HIGH-RESOLUTION AND HIGH SENSITIVITY MESOSCOPIC FLUORESCENCE TOMOGRAPHY BASED ON DE-SCANNING EMCCD: SYSTEM DESIGN AND THICK TISSUE IMAGING APPLICATIONS

By

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ABSTRACT

Optical microscopy has been one of the essential tools for biological studies for decades, however its application areas are limited to superficial investigation due to strong scattering in live tissues. Even though advanced techniques such as confocal or multiphoton methods have been recently developed to penetrate beyond a few hundreds of microns deep in tissues, they still cannot perform in the mesoscopic regime (millimeter scale) without using destructive sample preparation protocols such as clearing techniques. They provide rich cellular information; however, they cannot be readily employed to investigate the biological processes at larger scales. Herein, we will present our effort to establish a novel imaging approach that can quantify molecular expression in intact tissues, well beyond the current microscopy depth limits.

Mesoscopic Fluorescence Molecular Tomography (MFMT) is an emerging imaging modality that offers unique potential for the non-invasive molecular assessment of thick in-vitro and in-vivo live tissues. This novel imaging modality is based on an optical inverse problem that allows for retrieval of the quantitative spatial distribution of fluorescent tagged bio-markers at millimeter depth. MFMT is well-suited for in-vivo subsurface tissue imaging and thick bio-printed specimens due to its high-sensitivity and fast acquisition times, as well as relatively large fields of view.

Herein, we will first demonstrate the potential of this technique using our first generation MFMT system applied to multiplexed reporter gene imaging (in-vitro) and determination of Photodynamic Therapy (PDT) agent bio-distribution in a mouse model (in-vivo). Second, we will present the design rationale, in silico benchmarking, and experimental validation of a second generation MFMT (2GMFMT) system. We will demonstrate the gain in resolution and sensitivity achieved due to the de-scanned dense detector configuration implemented. The potential of this novel platform will be showcased by applying it to the longitudinal assessment of Ink-Jet Bio-Printed tumor models. This preliminary investigation focuses on monitoring four patient-derived glioblastoma multiforme (GBM) spheroids within their
bioreactor for up to 70 days and following their volume change prior to and after exposure to a cytotoxic drug.

Overall, our studies indicate that 2GMFMT is a powerful technique for *in-vitro* and *in-vivo* thick tissue molecular imaging applications due to its high resolution, fast tomographic imaging capability, and high sensitivity.
Chapter 1
Introduction

Regenerative medicine is an emerging research field that aims at building functional tissues for repair or replacement of *in-vivo* damaged tissues and/or organs [1–3]. One major challenge in regenerative medicine is spatial and temporal assessment of functional and molecular cellular states throughout a biodegradable scaffold. The traditional approach to quantify 3D cell distribution in thick scaffolds involves Laser Scanning Confocal Microscopy (LSCM) and cryo-sectioning of samples, after which data from the two modalities is compiled to form a 3D image of the whole tissue [4]. This is a robust method, but it is destructive, time-consuming, and registration can be challenging. Therefore it is not appropriate for longitudinal inspection of a large set of samples and/or for assessment of tissue maturation prior to implantation. Thus, there is a critical need for the development of methods that can image and analyze the structure and function of engineered tissue in a non-destructive manner with high resolution.

In addition, constructing a 3D tissue and maintaining its vitality often requires preservation of a tissue construct in a bio-reactor, which places considerable challenges on tissue characterization methodologies such as imaging techniques. Characterization of the engineered tissue may be performed at the morphological or molecular level, where the former delivers structural information and the latter helps to extract functional information. Delicacy of live cells and the extracellular matrix requires the imaging technique to be non-contact and in reflectance configuration for minimal interference and ease of operation. Moreover, as the microenvironment is typically precisely controlled and should not be perturbed, the imaging technique should be able to directly capture information while the tissue is embedded in a

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sealed bio-chamber.

Optical imaging enables the non-destructive quantification of both scaffold architecture and cell distribution at high resolution. Tissue engineering scaffolds are typically constructed from laden hydrogels, electronspun fibers, porous scaffolds, or 3D printed scaffolds [5-8]. These materials can exhibit significant scattering such that light penetration into the samples is limited. Available optical modalities include conventional microscopy, non-linear optical techniques, and tomographic techniques [9]. The most popular technique, LSCM, can visualize cells and molecules via a wide variety of fluorescent probes at sub-cellular resolution. However, LSCM has a limited imaging depth of 100-200 $\mu m$. For deeper investigation of engineered tissues, Two-Photon Microscopy (TPM) is preferred [10], but the depth penetration is typically still limited to 500 $\mu m$ [11–13]. Note that by leveraging the lower scattering in longer wavelengths (>1200 nm), TPM may enable imaging up to 1.5 mm, but this approach is not yet widely available [14, 15]. Hence, although these microscopic methods offer high resolution, they typically provide only a partial picture of the tissue construct (small field of view and limited penetration depth) and require long imaging times [9,15]. Thus, methods other than optical microscopy need to be employed in order to image at depths of a few millimeters.

For instance, Optical Coherence Tomography (OCT) can perform subsurface tomographic imaging of the microstructure of engineered tissues with high resolution. OCT has been used for imaging cell formation within a tissue construct with 1 − 5$\mu m$ resolution [16, 17] and is able to quickly deliver 3D structural information of tissues up to 1-2 mm thick. However, OCT image formation relies on back-scattered photons and provides mainly morphological information. Recently, Spectroscopic OCT (SOCT) [18, 19] has enabled imaging of molecular signatures [20], and has been successfully applied to imaging of engineered scaffolds to discern cellular phenotypes [21]. However, this approach does not rely on the wide libraries of fluorophores available. For many applications, fluorescence techniques based on reporter genes or established immunostains are required.

Laminar Optical Tomography (LOT)/Mesoscopic Fluorescence Molecular Tomography (MFMT) is an emerging optical tomographic imaging modality that re-
lies on fluorescence signals similar to LSCM but with the unique combination of millimeter-depth imaging and relatively high tomographic resolution [22]. Similar to Diffuse Optical Tomography (DOT) [23, 24], LOT/MFMT is based on multiple detectors with millimeter-range separation from the illumination source and image formation via an optical inverse problem [25, 26]. The combination of dense spatial data sets with an accurate forward model enables 3D reconstructions of fluorophore distributions with a resolution of 100-200 nm at imaging depths of ∼3 mm [27]. Initially, LOT/MFMT had been developed to image absorption contrast for hemodynamic imaging [28, 29] and cancer detection [30]. Then, it was rapidly adapted to molecular imaging (fluorescent LOT or FLOT) using fluorescent contrast agents [31–34]. The technique has since been employed for diverse in-vivo applications as well as tissue engineering applications, and with different names such as Mesoscopic Epifluorescence Tomography (MEFT) [35–37] or Mesoscopic Fluorescence Molecular Tomography (MFMT) [27,38,39].

In this report, we will first cover the physical principles and techniques that enables depth-resolved imaging. In Chapter 2, different tissue bio-printing techniques on which we conducted our experiments will be explained, namely Ink-Jet Bio-printing and Laser Direct Write. Chapter 3 will discuss current deep tissue imaging modalities from Confocal Microscopy to Photoacoustic imaging. In Chapter 4, we will introduce the formulation of the optical inverse problem and summarize current algorithmic implementations. Chapters 5 and 6 will then recapitulate the overall designs and sub-system components of typical instrumentation, respectively. The experiments, conducted in-vitro and in-vivo settings will also be explained in those chapters. Finally, we will present an experimental study where we compare the 3D visualization performance of second generation MFMT system with that of conventional confocal microscopy.
Chapter 2
Tissue Engineering

2.1 Introduction

Tissue engineering aims at regenerating or replacing malfunctioning/missing organs or tissue components [40]. Three main strategies are used to actualize this goal: (i) introduction of a scaffold into the host environment to guide cells to stimulate regeneration, (ii) construction of an environment to deliver repaired cells, growth factors etc. into the damaged/malfunctioning location, (iii) construction of a scaffold for the cells to cultivate in a culture system (bio-reactor) where environmental conditions can be engineered and maintained in favor of cell growth or cell death, depending on the application [40]. In terms of imaging, the former two strategies (i and ii) are considered in-vivo applications, and the last one (iii) is considered an in-vitro application. For brevity and for the purpose of our experimental studies, herein we will focus on the in-vitro tissue engineering strategy with emphasis on Ink-Jet and Laser Direct Write for constructing tumor microenvironment. We will conduct our experiments on patient-derived Glioblastoma Multiforme (GBM).

The gold standard to study GBM is through animal models, which typically involve injecting GBM cells into the mouse brain. However animal studies are extremely long, expensive and suffer from large variability. Those limitations leads to poor predictive power for clinical outcome. Similarly, 2D culture studies do not have the capacity to mimic the 3D tumor microenvironment. Hence, a major focus of research in brain oncology is to develop 3D GBM models in bio-engineered microenvironments. In this section we will discuss two major technologies to print such models. A multimodal longitudinal study of GBM will be conducted using our

novel platform.

In the following section, we first review the usage of aforementioned bio-printing technologies for cell and tissue engineering applications, namely ink-jet and Laser Direct Write printing. We address advantages and limitations of each bio-printing technology and imaging system, and suggest a perspective on integrating multiple techniques for optimal performances in replicating the tumor microenvironment.

2.2 Tissue Printing Techniques and Their Applications

Three-dimensional (3D) bio-printing is a rapid prototyping method to construct complex 3D structures through a layer-by-layer approach, allows depositing various types of cells and scaffold materials in the desired 3D pattern, and thus has a great potential in cell and tissue engineering applications. An important advantage of this technique is its capability to simultaneously deposit live cells and biochemical molecules (e.g., growth factors) along with biomaterial scaffolds at the desired location to mimic the native tissue architecture or to create a specially-designed 3D microenvironment.

With its flexibility and robustness, the 3D bio-printing technology has been considered as a versatile tool for controlling cell proliferation and cell migration. 3D bio-printing system capable of precisely deposit biomaterials in desired 3D pattern, allows fine-adjustment of microenvironment where cells are embedded.

Meanwhile, 3D printed structures often incorporate thick opaque scaffold combined with dense population of cells or cell aggregates. Therefore, there are significant difficulties in visualizing the 3D constructs with current imaging modalities. Biological understanding of cell biomechanics and functions has been mainly achieved in cell culture and tissues via destructive techniques such as western blots, immunohistochemistry or quantitative polymerase chain reaction (qPCR). However, to elucidate the interaction of stem cells with the microenvironment in tissue engineering applications, it is necessary to non-destructively monitor the spatio-temporal proliferation and/or differentiation of these cells in an unperturbed environment. To this end, developing novel molecular imaging techniques is critical to observe stem
cell fate, cell-cell interactions, and/or structural features of an engineered tissue [41]. A detailed survey of different imaging modalities will be discussed in Chapter 3.

2.2.1 Inkjet-Based Printing

In early stages, bio-printing was converted from a commercial 2D inkjet printer by replacing the printer ink with biological ink [42, 43]. While the first bio-printing approach is in use for many tissue engineering applications [44–46], recent development on inkjet-based bio-printing systems facilitates a wide range of biomaterials with improved resolution and speed. Inkjet-based printers commonly use thermal [47], piezoelectric [48–50], or microvalve-based [51, 52] drop-on-demand methods. These methods dispense picoliter or microliter volume of aqueous biological media in droplets, Fig.2.1(a-c). The ink-jet bio-printing technique constructed the tissue engineered samples used in this study. The technique developed by Dr. Guohao Dai at Rensselaer Polytechnic Institute [38]. The ink-jet printing system at RPI prepared tissue construct experiments for most of the first generation and the second generation system experiments.

