tumor slices acquired 24hs post AngioSense®680EX injection, acquired with 20× magnification. DAPI staining for DNA is shown in blue, AngioSense®680EX signal is shown in red. (a) Unfixed slice of a C51 tumor, (b) Unfixed slice of a 4T1 tumor, (c) PFA-fixed slice of the same 4T1 tumor shown in (b). AngioSense®680EX 24hs post injection is contained in the vessels and in macrophages (red dots between cell nuclei). As AngioSense®680EX does not contain fixable groups, it is washed out from the vessels post PFA fixation, but not from the macrophages. Images courtesy of Dr. Steffi Lehmann.
Bibliography


The years spent doing my PhD thesis have been a very important time for me – always challenging, mostly exciting, very often fun and occasionally frustrating, too. The people mentioned here have been indispensable to me throughout it.

My supervisor, Markus Rudin gave me the opportunity to work in this group and field, and showed me great trust. I felt deeply appreciated and supported as a scientist and as a person, and learned a great deal; from MR physics and biology to how research scientists think, and from being realistic to keeping a cool head. Even when I was overwhelmed, coming out of his office found me calmer. The importance that he placed on scientific exchange and his generosity gave me the opportunity to attend many conferences all over the world during these years, from which I greatly profited.

I wish to thank the professors Vasilis Ntziachristos and Thomas Roesgen for agreeing to be members of my PhD committee. I appreciated their input and suggestions and the fresh view they brought to this work.

Dr. Florian Stuker was a great coworker in the optical lab during the first three years of my thesis. Our complementary skills and the lively, loud, free and collegial spirit of our collaboration made work a lot of fun.

Markus Kuepfer at the workshop provided excellent mechanical assistance, pivotal to this work. His talent and desire to share his knowledge have given me the delight of understanding how mechanical things work.

Dr. Divya Vats was the first biologist I worked extensively with during my thesis. Her motivation, her positive spirit and her gentleness made work with her a sheer pleasure.

Dr. Jan Klohs has been an invaluable colleague; his critical thinking, planning, writing and personal skills belong to a true scientist and have contributed to my thesis greatly. His personal views and interests have made for stimulating conversations inside and outside the
Acknowledgments

Catherine Germanier, Giorgos Batsios, Aline Seuwen and Dr. Aileen Schroeter have been great colleagues and friends. At critical times of this project, they jumped onto the boat with me unhesitatingly. For everything, I thank them.

Dr. Steffi Lehmann has been an inspirational colleague; her optimism, hard work and passion for finding biological questions worth answering engaged me and helped me see the big picture of this project.

Manoj Desai taught me microscopy in his characteristic positive, fun and relaxed way. My coworker at the optical lab, Andreas Elmer assisted in hardware maintenance and optical experiments. I wish to thank him for his hard work. Ruth Keist has been an indispensable biology partner at the Irchel part of the group. I greatly appreciated her accuracy and reliability. Diana Kindler taught me how to cannulate mice. Dr.med.vet Gisela Kuhn ensured the well-being of all animals in our facility, from which I profited greatly for my experiments. Mark Migueis performed very good work as a semester student, assisting in preliminary experiments for the tumor study presented here. Supervising him was a rewarding experience. Dr. Christof Baltes and Dr. David Ratering taught me a lot about MR, along with Dr. Thomas Mueggler. I deeply appreciate their professionalism and sense of responsibility. Assistant Professor Jorge Ripoll kindly provided the original FMT acquisition and reconstruction software, parts of which were modified in this work. Especially in the beginning of my PhD, I benefited greatly from his input and explanations. David Beyeler from CSEM in Zurich, as well as Matt Fishburn and Yuki Maruyama from the Aqua Group in EPFL, have provided us with novel, MR-compatible cameras. I wish to thank them for their innovative work, but also for their immediate responses whenever problems occurred. Through our collaboration with Scanco, Dr. Kai Hassler and Martina Bucher gave me the opportunity to see a hybrid system – FMT/XCT – outside the university world.

This project was financially supported by the European FP7 project FMT/XCT (grant agreement 201792).

My first office mates at the legendary G305, Felicitas Princz-Kranz, Florence Razoux and Rudolf Fischer, have been much more than work colleagues. Luckily, their humor, cultural interests and clarity of thought accompanied me also after they left the group, in our regular get-togethers.

My housemates in our beautiful soon-to-be-demolished house in Zurich Sofia, Claudio and Bims, but also Django, Noemi and Bruno, have been the best I could have wished for, and
more. This has been a true home and the closest to a family away from a family.

Some of the great friends I made in Zurich (in order of appearance: Giorgos, Maria, Emily, Andrea, Chie, Cristina, Johanna, Marta, Lucy, Tim, Martina, Ric, Steffi, Graeme, Axel) are now scattered in all continents, and some are still here. The chats, the gigs, the hikes, the swims, the books, the food, the drinks and the teas have been amazing.

Without my family, I wouldn’t be where I am now. Athanasia, Giorgos, Dionisis, Christina, thanks for all your love and support, which you showed in numerous ways at all times.

My maternal grandparents, Giorgos and Vassiliki, have surrounded all of us grandchildren with their unconditional love (and food) during our childhood. A long time ago, they came to Athens from their small villages to find work; the importance that they placed in studying and being independent has influenced us all. This thesis is dedicated to their memory.