2.2.1.1 Printing Mechanism

Thermal bio-printers dispense droplets by increasing temperature of the heating element to produce pulses of pressure, Fig.2.1(a). The temperature the heater increases for short time, forming a bubble that forces the bio-ink out of the printer head. Although the temperature of the heating element reaches 200 – 300°C during this procedure, it does not have a substantial influence on post-printing viability of mammalian cells [47, 53]. Piezoelectric bio-printing systems utilize piezoelectric actuators or piezo-crystals, Fig.2.1(b).

Commonly-used printers include valve coils, a valve ball, and closing elements in the printer head, Fig.2.1(c). Unlike thermal or piezoelectric systems, additional pneumatic or piston-derived pressure is applied on the biomaterials loaded inside. The valve coil is triggered by electrical signal, lifting the valve ball, consequently dispensing a droplet. Then, valve orifice is quickly blocked by closing elements [52,54,55]. The droplet size is determined by the system and the material properties such as the actuation frequency, viscosity of the material, and the applied pressure.
Figure 2.1: Bioprinting Techniques. (a-c) Inkjet-based bioprinting technologies (a) Thermal inkjet printing system (b) Piezoelectric inkjet printing system (c) Microvalve-based inkjet printing system (d) Extrusion-based printing system (e) Laser direct-write schematic for cell deposition.

2.2.1.2 Resolution and Patterning Capability

Inkjet-based printers create patterns in drop-by-drop manner, in which a series of droplets are closely deposited to form line and surface patterns [56]. Therefore, the resolution of inkjet-based printer is determined by the minimum size of droplet that the printer can generate. The actual resolution of inkjet-based printer tends to be lower than the minimum droplet size because the contour of printed pattern often becomes enlarged by merging of closely printed droplets. Droplet size is depending on numerous parameters, including nozzle diameter, printing mechanism, material
viscosity, and substrate properties (e.g. hydrophilicity). Droplet size and printing speed can be controlled electronically, and can range from picoliter to nanoliter in volume with dispensing frequency of 1-10,000 droplets per second. The spatial resolution of inkjet-based bioprinters ranges from $50\mu m$ to $1mm$ (Table 2.1). Even though these technique does not have comparable single-cell level of spatial resolution, printing of single cell can be achieved by adjusting cell suspension density and droplet size [57].

2.2.1.3 Available Materials

Materials used for bioprinting applications must be bio-compatible and cytocompatible. Also they must provide appropriate structural integrity and functional properties during post-printing maturation [3, 58]. Considerations on the viability and functionality of printed bio-structures, limit the range of available chemistries, operating temperature, mechanical and rheological properties. Naturally-derived hydrogel (including collagen, fibrin, chitosan, alginate) and synthetic polymer such as polyethylene glycol (PEG) are commonly-used scaffold materials in the field of tissue engineering [58]. The hydrogel polymers are printed in aqueous precursor form, and then solidified by post-printing crosslinking or gelation process (including enzymatic crosslinking, photo-crosslinking, pH- or temperature-sensitive phase transition). Naturally-derived hydrogels have advantages in supporting biological functions. On the other hand, synthetic polymers are more beneficial for functionalization or tailoring of scaffold material, cost efficiency, and reproducibility.

Hydrogel polymers serve as scaffolds to support 3D structure created via layer-by-layer approach. Thus, the hydrogels used for 3D bioprinting applications are required to have proper mechanical strength in order to maintain the structural integrity during and after printing procedure. The required mechanical strengths often correlate to the concentration and viscosity of polymer. Higher concentration of hydrogel provides more sturdy structure, increased stiffness, and in some cases, better resolution or patterning capability. However, this condition may not be beneficial for embedded cells since they need to degrade the matrix to migrate and proliferate. Hence, material properties need to be specifically optimized for each cell
and tissue engineering applications to find balance between structure integrity and preferred cell culture condition.

Inkjet-based printing systems are capable of dispensing most of the hydrogel polymers stated above, but certain materials are not suitable for this printing system due to the limitation of printing mechanism. The use of gentle ejection force created by a bubble, vibrations, or pneumatic pressure (< 10 psi) minimizes post-printing cell death, phenotype alteration, or functionality loss, thus has a great advantage in viable cell printing. However, it has a limitation in dispensing highly-viscous materials or high-density cell suspensions that often cause printer head clogging issues and irregular printing patterns. In general, microvalve-based bioprinters have advantages in handling viscous materials compared to the thermal or piezoelectric bioprinters since the additional pressure applied on the loaded materials can be adjusted to create proper ejection forces.

2.2.1.4 2D and 3D Cell Printing Applications

Inkjet-based bioprinting systems are more beneficial for dispensing aqueous materials such as cell suspensions (with low cell density) and soluble growth factors. The printing technique can conveniently introduce gradient patterns of cells and growth factors in 3D space by altering droplet size, spacing between droplets, or number of printing times [59–61]. Inkjet-based bioprinters are also widely used for direct cell dispensing to generate 3D in-vitro co-culture models [55, 62], vascular tissue models, and cell aggregates/spheroids [63,64] maintaining high cell viability.

2.2.1.5 Advantages and Disadvantages

Advantages of inkjet-based bioprinting technology include wide availability, high cell viability, low cost, and high printing speed. The printing mechanism allows utilizing various biological materials, especially materials with lower viscosity. The use of gentle pressure guarantees high post-printing viability, showing a great potential of the technique in handling delicate bio-structures such as stem cells, progenitor cells, and embryonic bodies [50,64,65]. However, there are some concerns regarding the instant heat exposure in thermal inkjet printers, 15-25kHz frequencies used in piezoelectric printers, and shear stress caused by microvalve dispensing.
These factors may induce cell damages, phenotype alteration, or loss of functionality. Though, the strength of these stresses is minimal in comparison with other commonly-used bioprinting technologies.

Due to the material viscosity limitation, the aqueous form of hydrogel precursor is widely used for inkjet-based bioprinting platform. Post-printing crosslinking or gelation process is required in this case. These produces include UV irradiation, temperature changes, or the use of acidic/basic solutions, and may induce various cell damages. Another limitation of inkjet-based printers is the difficulties in achieving physiological cell density and matrix density. High concentration of hydrogel polymers or high density cell suspension often cause issues including nozzle clogging, irregular droplet size, irregular dispensing trajectory, and premature gelation.

2.2.2 Laser Direct-Write

Laser Direct-Write system at RPI was built by Dr. David Corr’s lab. High precision tissue printing enabled us to characterize our reconstruction performance and sensitivity.

2.2.2.1 Printing Mechanism

Laser Direct-Write (LDW) is a non-contacting method of material deposition that utilizes laser energy absorption to propel a cell-suspended hydrogel droplet to a growth surface. This technique is comprised of two major components: a laser-transparent print ribbon and a receiving substrate. The print ribbon contains both a sacrificial and a transfer layer of material. The laser is pulsed with a configurable energy and repetition rate through the transparent ribbon. The sacrificial layer absorbs the transmitted laser energy, volatilizes, and forms a vapor pocket at the ribbon-material interface. This vapor pocket rapidly expands and ejects a droplet of the transfer layer to a receiving substrate. The amount of transferred material can be adjusted with modifications to the laser energy profile. Notably, the rate of mass transfer exceeds the transfer of heat and thereby only negligible amounts of laser thermal energy is transmitted to the deposited transfer material, Fig.2.1(e).
For print ribbon preparation, a sacrificial and a transfer layer of material are coated onto the ribbon. First, the ribbon is coated with a thin sacrificial layer. This sacrificial layer will be the only material that will interact with the laser during the transfer step and needs to be able to adhere to the transfer layer material. For recent studies involving transfer of viable cells, a cellular suspension is prepared through the use of cultured mammalian cells re-suspended in media or a non-cytotoxic hydrogel. The cellular suspension is then distributed evenly onto the sacrificial layer. The receiving substrate is initially prepared with a hydrogel layer to dampen the kinetic energy of the falling droplet of transferred material. This method allows for the deposition of a high-resolution, 2D pattern of cells, or other bio-payload, on the receiving substrate. A recent study involving alginate deposition and a calcium chloride-coated receiving substrate has also demonstrated in situ cross-linking of the hydrogel cellular suspension into a 3D microbead [66]. This envelops cells within a 3D isolated microenvironment, and allows custom placement of cells or other bio-payloads on a planar surface. Typically, this surface is a controlled hydrogel microenvironment, which enables delivery, release, or sequestration of the bio-payload. Direct-written microbead fabrication allows media, growth factors, and waste products to diffuse in and out of each microenvironment on the receiving substrate.

Dr.Corr’s lab utilized the Laser Direct-Write technique for building high precision tissue samples.

2.2.2.2 Resolution and Patterning Capability

The LDW system has a camera lens that is coincident with the path of the laser. This setup allows direct visualization of either the transfer layer or receiving substrate. The visualization capability also allows control of which regions of the transfer layer are deposited and placed on the substrate. Furthermore, the size of transfer material that is deposited can be precisely controlled through laser energy adjustment via the beam size. In other bioprinting techniques (e.g. inkjet printing), this step is dictated by the limiting size of the nozzle. For LDW, the printed droplet size is controlled by the selected level of transmitted laser energy. To achieve high
spatial patterning resolution, the LDW system has the capacity to independently automate movements of the receiving substrate and ribbon platforms. Thus, highly specific and precise spatially patterning can be achieved and programmed through the use of computer-aided design and computer-aided manufacturing (CAD-CAM) technology. LDW resolution and patterning capability are therefore within the microscopic scale ($\mu$m) for the precise spatial patterning of cells.

### 2.2.2.3 Available Materials

For LDW deposition, a variety of biological materials have been successfully printed. These biomaterials include proteins [67], nucleic acids [68], polymer biomaterials [69], and live cells [70]. To enable material deposition, a sacrificial layer is used to absorb energy from the laser. A vapor pocket forms and ejects the desired material (i.e. transfer material) to the receiving substrate. Sacrificial layers can be created from several different biomaterials such as metals [70, 71] and hydrogels such as matrigel and gelatin [72, 73]. Each has been successful for multiple cell types. Typical transfer materials include media, glycerol, and various hydrogels (e.g. alginate, gelatin, etc.). Notably for the printing of cells, cytocompatible materials are needed. For the substrate, a large range of materials can be used. However, a softer hydrogel layer is often desirable to cushion cells during bioprinting. Matrigel has been a typical selection for a substrate coating, because it meets this requirement and provides a growth surface [70, 71, 74].

### 2.2.2.4 3D Cell Printing Applications

While 2D patterning has shown utility in elucidating cellular behavior and improving understanding of stem cell fate decisions, patterning a 3D microenvironment is closer for mimicking a true in-vivo microenvironment. LDW offers some capabilities for 3D patterning of both cells and biomaterials.

MSCs have been successfully printed in 2D [75] and in grid patterns to promote the formation of MSC-derived osteogenic and chondrogenic lineages [76]. The same technique has been also used in a layer-by-layer fashion utilizing fibroblasts and keratinocytes [77], and for endothelial and smooth muscle cells to be printed into 3D scaffolds [78]. For the layer-by-layer approach, cells were suspended in alginate, and
printed onto a substrate with a mixture of alginate and blood plasma. Each layer was cross-linked \textit{in situ} by wetting with calcium chloride after printing. Alternatively, cells were suspended in a collagen/media/hydrogen carbonate mixture, and then printed. This approach yielded up to 40 layers, with 500 $\mu m$ of total thickness. However, this structure exhibited shrinking, which can be expected with collagen gels. Layer-by-layer printing approaches have shown utility for stem cells, since stem cells can be deposited in co-culture with other cell types in a controlled fashion.

Another LDW-based approach for patterning cells in 3D microenvironments involves printing microbeads that encapsulate cells. Using LDW, alginate microbeads were fabricated in a single step by depositing cells suspended in alginate to a calcium chloride/gelatin mixture \cite{66}. The calcium chloride on the substrate cross-linked the alginate into 3D microbeads in situ, with excellent control of both microbead size and position, as well as high cell viability. Further applications of this technique include polymer processing of the microbeads for creating hollow shelled structures, or bead-by-bead fabrication for the use of more complex structures. This is especially relevant for stem cells, as printing volume pixels, or voxels of stem cells in microbeads enables studying the effects of geometry-based stem cell interactions in a novel fashion.

\subsection{Advantages and Disadvantages of LDW}

LDW has a number of advantages over contact-based approaches, including the ability of LDW to directly pattern cells on a homogeneous planar surface. Of the non-contact cell printing approaches, LDW offers the finest resolution, as it can deposit very small droplets of cells with high-level, potentially sub-10$\mu$m, accuracy \cite{73,74}. LDW systems can be set up to visualize cells in real time before and after they are deposited, which no other approach offers. This capability ensures specific cells can be chosen for transfer and confirmed visually post-transfer. By contrast, if a smaller number of cells is desired to be printed using an inkjet technique, cells are randomly dispersed in a volume. The number of cells deposited is therefore a function of the probability of the number of cells present in the dispensed volume. Furthermore, because LDW is a nozzle-free printing approach, it may be possible
to print a larger range of materials that may otherwise clog a nozzle, such as more viscous hydrogels. However, LDW may not be appropriate for every application, and its limitations should be considered with other printing approaches. Compared to inkjet printing, LDW has lower throughput, as printing multiple droplets requires movement of the ribbon and receiving stages, and pulsing the laser. The speed of stage movement can limit the rate at which single droplets are deposited. Droplet volume is also generally smaller than droplets printed using inkjet techniques. Smaller droplet volume requires more droplets to cover the same area, and this is also linked to throughput, especially for larger areas.

Table 2.1: Performance comparison between Ink-Jet printing and Laser Direct Writing technologies.

<table>
<thead>
<tr>
<th></th>
<th>Ink-Jet Based</th>
<th>Laser Direct Write</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution/Droplet Size</td>
<td>50µm – 1mm</td>
<td>&gt; 10µm</td>
</tr>
<tr>
<td>Material Density</td>
<td>Low</td>
<td>Low-High</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Single Cell Control</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Capability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Printing Speed</td>
<td>Fast</td>
<td>Medium-Fast</td>
</tr>
</tbody>
</table>
2.3 Summary

3D tissue-engineering by bioprinting has the potential to generate large structures that may be difficult to image with superficial imaging modalities. In particular, imaging tissue-engineered constructs at the millimeter scale or larger is necessary for multi-layer printed structures, and for when a construct is implanted \textit{in-vivo}. Three-dimensional imaging of an engineered tissue has great impact on the assessment of structure and 3D cellular interactions after printing. Engineered tissue may be sustained in a bioreactor, which provides features of the \textit{in-vivo} environment, such as temperature, fluid flow, and nutrients. However, the bioreactor puts certain requirements on the imaging modality such as deep and non-contact imaging with fast acquisition speed. Therefore, it is necessary to develop new imaging modalities to monitor fluorescence markers (e.g., protein markers, reporter genes, etc.) within thick tissues in real time in a non-contact manner.

To date, different imaging modalities were used within the above-mentioned limitations. Among them, confocal and multiphoton microscopy yielded high-resolution images but were limited by a maximum detectable depth of $\sim 1mm$. While these techniques are suitable for fluorescence imaging with high resolution, they cause fluorophores to photo-bleach in a short time, which hinders longitudinal studies. Moreover, this technique may not be suitable for large, multi-layer bioprinted constructs, or to assess a tissue-engineered construct after implantation.

Optical Coherence Tomography (OCT) is another deep tissue imaging technique, widely used for \textit{in-vivo} ophthalmology studies, that can produce high-resolution 3D images for tissue with no requirement for sample preparation. Despite its standardized technology, OCT is limited to structural imaging. Cell shape changes or apoptosis may be detected by OCT, but molecular imaging is difficult because no markers are used. Overall, OCT is the standard tool for characterization of structural properties of scattering tissues but it is not suitable for molecular imaging.

To address above mentioned issues, we implemented the Mesoscopic Fluorescence Molecular Tomography (MFMT) to tissue-engineered construct with reporter gene expressing cells. Since MFMT does not require a tight focus on the imaging spot, it relieves the excessive excitation on imaging area. At the same time, MFMT
was shown to be effective up to 3\( \text{mm} \) deep in scattering media with multiple markers. Therefore, this imaging modality may be useful assessing large, 3D printed structures. Using multiple markers yields both functional information, from different cell types, and structural information. Despite having lower resolution than multiphoton imaging, MFMT may be useful for rapid, close to real-time imaging and analysis of tissue engineered constructs \textit{in-vitro} and \textit{in-vivo}.

As emphasized above, in order to have a complete picture, a multi-modal approach is strongly recommended. Future development in multi-modal approaches may prove useful for structural and functional real-time assessment of 3D bioprinted tissues and may become an invaluable tool for tissue engineering applications.
3.1 Governing Optical Properties in Tissue Imaging

Light tissue interaction parameters have a paramount effect in imaging as those parameters govern photon propagation in tissue. The three main parameters are the anisotropy factor and absorption/scattering coefficients. However, all of these parameters are wavelength-dependent and each tissue type is characterized by a range of values as reported in extensive surveys for \textit{in-vivo} as well as \textit{ex-vivo} samples [79–82].

The underlying physics of those parameters are well established. A brief review of their definition is provided below. Absorption of a photon occurs when the photon excites a molecule (ground state to excited state). The electron may follow different relaxation paths after dwelling on the excited state: radiative or non-radiative relaxation. The former dissipates the energy as heat and the latter one will dissipate excited state energy through electromagnetic radiation [83]. The absorption coefficient is the probability of a photon absorption event per unit length (cm$^{-1}$) and the tissue absorption coefficient is defined by

$$\mu_a = N_a * \sigma_a$$

where $N_a$ is the absorber density and $\sigma_a$ is the absorption cross section. In the case of material with only absorption, Beer-Lambert Law describes the photon propagation in tissue:

$$I(x) = I_0 exp(-\mu_a x)$$

where $I$ denotes the intensity of the light after travelling $x$ distance away from
the injection point with an initial intensity of $I_0$ [83].

The other main kind of interaction of a photon, as it propagates in a sample is scattering. Scattering is characterized by two parameters: the scattering coefficient and anisotropy factor. The scattering coefficient is the probability of a photon scattering event in a unit length ($cm^{-1}$). The scattering coefficient is defined by the density of scattering particles ($N_s$) and the scattering cross-section ($\sigma_s$):

$$\mu_s = N_s \sigma_s$$

Anisotropy factor ($g$) is the average of $\cos \theta$, which changes between -1 and 1 where $\theta$ is the scattering angle. A sample type with $g = 0$ express an isotropic scattering, while $g = 1$ exhibits only forward scattering and the values changes accordingly depending on the tissue type. The anisotropy factor is less sensitive to the wavelength than the absorption and scattering coefficients.

Reduced scattering mean free path is defined as: $l_s' = 1/(\mu_s(1 - g))$ [83] which is the average distance a photon travels without being scattered by the media. The term in the denominator is also called as reduced scattering coefficient ($\mu'_s$), which accounts for number of scattering events and the scattering direction.

The absorption coefficient and the scattering coefficient together defines the total extinction coefficient, which represents the resultant interaction of photons with media while taking the photon propagation ($\mu'_s$) and the decay of photon energy ($\mu_a$):

$$\mu_t = \mu'_s + \mu_a$$

All of those parameters change the initial state of the injected photons by affecting the intensity, state of polarization, coherence etc. After numerous interactions, these initial states will be lost and the characteristics will be fully randomized. Thus, image formation is directly related to photon properties (coherence, polarization, direction etc.), which are prone to change as soon as photons enters a medium. Conventional optical microscopy techniques deal with either thin (a few micron) or transparent samples. Advanced optical imaging methods (i.e. LSCM, MPM) either
use components that rejects scattered photons (pinhole) or operate with non-linear effects to overcome scattering to a certain extent. When the sample imposes multiple scattering events, the vast majority of collected photons are diffused. Therefore direct imaging does not work so tomographic imaging techniques are required to form an image.

Here, we will review imaging modalities operating on predominantly scattering media and classify them into two groups: modalities that reject the scattered photons and modalities that harness the scattered photons. Depending on the scattering range, number of methods were proposed to cope with the randomization of photons, and different contrast molecules. Laser Scanning Confocal microscopy (LSCM) and MultiPhoton Microscopy (MPM) are suitable for fluorescence contrast, where the latter has deeper imaging range than the former. Selective Plane Illumination Microscopy (SPIM) and Optical Projection Tomography (OPT) target transparent samples and both are compatible with fluorescence imaging. Optical Coherence Tomography (OCT) on the other hand relies on long wavelength light (typically > 900 nm) to maintain the coherence between the reference and the reflected signal from the sample, which is inherently sensitive to refractive index change. Recent efforts focus on implementing molecular sensitivity into OCT but research is still ongoing. Laminar Optical Tomography (LOT), or Mesoscopic Fluorescence Molecular Tomography (MFMT) exploits backscattered photons from multiply scattering media and it is suitable for both absorption and fluorescence contrasts. Finally, Photoacoustic (PA) imaging is a multiphysics approach, which utilizes chromophore contrast while benefiting from acoustic wave propagation in tissue.

3.2 Scattering Rejecting Imaging Modalities

3.2.1 Laser Scanning Confocal Microscopy (LSCM)

Laser Scanning Confocal Microscopy is a ubiquitous imaging modality in tissue engineering. LSCM can be used to form depth resolved images of samples less than a few hundred microns. Use of LSCM has helped in the understanding of many complex effects, such as differentiation, proliferation or migration of cells in bioengineered scaffolds [9]. Stains and dyes provide contrast for the images, and can be
used to observe the extracellular matrix or proliferation of cells. It is a noninvasive method that allows for visualization of various interactions and processes in-vivo or ex-vivo. However, LSCM is limited in imaging a couple of hundreds of microns in depth. Hence, it has very limited utility in imaging in-vitro thick scaffold and for in-vivo applications besides epithelial tissue imaging.

3.2.2 Multiphoton/Two-Photon Microscopy (MPM/TPM)

MultiPhoton Microscopy (MPM) produces images by scanning a sample with femtosecond pulses of light with wavelengths in the near-infrared region [9, 84, 85] to produce two- or three-dimensional images. If multiple photons at a fraction of the wavelength required for fluorescence are absorbed simultaneously, then this is equal to the amount of energy required for a single photon of full wavelength [9, 84]. The use of multiple photons results in higher penetration depth (up to ∼300µm in epithelia tissues with native fluorophores [86]), but the resolution degrades as the penetration depth increases due to an increase in scattering events and attenuation of the signal. Besides its increased depth of interrogation, multiphoton microscopy is preferred over single-photon techniques such as LSCM, because of the reduction in photobleaching and damage to the sample, which allows for increased viability of the sample for time-course imaging sessions [9, 87]. Photons in the near-infrared (NIR) wavelength region are able to excite exogenous fluorescent probes at greater depth, which allows researchers to analyze structure and function of the tissue in-vivo [85]. Increased penetration into the tissue allows for imaging of extracellular matrix or molecular factors within cells [9, 88]. Since scattering and absorption are reduced in the NIR range (therapeutic window) these processes can be observed even a few hundreds micron deep in the tissue while still maintaining cellular resolution. This is especially useful in 3D tissue engineering applications, since it is necessary to visualize cellular activity at the sub-cellular level [87, 88]. One main draw back for both CM and MPM is the limited Field of View (FOV), typically a few hundreds of microns in lateral plane. Though, MPM was reported to reach >1mm for in-vivo open skull brain imaging, phototoxicity still the limits the operation of advanced microscopy techniques for live tissue samples.
3.2.3 Selective Plane Illumination Microscopy (SPIM)

Selective Plane Illumination Microscopy (SPIM) (a.k.a. Light Sheet Microscopy) creates images by using a thin sheet of laser light to illuminate the sample. The illumination axis is orthogonal to the axis of detection, and only a section of the tissue is illuminated at a time [85,89]. The final image is composed of many images taken by moving the sample in relation to the light plane until the entire sample is imaged. This technique requires long time time for completion of the image if a large sample is used. Even though only portions of the tissue are illuminated at a time, SPIM is still affected by light scattering as the light propagates through the tissue [85]. Thus, this imaging method results in lower penetration depth into the sample.

SPIM has recently been used to visualize embryos as well as in time-course experiments. This means that the data can be collected in real time, resulting in four-dimensional imaging [90,91]. This is especially helpful when observing development or cell differentiation. For instance, Swoger et al., were able to trace cell lineages in the embryos of zebra fish throughout development by using dyes to track movement of cells throughout the development process [91]. It was simple to visualize the dyes in the clear embryos, but at times tissue clearance can be necessary to increase the penetration depth into the tissue, making it impossible to image in-vivo in those cases. Moreover, the geometry of SPIM (perpendicular illumination) makes it difficult to employ it with bio chambers and also in-vivo beside developmental biology samples.

3.2.4 Optical Projection Tomography (OPT)

Optical projection tomography (OPT) is an imaging method that can only be used on transparent or thin media. It is based on light trans-illumination over multiple projections through the tissue, and reconstruction is conducted to get a three-dimensional image [85,92–94]. OPT can be used to produce images of structures and function within tissues without altering its morphological characteristics. Typically, the tissue first needs to be subjected to optical clearing, a process by which the tissue is treated with chemical solvents until it is rendered transparent
This decreases but does not eliminate the effects of photon scattering, which results in a limit of penetration depth but higher resolution. However, clearing protocols can be detrimental to the biomaterials employed. Moreover, due to its trans-illumination and multi-view requirements, OPT is not easily applied to tissue within a bioreactor. Since this method is most effective on clear tissues, OPT has mainly been used in developmental biology to visualize development and gene expression in embryos [95, 96]. Use of fluorescent stains or dyes allows for comparison of morphology and anatomy at different points in development. Researchers have also been developing a method known as flow-OPT, which would be used to visualize flow of fluids in transparent tissues [92]. The tissue clearance method is beneficial in these cases, since it would allow for more accurate visualization of the fluid flow or changes in development due to gene expression or blocking within the tissue. Recent studies extend OPT into lifetime imaging as well which will explore the metabolic activities beyond the fluorescence intensity [97].

3.3 Scattering Harnessing Imaging Modalities

3.3.1 Optical Coherence Tomography (OCT)

Optical coherence tomography (OCT), is sometimes referred to as the optical counterpart of the ultrasound scan [98]. This technique is based on optical interference of the backscattered signals from the sample with a reference beam [9, 85, 98]. The resolution of OCT is dictated by the low-coherence light of the light source and a few microns resolution can be achieved in tissues as deep as 1mm (depending on the transparency of the tissue) [9, 85, 99]. OCT is a structural imaging modality that is based on differences in refractive index within the tissues. OCT imaging can be enhanced using polarization contrast (birefringence collagen imaging), Doppler signals (fluid flow) and contrast agents (gold nanoparticles being the most common). However, OCT is not inherently sensitive to molecular signals. Hence, OCT is mainly used in tissue engineering applications for real-time monitoring of tissue engineering maturation via structural markers. Since OCT is a reflection-based modality, it can be used in-vitro specimens within a bio-chamber and as well as in-vivo samples. Clinical OCT, enables real-time in-vivo imaging, are commercially
available [100]. These systems are able to visualize anatomical features without the need for contrast agents or preparation of separate samples [99, 101]. In terms of tissue engineering OCT is a versatile tool to assess scaffold transplantation and degradation in clinical settings while lacking the capability of capturing molecular signals.

### 3.3.2 Photoacoustic Imaging

Photoacoustic is a multiphysics imaging modality that aims at providing the specificity of optical imaging techniques with the resolution of ultrasound imaging. This modality's resolving power can range from the macroscopic level, Photoacoustic Tomography (PAT), down to the microscopic level, Photoacoustic Microscopy (PAM) [102]. PAT capitalizes on the low scattering of sound waves in deep optically scattering media, which enables high resolution imaging that is otherwise very difficult to achieve with traditional optical imaging techniques [102]. Previous studies claimed a resolution as high as 1/200 of the penetration depth into the sample (up to 7 cm) [102]. PAM has been applied successfully to image 3D scaffolds [103] to characterized porous scaffold [104], melanin-labeled cells [105] and neovascularization [106]. Despite the capacity of high resolving power, the amount of absorption contrast provided cells is not sufficient for PAT. Hence nanoparticles are utilized to increase the contrast and specificity [107, 108]. Recently, studies have demonstrated that multimodal approaches using PAT (functional) with ultrasound (structural) imaging offers the possibility to image and track stem cells labeled with gold nanotracers both in-vitro and in-vivo [107, 109]. Although the requirements of labeling agents, the necessity for impedance matching with the ultrasound transducer, relatively low sensitivity, and the mitigation of image artifacts are still limitations for PAT, it is emerging as a promising new modality for tissue engineering and stem cell applications. Readers may refer to extensive review for potential molecular imaging venues for photoacoustic imaging [110].

### 3.3.3 Mesoscopic Fluorescence Molecular Tomography (MFMT)

Mesoscopic Fluorescence Molecular Tomography (MFMT) is a nascent depth-resolved imaging technique [25]. MFMT originates from the combination of two
well-established techniques: diffuse optical tomography (DOT) and LSCM. Radially spaced detectors are placed with increasing distance from the light source injection point in epi-configuration. As in OPT, MFMT is based on an inverse problem, but in which multiple scattering is modeled; this means that there is no need for clearing agents. Different source-detector distances enable projection to different depths within the target tissue (up to 3mm) [22] with reported resolution of 200 µm [111]. MFMT is able to reconstruct absorption contrast [29] or fluorescence contrast [27, 38]. Although MFMT has appeared in different instrumentation and under different names [27,36,112], the working principle is based on similar phenomena. MFMT and variations of MFMT perform in epi-configuration (illumination beam and detectors are on the same side of the sample) in non-contact fashion, which makes it a suitable imaging method for tissue engineering applications, even in bio-reactors. It has been shown that MFMT is a powerful technique to reconstruct fluorescence molecules [38,113] and reporter genes [27] in-vitro as well as hemodynamic response in-vivo in the brain [29]. A recent study brings MFMT into a new venue by increasing the resolution power below 100µm thanks to sparsity constraints [114], which will be able to further the use of MFMT [115].

3.4 Summary

Non-invasive imaging is essential for longitudinal assessment of engineered tissue constructs. Herein, we surveyed a broad range of techniques in two classes based on avoiding or exploiting scattering in the media. Special attention needs to be paid to select the appropriate technique depending on the type of information that is sought: structural, molecular, sub-cellular, tissue level etc.

Applications, seeking large sample investigation in a short time frame have limited options in terms of imaging. Recent progress in Mesoscopic imaging satisfies these requirements without compromising depth of imaging. Mesoscopic Fluorescence Molecular Tomography (MFMT) (a.k.a. Fluorescence Laminar Optical Tomography) offers high sensitivity and fast data acquisition over a large field of view. Even though it delivers lower resolution than microscopy counterparts, much lower power of excitation and rapid data collection put significantly lower stress on cells,
which is critical for longitudinal studies.
Mesoscopic Fluorescence Tomography Methods and Reconstruction Techniques

Mesoscopic Fluorescence Molecular Tomography (MFMT) is a non-contact laser-scanning imaging technique similar to LSCM. The difference between the two techniques stems from the configuration of data collection. MFMT collects backscattered/fluorescence light with its detectors, radially spaced from source (focal point), while LSCM executes direct imaging point by point. The projected depth of MFMT is proportional to the distance between the source and detectors. Because the photons, injected from the source and make their ways to the detector, form a "banana shape" distribution. This phenomenon results in a depth-resolved data set without the need of z-scanning. However, this implementation comes with the drawback of loss of resolution compared to LSCM.

Then 3-D reconstruction of the contrast (either absorption or fluorescence) is enabled by solving an optical inverse problem using mathematical models of light propagation through the sample. MFMT allows for acquisition of depth-resolved 3D quantitative images of molecular probes at depths of several millimeters (3−5mm) with high-sensitivity and relative high resolution (100−200µm) [25,27,33,36].

4.1 How to Form a 3D Image with a Tomographic System

4.1.1 Problem Types

Problem types can be classified into two groups: direct and inverse problems [116]. Two types of problem can be explained with the following example. Lets

Figure 4.1: Schematic depicts an input (INP) and output (OUT) for a tomographic imaging system. INP represents a source position, OUT represents a detector position. The spatially varying optical properties (either absorption, scattering, or fluorophore concentration) are represented by \( f(x) \) function (system response).

Assume an equation is given as:

\[
y = 2x + 5 = f(x)
\]

Finding the points on this function is called direct problem.

Let's assume a set of lines is given, \( y = mx + n = g(x) \) and a set of points: \((x_1, y_1) = (1, 5)\) and \((x_2, y_2) = (7, 9)\).

Finding \( m \) and \( n \) with given points is called an inverse identification problem. In this report, we will focus on the inverse reconstruction problem which can be defined as finding the \( x_1 \) given an equation (\( f(x) \)) and a measurement data (\( y_1 = 5 \)).

Here, \( f(x) \) is the mathematical model of the system [116]. In the context of the optical tomography, we have a black box system of tissue. The input is the light we inject into the tissue and the output is the light, collected at a particular point, away from the injection point, Fig.4.1. The linear relation between the specific source detector pair and the local variations in the objective function (\( f(x) \)) is rendered via sensitivity matrix (\( A \)), also called Jacobian matrix.
The system equation forms as follows:

\[ Ax = y \]

\( y \) is the measurement data collected through experiments, \( x \) represents the unknowns i.e. fluorophore/chromophore concentration, absorption/scattering coefficient etc.

### 4.1.2 Forward Model

Monte Carlo simulation has been a versatile tool to simulate photon propagation in scattering media since 1949 [117]. Decades after been introduced, it was applied to simulate laser-tissue interactions [118] and a series of seminal papers were presented since then [119–123]. Although diffusion theory is an established and convenient method for light transport in tissue, it fails when source detector separation becomes comparable to scattering mean free path [124]. Especially in the mesoscopic regime, small source detector separations are utilized where MC simulation becomes the preferred tool. The main challenge for MC is the computational cost. However, following the development of GPU computers, the computational cost can be mitigated [121, 125]. Moreover, efficient MC formulations such as perturbation MC or adjoint methods can further speed up computation [115, 126, 127]. This section will briefly explain the underlying principles for MC simulation and the method we used for this study.

The sensitivity matrix \((A)\) is generated by Monte Carlo simulation, based on tracking the random-walk trajectory of photons in tissue. For each photon entered into the media, a series of processes take place. The photon in the media moves with \( \delta s \) which has a value comparable to the mean free path of the medium \( (l_s = 1/\mu_t = 1/\mu_s + 1/\mu_a) \) [120]. \( \delta s \) is a distance over which photon will have a chance to observe absorption and scattering events. A generic flow of the MC simulation is represented in Fig.4.2. A photon is launched at an initial position \((p_1)\), with a predefined direction \((d_1)\). This photon travels a distance \((L)\), then it experience absorption \((\mu_a)\) and scattering \((\mu_s)\) through the tissue. The photon intensity gets weighted by the attenuation \((\exp(-L\mu_a))\) [121] and the direction of the photon changed through probability distribution, dictated by Henyey-Greenstein function.
A generic MC flow chart shows the flow chart of generating sensitivity matrix. The program starts with launching photons from a particular location and save the path of the photon until it reaches a boundary, attenuates or travels more than a predefined duration. This process continues until the last photon.

Depending on the boundary conditions and the morphology of the sample, one might choose from the selection of MC methods. An extensive review of different MC methods was reported elsewhere [115]. In this study, we utilized the Adjoint MC method which exploit the reciprocity of photon propagation in tissue. In this model, a Green’s functions are generated for source and detector, ($G_x$ and $G_m$) respectively. This pair of Green’s function yield the forward and backscattered photon propagation in tissue (Eqn.4.1)

$$U_f(r_s, r_d) = \int_\Omega G_x(r_s, r)G_m(r, r_d)\eta(r)dr$$  (4.1)

gives the fluence for each source ($r_s$) and detector position ($r_d$) through integrating the source ($G_x$) and the detector green function ($G_m$)over solid angle ($\Omega$). The detected energy will be proportional to the efficiency ($\eta$) of the fluorophore which has a spatial distribution.
4.1.3 Inverse Problem Solvers and Regularization

The goal is to find the $x$, through inverse operation:

$$x = A^{-1}y$$

Since the optical tomographic data reconstruction is an ill-posed problem, standard inverse operation does not work for the sensitivity ($A$) matrix. Thus, typically, a pseudo-inverse of the system equation is applied [128]:

$$A^* = (A^TA)^{-1}A^T$$

which requires regularization to solve it. Traditionally, Tikhonov regularization is used:

$$x = ((A^TA) + \lambda I)^{-1}A^Ty$$

where $\lambda$ is the regularization parameter and $I$ is an identity matrix. However, it is not the best suited in MFMT as a large dynamical range is observed in the sensitivity profiles. Hence, in this study, we used a spatially variant regularization parameter ($\lambda(r)$) to mitigate the surface weighting on reconstruction [129]. Hence, we used the modified version of Tikhonov regularization (Eqn.4.2):

$$x = ((A^T A) + \lambda(r)I)^{-1}A^Ty$$ (4.2)

where $\lambda(r)$ is the diagonal elements of $(A^T A)^n$, and $n$ is the weighting factor, typically 0.5.

Estimation of $x$ is an iterative process. We used $l_2 - norm$ and $l_1 - norm$ regularization by using $l_p$ code, explained elsewhere [130]. Our results indicated that due to sparsity of data $l_1 - norm$ ($p = 1$) over performed $l_2 - norm$ [114].
Chapter 5
The First Generation Mesoscopic Fluorescence Molecular Tomography and Biological Applications

The first generation MFMT system was built primarily for tissue engineering applications with two spectral channels [38]. Later, the system was upgraded to 3 spectral channel and optimized for minimized chromatic aberration for visible spectrum. Then, they system was used for both tissue engineering applications (in-vitro) [27] as well as animal studies (in-vivo) [39]. In this section, we will explain the basic working principle of the first generation MFMT, and related studies will be explained in detail.

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Figure 5.1: MFMT system utilized 1by7 APD detector array for detection. The excitation signal (Orange Color) linearly polarized (P) and reflected from two different Galvo mirror (GM) to execute raster scanning through focusing with a Scanning Lens (SL). Fluorescence emission (Red Color) follow the same path due to de-scanning mode until the Beam Splitter (BS) and then emission light focused onto the detector array through a tube lens (TL), which is color filtered (F) after and cross polarized (A) to enhance the signal to noise ratio.

5.1 System Properties

MFMT imaging system is based on raster scanning a focused laser beam over the sample, Fig.5.1. The fluorescence light is then detected at 7 radial positions ($d_i$) away from the illumination spot in an epi-configuration ($d_1 = 800\mu m, d_7 = 3,900\mu m$).

The optical setup is similar to the one in Zhao et.al, [38]. We have replaced the lenses with anti-reflective coated achromatic ones and added one spectral channel. We have used emission filters (XB97/560BP10, Omega Optics; FF01-650/60-25, and FF01-700/13-25, Semrock) for corresponding Diode Laser source (of 488nm, 589 and
Figure 5.2: Schematic of tissue engineering imaging is depicted. Excitation signal (solid yellow), passes through L1 for collimation and raster scanned over the sealed chamber. Emission signal (solid red) de-scanned and imaged on to detectors after filtering excitation signal. Sealed chamber can be perfused with injection inlets and outlets.

658nm, respectively). The focused beam impinges 17mW on a circular area of 375m diameter on the chamber. Emission signal and a matching background signal acquired during raster scanning of the flow chamber over $3 \times 7 \text{mm}^2$ area, $x - y$. The dwelling time at each collected source point was $\sim 8.3 \mu\text{sec}$. The $3 \times 7 \text{mm}^2$ area was imaged in this work lead to $80 \times 200$ source positions ($\delta x = 37 \mu\text{m}, \delta y = 35 \mu\text{m}$) and a total acquisition time of $\sim 132.8 \text{ msec. per frame}$ (16,000 source position; corresponds to $\sim 7.5 \text{ fps frame rate}$). Data acquisition was averaged over 1 minute ($\sim 450$ frames) to obtain fluorescent data with good signal-to-noise ratio. Averaged background signal was subtracted from averaged emission signal to minimize the autofluorescence and the bleed-through effect a posteriori. Reconstructions were performed using 14,400 laser beam positions as artifacts, originated from the soft-
ware, were observed on the border of the imaging area. Overall, the 14,400 source positions and data gathered from 7 detectors for each laser beam position led to a total of 100,800 spatial data points. We used a CPU based Monte Carlo photon propagation model to compute the Jacobian matrix. The forward simulations were carried with \(10^5\) photons and \(0.2 \times 0.2 \times 0.2 mm^3\) voxels. The optical properties, used to compute the Jacobian, were set at: \(\mu'_s = 0.5 mm^{-1}, \mu_a = 0.002 mm^{-1}\) and \(g = 0.9\). These values were selected based on previous studies [38] and reported values for collagen in the literature. Note that the optical properties were estimated as same for all wavelengths investigated herein.

This configuration was used throughout the experiments pertaining to the first generation MFMT system [27, 39].

### 5.2 3D Reconstruction of Vascular Channels in Bio-Printed Tissue Construct

#### 5.2.1 Sample Preparation and Methods for Analysis

##### 5.2.1.1 Cell Labelling

Human umbilical vein endothelial cells (HUVEC) were labeled with 3 different colors: GFP (\(\lambda_{ex}/\lambda_{em} = 488/507\text{nm}\)), mCherry (\(\lambda_{ex}/\lambda_{em} = 587/610\text{nm}\)), and Far-red (\(\lambda_{ex}/\lambda_{em} = 650/670\text{nm}\)). Reporter genes were used for GFP and mCherry labeling (lenti-viral transfection). For Far-red, HUVECs were labeled with 5\(\mu M\) Far-red cell tracker (DiIC18(5)-DS, Invitrogen). The labeled cells were cultured at 37°C in 5% \(CO_2\) in Endothelial Cell Growth Medium-2 (Lonza).

##### 5.2.1.2 2D Letter Bio-Printing

To validate the MFMT capability on reconstructing complex structures, 2D letters were printed on top of collagen. Three letters were printed on collagen matrix (type I, 3.0 mg/mL, BD Bioscience) (Fig.5.5). Far-red, GFP, and mCherry-labeled HUVECs were used to print R, P, and I, respectively.
Figure 5.3: 3D bio-printing steps are depicted. Ink-Jet printing starts with a base layer of collagen, then it is followed by gelatin printing for channel formation. After liquifying gelatin, Human Umbilical Cord Endothelial Cells expressing mCherry (Purple, bottom channel) and GFP (Green, top channel) are seeded into the channels. Bottom channel was perfused with Far-red fluorescent beads (Red, bottom channel).

5.2.1.3 Vascular Channel Bio-Printing

Through a layer-by-layer approach two vascular channels were constructed inside of a 3mm-thick sealed chamber (Fig.5.3) [61]. GFP- and mCherry-expressing cells were seeded on the inner surface of top and bottom channel, respectively. The distance between orthogonally located channels was varied (100 and 1300µm) to test the imaging capability in different configurations. In order to visualize the channel structure, Flash Red beads (Bangs Laboratory Inc.) were perfused into the lower channel (mCherry cells seeded) at 0.1mL/min flow rate.

5.2.1.4 Objective Metrics for Comparison

We benchmarked the 3D reconstructions (RECONS) against two classical microscopic modalities (maximum intensity projection): Phase Contrast Microscopy
Figure 5.4: Color scale represents the result accuracy. The perfect agreement between different imaging modalities will yield 1 (green area) any deviation between them will result away from 1 and will move toward the red area.

(PCM) and WideField Fluorescent Microscopy (WFFM) (Eclipse Ti, Nikon). Accuracy of the reconstruction was quantified by segmentation of WFFM, PCM and reconstruction images by an ImageJ plug-in (Mixture Modeling). Two metrics for objective image analysis were derived: Area of segmented image (mm$^2$) and Agreement Factor (AF). First, Areas ($A_{WFFM}$, $A_{PCM}$, $A_{MFMT}$) and Area Ratios ($A_{MFMT}/A_{WFFM}$ and $A_{MFMT}/A_{PCM}$) were calculated. Then, Overlap Ratio was estimated: $A_{MFMT} \cap A_{WFFM}$ and $A_{MFMT} \cap A_{PCM}$. Both Area Ratio and Overlap Ratio were lumped together in the Agreement Factor:

$$AF = 2^{1 - \text{Area Ratio} + |1 - \text{Overlap Ratio}|}$$

If areas are the same and locations are overlapped that mean two modalities are matched, $AF = 1$. Any discrepancy will result as a distance from 1, as shown in Fig.5.4

5.2.2 Results

5.2.2.1 MFMT Imaging of Printed Letter Patterns

MFMT successfully reconstructed the printed patterns regardless of the spectral range (green, red, and far-red) and labeling methods (cell tracker and reporter genes), Fig.5.5(a). The MFMT reconstruction results, Fig.5.5(b-d) matched the wide field images, demonstrating the ability of MFMT to recover the shape of the letters with accuracy. Area values for WFFM, PCM, and MFMT reconstruction are: 6.3, 6.86, 4.6 mm$^2$ for R; 5.44, 5.97, 4.33 mm$^2$ for P; 1.63, 2.78, 1.98 mm$^2$ for I. AF values of R, P, I are 1.33, 1.31 and 1.25.
Figure 5.5: (a) WFFM image of R, P, I letters are expressing, Far-red cell tracker, GFP and mCherry, respectively. 3D reconstruction is shown from top (b), from perspective (c) and side view (d) image shows the letters printed on the top of the collagen block sample (thin sample). Results show the accuracy of MFMT reconstruction as it is benchmarked against WFFM. Scale bar, 1mm.

5.2.2.2 MFMT Imaging of 3D Two-Channel Construct

Two-channel collagen structures were formed with varying channel-to-channel distance were constructed: orthogonal channels printed closely (∼100µm), and far apart (∼1.3mm). In PCM imaging, the channels exhibited elliptic cross-section estimated to be around 0.7 – 1.3mm in width and 0.4 – 0.8mm in height. Reconstructions for both cases and benchmarking against PCM and/or WFFM images are depicted, in Fig.5.6 and Fig.5.7. The populations of GFP and mCherry cells were distinguished accurately in both cases. The estimated channel diameters were ∼0.6mm and ∼0.8mm for GFP and mCherry, respectively (Fig.5.6). Far apart channels had a diameter ∼0.5mm (Fig.5.7). Overall, these dimensions were consistent with the values estimated by PCM. However, changes in temperature or inner-channel pressure caused slight expansion of channel edge on the MFMT reconstruction. Area values for WFFM, PCM, and reconstruction are: 3.93, 3.31, and 4.09mm² for GFP; 6.13, 7.43 and 5.75mm² for mCherry. AF values for WFFM and PCM are: 1.21, 1.49 for GFP; 1.16, 1.29 for mCherry. WFFM images are in better agreement with reconstruction values. This is an expected outcome as both WFFM and reconstruction are sensitive to the same contrast function.

Additionally, Far Red fluorescent beads perfusion through lower channel (mCherry) was reconstructed in 3D (Fig.5.7). The reconstructed beads flow was co-
Figure 5.6: Phase Contrast Microscopy image (a) shows the shape of both channels and Wide Field Fluorescence Microscopy image (GFP is green, mCherry is red) (b) shows the gene expression signal. Maximum intensity projections from two MFMT channels reconstructions are merged (c). 3D perspective image (d), top view (e) and side view (f) images, obtained from MFMT, show the respective positions of vascular channels. GFP and mCherry channels are printed adjacent in 0.5mm and 1 mm in depth, respectively. Scale bar, 1mm.

localized with lower channel reconstruction (Fig. 5.7(d-f)). mCherry and beads flow reconstruction showed a slight discrepancy in the overall size. This may be due to the beads accumulation on the centerline of the channel during gentle beads perfusion (0.1mL/min). The problem can be addressed by applying higher flow rate or using larger beads. A slight overlap was observed where two channels are adjacent (∼100µm) (Fig.5.6), this is attributed to voxel size of 200µm. With higher computational cost, the voxel size can be decreased to increase the image space resolution and, hence, separate channels more accurately. We also note that the optical properties were kept constant for all reconstructions. Hence, a slight mismatch between true and simulated optical properties over the spectral range of the system was induced. Impact of optical property mismatch between the light propagation model and the sample has been studied for DOT [131] and we are currently investigating the effect of this mismatch on MFMT performance. We expect it to be the
Figure 5.7: Phase Contrast Microscopy (PCM) image (a) shows the shape of both channels and Wide Field Fluorescence Microscopy (WFFM) image (GFP is green, mCherry is blue) (b) shows the gene expression signal. Maximum intensity projections from two MFMT channels reconstructions are merged (c). 3D perspective image (d), bottom view (e) and side view (f) images, obtained from MFMT, show the respective shapes and positions of vascular channels. GFP and mCherry channels are printed in 0.5mm and 2 mm in depth, respectively. Black square in (b) is a WFFM defect that occurred while acquiring the data. Scale bar, 1mm.

main source of mismatch between microscopy and MFMT reconstructions observed in this study.

5.3 Summary

In this study, we have demonstrated 3D fluorescence reconstruction of live, reporter gene expressing cells, and perfusion through vascular channels in 3mm engineered tissue constructs within a bio-reactor. The whole imaging, data acquisition and reconstruction, was completed in less than 5 minutes for each fluorophore.

Our results, as well as the previous studies [38], show the potential of MFMT as a powerful tool for tissue engineering applications. We believe MFMT will have an important role to localize and monitor live fluorescent cells in thick tissue. This is a cost- and time-effective technique to evaluate the dynamic progression and
functional status of engineered tissues, prior to host implantation.

Especially, the ability of MFMT to image multiple bio-markers paves the way for imaging cellular heterogeneities as well as different bio-markers simultaneously. In combination with novel hyper-spectral methods [132], the MFMT should enable a comprehensive molecular assessment of live bio-printed tissues.

5.4 3D Bio-Distribution of Photodynamic Therapy Agent for in-vivo Mouse Model

The standard-of-care cancer treatments include surgery, radiation therapy, chemotherapy, immunotherapy, and monoclonal antibody therapy. Depending on the patient and cancer stage clinicians decides one the above mentioned techniques or combination of multiple of them. While improvement of these treatment modalities to enhance their tumor selectivity is undergoing, development of novel alternative treatment approaches that may be safer or reduce functional tissue damage, and improve patients quality of life is a major focus area of research. Among all new modalities investigated, photodynamic therapy (PDT) emerges as a promising alternative treatment strategy [133]. PDT is a treatment aims to avoid surgical operation by a photosensitizer (PS) drug and drug activating light and oxygen [134]. Activation of a PS results in cytotoxic reactive oxygen species, which annihilate cells locally. Due to high vascularization, accumulation will occur dominantly at the tumor site [134].

The effect of PDT was investigated for in cancer treatment and various PS have been approved for clinical use in the United States and other countries [135, 136]. Currently, PDT is being used mainly to treat skin cancer and other easily accessible lesions [134,137,138].

PDT is a local therapy which has minimal toxicity in the absence of light. PS accumulates minimally in non-cancerous tissues, therefore it imposes minimal toxicity for healthy tissue. Furthermore, light activation is performed below the Maximum Permissible Exposure limit and at wavelength that do not harm the tissues. Hence, a repeated treatment is possible for PDT if the initial treatment fails. The efficiency of PDT is strongly related to the functional state of the tissue,
the PS uptake and light fluence delivered locally. If these parameters can be controlled/assessed relatively easily in superficial lesions, it is a significant challenge for pathologies that are thick or located a couple/few millimeters deep. In such scenarios, the PS bio-distribution profile in tissue is not homogeneous, even in the case of local topical application. Second, light propagation in tissues is highly affected by the optical properties distribution in the sample. Hence, PDT efficiency can be hampered by intra-lesion PS heterogeneous bio-distribution and light flux profiles.

The most accepted method to measure PS concentration and distribution in tissue has been by biopsy sampling of the tissue and chemical extraction of the compound for spectrophotometric assay quantification [139]. However this is an invasive, time consuming and expensive method that is prone to sampling errors. To provide a direct mean to image the PS bio-distribution, there is a critical need to develop new imaging modalities that could provide the 3D PS bio-distribution on the bedside for optimal therapy planning, and potentially, during treatment to assess therapy efficacy [140–144].

One key property of the main PSs is their ability to fluoresce. Therefore fluorescent signals can be used to directly measure the bio-distribution and concentration of the PS in-vivo [145,146]. However, 2D surface measurements have been shown to be unreliable due to variations in tissue optical properties and heterogeneous drug depth distribution [147]. Researchers have proposed either spatially-resolved spectroscopic and/or tomographic methods to overcome these issues [148]. In addition, some alternative approaches, such as small fibers probes, have been developed to limit the effect of optical properties on the fluorescence signal. However, such probes still lead to local measurements prone to error sampling and could require minimally invasive protocol to obtain depth profile [149, 150]. For non-invasive 3D imaging, classical fluorescence microscopic techniques have been employed to image the PS bio-distribution and/or assess therapy efficacy. For instance, LSCM (CM) was used for imaging PpIX accumulation in the target tissue [151] whereas two photon microscopy was used for post-treatment imaging [152] to assess cell death after radiation. Although these latter two imaging methods offer high-resolution performances and great sensitivity, they are restricted in their depth penetration (<1mm) and field
of view (< 1mm²), limiting their usefulness for fast imaging of deep pathologies. To probe bio-tissues beyond the traditional microscopic depth penetration a novel method known as Mesoscopic Fluorescence Molecular Tomography (MFMT) has been developed recently.

MFMT is an optical tomography technique which can reconstruct absorption or fluorescence contrast over relatively large areas in a fast manner. MFMT is able to image highly scattering tissues that are a few millimeters thick (3 – 5mm) with a relatively high-resolution (100 – 200µm). Similarly, to Diffuse Optical Tomography (DOT) or Fluorescence Molecular Tomography (FMT), MFMT utilizes several detectors to collect scattered photons at multiple distances away from the illumination spot, though at distances of a few hundred microns to interrogate tissues at the mesoscopic scale. MFMT uses discrete detectors analog to the pinholes in LSCM. Smaller or larger optode separations lead to shallower or deeper photon collection, enabling depth sensing. Combining these depth sensitive measurements with an optical inverse formulation allows retrieving 3D quantitative image of the fluorescence bio-distribution. This unique combination of small source detector separation, discrete detectors and optical tomography approach offers unique capabilities for thick tissue imaging. A detailed explanation of the inverse problem and reconstruction schemes can be found in the Method section.

MFMT works in epi-configuration and it is well suited for in-vitro tomographic imaging [38], as well as for in-vivo preclinical [33] and clinical imaging scenarios [30]. Furthermore, it can be fused with other traditional optical imaging modalities such as OCT [33] to obtain molecular, function and structural information. One key advantage of MFMT is its ability to image a large number of readily available fluorescent probes at fast acquisition speed [27]. Herein, we apply MFMT to retrieve the distribution of 2-[1-hexyloxyethyl]-2 Devinyl Pyropheophorbide-a (HPPH), a PS that has been used in patients since 1999, within skin cancers in preclinical models. This is an early first step towards establishing MFMT as a bedside imaging modality to assess non-invasively the PS bio-distribution prior treatment for best therapy planning, and potentially during therapy for light dose delivery optimization. This article summarizes the imaging platform characteristics, the image formation al-
5.4.1 Materials and Methods

5.4.1.1 Preclinical Model Protocol

All animal experiments were conducted according to the IACUC of Roswell Park Cancer Institute (RPCI) protocols. Transgenic K5-Gli mice were a domestic colony located in RPCI. These mice hosted an activated Sonic hedgehog (Shh) signaling pathway and with an over expressed Gli2 downstream transcription factor, which lead to spontaneous multiple basal cell carcinoma (BCC). The significance of these spontaneous BCCs stems from histological similarity with nodular BCCs that is observed in human patients, which is the most common skin cancer in human with increasing incidence over the last decades [153]. Three Gli mice (6-12 months old) from the breeding colony were selected.

As soon as the mice developed visible BCC tumors on their tails, we initiated the imaging process. All mice in this study were euthanized immediately prior to imaging. If live imaging sessions under anesthesia (isoflurane) will have been preferable, working with freshly euthanized subject allowed avoiding any movement during the imaging session for seamless non-concurrent registration between MFMT and Ultra Sound (US) imaging, but still to work under similar conditions than live studies. Of the three mice imaged: Mouse A had 3 lesions imaged, Mouse B had 4 lesions imaged and Mouse C exhibited no lesions based on visual inspection. All mice were injected intra-peritoneally with 0.47µmol/kg (∼ 0.3mg/kg) of HPPH (Roswell Park Cancer Institute) and were kept under reduced light conditions (< 60lux) until being sacrificed. Mouse A and C were imaged 20 hours post injection, whereas Mouse B were imaged 40 hours post injection. Note that in clinical settings, HPPH treatment is commonly applied 24 hours and 48 hours after injection [154].

5.4.1.2 Tumor Imaging

Areas of the tail that exhibited lesions based on visual inspection were selected and then imaged sequentially with MFMT and high-resolution US A-scan imaging, Fig.5.8(a). Imaging sessions were performed within 30minutes. Both data sets were
Figure 5.8: A visible tumor was selected for imaging. (a) The tail of the mouse was immersed in a matching liquid. Raster scanning was applied on the tumor area (shown in inset) (b) Laser diode excites the fluorophore by raster scanning over 6mmx3mm area. Emission signal de-scanned and directed onto detector array with a beam splitter (BS) followed by an emission filter.

registered a posteriori using commercial software. To mitigate for the curvature of the tail over the area imaged, a matching liquid solution was employed during the imaging sessions, Fig.5.8(a). A diluted intralipid solution (30% Fresenius Kabi) matching the optical properties of the mouse skin [155,156] was created and the tail immersed halfway to generate a planar boundary condition.

5.4.1.3 Mesoscopic Fluorescence Imaging System

The MFMT system operated at 658nm (Laser diode L658P040, Thorlabs) to match HPPH excitation spectra. A high pass emission filter (FF01-692/LP-25, Semrock) is used in the detection channel to collect HPPH emission light. To image large area, the illumination beam is raster scanned over a 6mm by 3mm surface on the selected area of the tail. The overall light dose applied during the MFMFT imaging sessions is 15J/cm², which is significantly below the light dose applied for PDT treatment (50 – 200J/cm²) [154]. Both an emission signal and a matching background are sequentially acquired (both with the same emission filter). Data
acquisition over the above-mentioned area took 1 minute at a $\sim 7.6\text{fps}$ rate. Following the post-processing methodology described in [32], averaged background signals were subtracted from averaged emission signals to minimize the auto-fluorescence and the bleed-through effect \textit{a posteriori} and to render the system more sensitive to deep-seated fluorescence signals.

5.4.1.4 MFMT Reconstructions

MFMT is a subsurface sensing modality that requires solving an optical inverse problem to retrieve the bio-distribution of the fluorescent probe in 3D. Due to the limited volume probed with this mesoscopic imaging modality, the classical diffusion equation forward model employed in fluorescence molecular tomography cannot be used. Herein, we used a Central Processor Unit (CPU) based Monte Carlo (MC) photon propagation model to compute the Jacobian for such shallow interrogation volume. The forward simulations were carried with $10^4$ photons and $100 \times 100 \times 100\mu m^3$ voxel size. The MFMT image reconstruction process can be expressed using the following equation:

$$x' = ((A^T A) + \lambda D(r))^{-1} A^T y$$  \hfill (5.1)

where b is obtained from the fluorescence measurement. $x$ is the reconstructed fluorescence distribution; $\lambda D$ is a regularization depth-dependent parameter; A is the whole Jacobian that is computed based by the aforementioned Monte-Carlo forward model. To gain from the computation time, we exploited the planar boundary and image space symmetry. So instead of simulating photon propagation for all source-detector pairs for all source locations; we generated one optode set (1 source and 7 detectors) and populated this matrix for all of the source locations.

Optical properties, $\mu'_s$ and $\mu_a$ for Jacobian (A) matrix are chosen as $3mm^{-1}$ and $0.002mm^{-1}$ [155, 156], respectively. Both 3D reconstruction of absorption and/or fluorescence contrast can be performed using this model [157]. A depth-dependent regularization technique is employed to mitigate the ill posedness of the reflectance geometry. D is a diagonal matrix whose elements are the square root of the corresponding diagonal elements of $A^T A$. The scaling factor ($\lambda$) for this diagonal matrix is
selected based on the L-curve analysis [158]. The conjugate gradient method is applied to solve this linear system (function cgs, MATLAB). The iterative algorithm was stopped if 100 iterations or if a tolerance of $10^{-2}$ was reached. Overall, the Monte-Carlo forward model and optical reconstructions were computed in less than 4 minutes for the typical 106,400 data measurements (15,200 sources positions × 7 detectors) on a PC (Intel Core i7-3820 CPU, 3.6 GHz, 64 GB).

5.4.1.5 Ultrasound Imaging

The preclinical US system used in this study, is a one-dimensional A-scanner, which operates at 50 MHz (Longport Episcan). The US probe allows investigating tissue along 14.9 mm and 5.39 mm depth with a resolution of $\sim 30 \mu m$. Ultrasound scans were taken immediately after MFMT imaging without altering the position and the condition of the mice. The US probe was wrapped in a sealing membrane to operate the US transducer in water for minimal impedance mismatch. A-scans were taken along the two sides of the tail with 100 $\mu m$ steps, leading to 30-60 slices acquired depending on the broadness of the tail. A-scan slices provided both the geometrical information about the tail boundaries and about the tumor location and structure.

5.4.1.6 Co-registration of US and MFMT Data Sets

The MFMFT and US data sets were co-registered a posteriori. First, to validate our approach, phantom experiments were conducted. 3% Agar (Sigma Aldrich, USA) was mixed with 2% Intralipid suspension (20% emulsion, Sigma Aldrich, USA) in deionized water. Solution was kept at 80 °C on a hotplate (Isoterm, Fisher Scientific) and stirred at 350 rpm (Magnetic Stirring Bars, Fisher Scientific, USA) for 1 hour. We aimed to produce homogeneous turbid matrix with optical properties similar to the one employed in the optical forward model for tail imaging. Prior to solidification of the agar, we placed a cylinder-shaped clear solid inclusion (PDMS, Sigma Aldrich), $\sim 4 mm$ in diameter and $\sim 4-6 mm$ in height, just beneath the surface of the matrix. This inclusion, placed inside the matrix, mimics a tail immersed in intralipid as employed in the tumor imaging cases. The PDMS inclusion, shown in Fig.5.9(a), allowed inserting a 1.5mm-diameter capillary, filled with fluorophore
Figure 5.9: A clear inclusion (PDMS) was depicted in (a), cylinder inclusion allowed a hollow opening to host a capillary. Depending on the depth of the hole, we arranged the fluorescence inclusion position in the inclusion. (b) Phantom was cut with a scalpel over the scanning area (red rectangular). Inclusion was positioned (dashed rectangular) prior to solidification of agar solution. (c) Raw data demonstrates three different localized fluorescence signals in the phantom, through the inclusion.

(Alexa 660, Life Technologies, USA). The phantom was imaged non-concurrently with ultra-sound and MFMT. The border of the scanning areas was marked via cutting the matrix with a scalpel leading to strong contrasts in MFMFT raw data Fig.5.9(b), analogous to tail immersed in intralipid, Fig.5.9(c).

These cuts were used as 2D fiducial markers for registration between the two modalities. 2D MFMT measurements from the first detector were matched with the 2D corresponding US projection. The US data were rescaled through linear scaling and resampled to match the MFMT images. Registration was performed by pixel-to-pixel matching. We applied this co-registration scenario to three different cases: fluorescence capillary within the PDMS inclusion, fluorescence inclusion next to the PDMS inclusion, and fluorescence inclusion at the top part of PDMS cylinder. A similar registration methodology was employed to co-register the MFMFT and US data for tumor imaging. The boundaries of the tail were clearly delineated on both imaging modalities and used as 2D fiducial markers for rigid registration.

5.4.1.7 Image Analysis

Estimation of the tumor volume and PS bio-distribution volume were calculated from US and MFMT respectively. Tumor volume was obtained via image segmentation of the 3D US data. Prior to creating a volume for tumors; A-scan im-
Figure 5.10: (a) Ultrasound B-mode image shows the membrane that separates transducer media from the outside (membrane), tumor area and the skin of the tail, scale bar 1mm. (b) Segmentation was performed based on thresholding at 10% of maximum intensity thanks to high contrast between tumor area and the surrounding. (c) Same slice of US and MFMT reconstruction was overlaid to show the correspondence between the modalities. (d) Cross section of the reconstructed images was shown together; tail (gray), US (blue) and MFMT (red), white border shows the segmentation boundary for US slice (a) and segmented slice (b).

Images were segmented and inter-spacing between slices was adjusted to the same scale with 2D images. Due to high contrast, simple intensity threshold at 10% of maximum was performed and enclosed volume was calculated by an embedded software function (function SurfaceArea/Volume, Amira 5.2). PS bio-distribution volume was estimated using same protocol, though with various threshold values (see below). An example of US data, segmentation and overlay with MFMT data is provided in Fig.5.10. Due to the regularization term and diffuse nature of the optical reconstructions, multiple isovolume thresholds were used to compute the PS bio-distribution volume (10 – 40%).
5.4.2 3D Reconstructions and Analysis

5.4.2.1 Phantom Study Results

MFMT raw data presented in Fig.5.9(c) depicts fluorescence signals acquired by the first detector of the optical imaging platform for various position of the capillary. By design, the fluorescence signal was constrained within the capillary and MFMT captured those localized signals effectively, Fig.5.11(a-c). US images were segmented via software (Amira 5.2, Visage Imaging Inc.) and fused with MFMT data as described in the Co-registration of US and MFMT data sets section. In all three cases investigated herein, successful non-concurrent registration was obtained between MFMT data and US data as shown in Fig.5.11. MFMT data and US data were positioned at their respective correct location as expected from the phantom design.

5.4.2.2 Tumor Imaging Results

MFMT data acquired on all 7 tumors and a tail without lesions are provided in Fig.5.12. The measurements are provided for the first detector (800 $\mu$m offset), Fig.5.12(a) and the second detector (1,320 $\mu$m offset), Fig.5.12(b). In the case of no tumor, fluorescence signal was weak. On the contrary, in all tumors, strong fluorescence signals were acquired and the fluorescence signals were always confined.
Figure 5.12: Background subtracted raw data for the no tumor tail and 7 different tumors. (a) 1st detector raw data, b) 2nd detector raw data. Both detectors deliver high contrast and highly localized signal. Images show 3mm by 6mm scanning area.

within the area of the lesion. No remnant fluorescent signals were observed outside the lesion regions. However, the fluorescence signals captured by the first detector (which is close to epi-configuration) were highly heterogeneous over the tumor area. Strong focal signals were captured that were confined to small area of the tumor. Conversely, the fluorescence signals acquired from the second detector exhibited a greater spatial distribution. For this set of data, the fluorescence signals were acquired over the vast majority of the lesions spatial extent. These results are expected as when the detector offset increases, the fluorescence signal acquired originates from deeper tissue in the tail. The discrepancy between shallow fluorescence signals and deeper fluorescence signals, as seen in all cases herein, exemplifies the heterogeneous
depth distribution of the PS.

As a note, the 2D fluorescence map for the offset detectors can display signals outside the tail tissue. As the illumination beam is raster scanned, the offset detector can be located outside the tail tissue and still collect fluorescence signal emitted within the tail, thanks to the matching liquid. This location blurring is corrected when performing optical reconstructions as the photon propagation is modeled accurately.

Overall, these raw data demonstrate the potential of our imaging platform to sense HPPH fluorescence signals over a large area and with enough sensitivity to provide depth dependent measurements. Even after 40hrs., the captured fluorescence signals are still strong for all 7 detectors in this BCC model. Moreover, these data also support the need to monitor in 3D the PS bio-distribution prior and during PDT treatment with dense spatial sampling. Our MFMT system is based on a resonant galvanometer mirror for ultra-fast scanning. Such implementation allows collecting dense spatial measurements on the imaged surface.

In this specific study, measurements were collected each 100µm (laser beam positions) over the full area. Such dense spatial sampling allows revealing the highly heterogeneous distribution of PS in the tumor both in the illumination plane and in depth. Such information is not attainable using fiber based systems that are limited in their measurements channels and hence, prone to error sampling. To analyze the PS bio-distribution further, MFMT reconstructions were performed and co-registration with US was achieved. The demonstration of co-registration process was depicted in Fig.5.10.

5.4.2.3 MFMT Reconstructions and US Co-registration

An example of 3D co-registration between MFMT and US for Tumor 7 is provided in Fig.5.13. The co-registration allows visualizing the tumor volume and PS bio-distribution as estimated by the methods described above. The visualization is expected to provide the overall structure of the lesions via US and local drug delivery via fluorescence 3D imaging.

The same process was applied to coronal planes of US reconstructions, were
Figure 5.13: Both US (blue) and MFMT (red) was used to generate 3D image of the tumor. US data also gave tail structure (pink). MFMT data was acquired by raster scanning 6mm $\times$ 3mm area over the tail and 20% ISO value used to visualize the MFMT image (a). Top view (b) shows the overlay of two reconstructions and the scanning area (orange box). (c) Interrogation depth and depth co-registration is shown.

overlaid with first and second detector raw data (Fig.5.14), showing the heterogeneity within each tumor.

Also, sagittal planes for all tumors shows the depth profile (Fig.5.15). The corresponding estimated volumes are reported in Table 1. In all cases investigated, the PS bio-distribution was found to be located within the tumor volume as estimated by US. However, as described previously, the PS bio-distribution was highly heterogeneous. In the majority of the cases, the PS bio-distribution was limited to a sub-area of the lesions.

Only in the case of Tumor 1, which was a very small tumor (0.22mm$^3$), the PS bio-distribution volume was bigger than the tumor volume for isovolume threshold equal and larger than 20%. Also, the PS distribution had complex depth profiles. These results indicate, as expected, that there is no one-to-one match between tumor volume, as obtained via structural imaging, and PS biodistribution. The PS biodistribution is unique to each tumor and is highly heterogeneous in all dimensions. Even, in some cases, such as Tumor 2, the PS seems to accumulate in the
Figure 5.14: US reconstruction was overlaid with (a) first and (b) second detectors. Black lines are tail boundary. (a) and (b) shows the strong heterogeneity within each tumor. Scale bar, 1mm.

Figure 5.15: All tumors were shown in increasing volume order according to the US volume estimation. Tail (gray), US tumor (blue) and MFMT tumor (red) are all in shown in same scale. Scale bar, 1mm.
peripheral tissues.

5.5 Summary

This preliminary study aimed at investigating the potential of MFMT in retrieving the 3D bio-distribution of a PS prior to treatment. One key aspect of the investigation was to establish that MFMT had enough sensitivity to acquire fluorescence signals emitted by the PS over large surface, with high spatial sampling and at fast acquisition speed. Our results demonstrate that our MFMT platform is able to acquire such data sets within a minute and with a 100µm spatial sampling rate. Moreover, tomographic reconstructions were performed using these data sets to visualize the PS bio-distribution in 3D. The computation time required for computing the forward model and solve the inverse problem was < 4 minutes on a personal computer. The overall imaging session was achieved in 5 minutes, which is compatible with clinical practice. According to the American Cancer Society a PDT treatment takes 45-60 minutes, which indicates that our current system can be integrated into PDT patient work-flow without significant interference. We foresee that with the combination of more sensitive detectors to reduce the data integration time, and optimized reconstruction algorithms, that MFMT imaging procedure could be reduced to a few seconds. Then, MFMT could be also a promising imaging modality candidate to monitor PDT efficacy non-invasively during treatment.

Additionally, the data acquired on the 7 tumors imaged in this study demonstrated a highly heterogeneous PS bio-distribution. The overall PS volume did not match one-to-one with US data. This was expected as the contrast function difference between US and MFMT inherently yield different information. in-vivo HPPH distribution is expected to be affected by diffusion, localization, compartmentalization between blood vessels (HPPH was iv-injected) and tumor interstitial space, parameters that are both intra- and inter-tumor heterogeneous [159]. Our results comfort these anticipations and suggest that imaging techniques that have a high spatial sampling are required for mapping the PS uptake. Moreover, if anatomical data such as obtained by US could be useful to define the area to be imaged, they cannot be used as prior information for PDT planning and optimization [160].
Another point of importance is that: herein, we focused on the relative distribution of the PS agent, not on the quantification of the PS concentration. Quantifying fluorophore concentration absolutely is very challenging as it is greatly influenced by the optical properties of the tissue. Here, we employed a classical linearized tomographic formulation that employs the average optical properties of the tissue to compute the optical forward model. A more accurate approach would be to map optical properties at both excitation and emission wavelengths and provide the optical properties as *a priori* information for the fluorescence reconstruction [161]. Such an approach has been described by Rohrbach et al. [162] and Saager et al. [163] who employed spatial frequency domain imaging (SFDI) to estimate the optical properties of tissue over large areas, which in turn, allowed extracting absolute fluorescence concentration in 2D. Still, it is a very challenging task to be able to extend such work in 3D, though, Konecky et al. [164] demonstrated that SFDI data sets can be also employed for tomographic reconstruction of optical properties. This 3D map of the optical properties could then be used as *a priori* information to compute accurately the optical forward model for absolute reconstructions of the PS concentrations.

This work reports the initial evaluation of using MFMT for retrieving the 3D distribution of PS in skin cancer prior to PDT treatment. To this end, HPHH was injected intra-peritoneally into mice expressing spontaneously skin cancers. Tumors located in the tail of the animal were imaged using MFMT and US. In all cases investigated herein, all lesions exhibited strong fluorescence that allowed for mesoscopic optical reconstructions. Overall, the acquisition time of fluorescence signals over $6mm \times 3mm$ area was completed within 1 minute, whereas the optical reconstructions were performed within 4 minutes on a personal computer. This preliminary investigative study highlights the potential of MFMT to retrieve the 3D PS bio-distribution within a few minutes. Future study will focus on imaging the 3D PS bio-distribution changes over treatment and optimize the reconstruction algorithms for allowing near real-time feedback during therapy. In conjunction with active illumination strategies [130, 165, 166], we expect that MFMT will improve PDT efficacy for deep pathologies.
Chapter 6
The Second Generation MFMT (2GMFMT)

6.1 System Properties and Improvements on the System

The second generation MFMT (2GMFMT) focuses on improving two aspects of the first generation: developing a flexible and more sensitive optical system and achieving higher resolution in the reconstruction algorithm. Below, I present the target components and rationale to realize those goals:


Detector Selection

- APD arrays offer a limited number of detectors. Tomographic imaging using reflection geometry already suffers from limited projections compared to transmission geometry. To mitigate this limitation, we decided to switch to 2D detector arrays.

- 2D APD arrays are commercially available, but incorporating them requires a custom-made acquisition system. This system is prone to electrical noise, which can decrease the efficiency of APD detectors and increase the cost significantly.

- EMCCD cameras offers exceptional Quantum Efficiency (90-95% in visible regime). Industrial grade packaging reduce the system noise to the current technological limits. Electron Multiplying (EM) Gain enables to reach low level of signal without increasing exposure time. From tomographic aspect, it offers great flexibility on detector size (Binning) and detector locations (on-demand selection of detectors).

Galvo-Mirror Selection

- Resonant scanning galvo-mirrors give superior performance in terms of speed, but lack the flexibility in adjusting the dwell time on the sample. The fixed exposure time for resonant galvo-mirrors limits the minimum detectable signal level due to the minimal dwell time (exposure) of light. However 2D adjustable galvanometer mirrors can be adjusted depending on the signal level from the sample.

Reconstruction Algorithm

- The size of the measurement data will increase due to an increase in the number of detectors. This requires an optimization of the number of detectors and sources. In turn, this constraint put some stress on the size of the inverse problem to solve. Because, due to the diffusive nature of light in scattering
media, most likely only a subset of measurements will lead to adequate reconstruction. Hence, an important question to answer is: "What are the sufficient number of sources and detectors?"

Below, a brief description of the 2GMFMT system will be followed by detailed characterization studies.

The optical setup is comprised of an EMCCD camera, scanning galvo-mirrors and relaying optical elements, Fig.6.1. Excitation light is introduced into the system, passing through a linear polarizer (P). A polarizing beam splitter (PBS) reflects \( \sim 90\% \) percent of the S-polarized light onto the galvo-mirrors (GM). The excitation signal is then raster-scanned through a scan lens (SL) with a working distance of 25.4mm. Reflected excitation and backscattered emission signal follow the same path as the excitation light. The PBS transmits \( \sim 90\% \) of the P-polarized light while minimizing specular reflection of the S-polarized excitation light. In order to further minimize specular reflection, the collected light is led to another linear polarizer (analyzer, A). For spectral separation, collected light passes through a color filter. A relay lens system precedes the camera in order to place a reflection block (RB) on the image plane. The reflection block minimizes the source signal that will be propagated onto the detector and prevents the source signal from saturating the camera and hence, reducing the dynamic range. The reflection block was in place for all data collection including fluorescence, background and system reflection. The RB was used when higher binning (> 16) was used because the spatial integration that occurs during binning becomes a limiting factor for the dynamic range. RB is removed when low binning (< 16) was used. After RB, the image is relayed onto the camera with a 4F imaging system (4F).

6.1.1 Design Parameters

Prior to implementing the second generation MFMT system, we conducted an in silico study to investigate the main part of the system: the effect of detector number on optical reconstruction fidelity. The first generation system suffered from high electrical noise and fixed excitation dwell time (exposure time) for each scan point, which hindered the potential performance of the MFMT system. Hence, we com-
Figure 6.1: Excitation light (Ex) was transmitted into the system through a polarizer (P) and any stray light was cross-polarized (A). The reflected light and fluorescence emission, from the beam splitter (BS), executes raster scanning through the use of the galvanometer mirrors (GM), and the scanning lens (SL). The polarized emission signal passed through a color filter (F) and was imaged with an EMCCD camera using a tube lens (L). If the camera operates in high binning condition, then a reflection block (RB) is placed before the emission signal transmitted to the camera.

Compare two main types of detector configurations herein: 1D and 2D detector arrays, which simulates a comparison between Avalanche Photodiode (APD) and Charge Coupled Device (CCD), respectively. Inherently, both APD and CCD detectors may be utilized in 1D and 2D configurations. However, due to the complexity and cost of integration, APD was considered as 1D and CCD was considered as a 2D detector array. The reasoning behind such an investigation has multiple aspects:

1. Currently available APD detectors have a limited 2D array selection.
2. High quantum efficiency (QE) 2D APD detectors have asymmetrical detector placement [167].

3. Symmetrical 2D APD arrays exhibit dramatic wavelength sensitivity over the visible range \((20 - 75\%)\) [168].

4. APD arrays with a large number of detectors are still in the research level [169].

An \textit{in silico} model was designed to optimize the configuration of the optical system and evaluate the performance of the proposed method in terms of reconstruction accuracy and robustness to noise. First, a regular model representing a vascular tree, with main trunk and three groups of off-shoots with \(200\mu m\) separation along three coordinate axes was assembled (Fig.6.2). The diameters of the vascular tree components are \(200\mu m\) and \(400\mu m\) as shown in Fig.6.2. The uniqueness of this model is that the system reconstruction performance can be evaluated in all planes (i.e. \(x - y, x - z, y - z\))

The fluorophore concentrations are assumed to be homogeneous over the vessel with effective quantum yield equal to one. The simulated imaging domain has a \(6.2 \times 6.2 mm^2\) surface area with \(3mm\) depth. In this work, we discretized the imaging domain into \(31 \times 31 \times 15\) voxels with \(200\mu m\) voxel size, resulting a Jacobian matrix size of \(46,128 \times 14,415\) (961 source positions \(\times\) 48 detectors by \(31 \times 31 \times 15\) reconstruction volume). We also assume a homogeneous reduced scattering coefficient \((\mu'_s = 0.5mm^{-1})\) and endogenous absorption coefficient \((\mu_a = 0.002mm^{-1})\) over the entire sample. The Henyey-Greenstein phase function was used for all computation herein with \(g = 0.9\). These parameters were derived from the optical properties of the collagen scaffold employed in our bio-printing application at collagen density of \(9mg/ml\) [27,38]. The vascular tree was first employed to compare the performance of reconstruction when using 1D and 2D detectors to investigate the merit of using 2D detectors by placing the same number of radially spaced (1D) in a square grid formation.

Secondly, we observed the effect of detector number on the reconstruction. In this experiment, we kept the maximum source-detector separation fixed while
Figure 6.2: A numerical phantom was generated to mimic a vascular structure. Different branches were laid down in different planes to show the power of our reconstruction algorithm and effectiveness of data collection. The main trunk has a diameter of 400µm; the off-shoot branches are 200µm in diameter and separated by one voxel spacing.

Increasing the density of the detector array: 3 × 3, 4 × 4, 5 × 5 etc.

Next, the number of source positions was examined with regards to reconstruction performance while keeping the number of detectors fixed at 49 detectors (7 × 7). Even though these two experiments provided insight on how the data size affects the reconstruction, it is not clear how these two components (number of sources and number of detectors) of the measurement vector would have an effect with respect to each other. So, to address this question, we designed another experiment with approximately the same amount of measurement data. In each experiment we changed the number of detectors and number of sources, reciprocally, decreasing one while increasing the other. For all of the above experiments, we kept the noise at 10dB.

Then, we tested the system under increasing noise levels (5 − 25dB with the steps of 5dB) with the optimal number of sources (31 × 31) and detectors (7 × 7). Then, the we compare the reconstruction with numerical phantom for each of the noise levels. For all these characterization experiments we use evaluation metrics,
described below.

### 6.1.1.1 Evaluation Metrics

We adopted merit functions from the registration literature [170,171] to compare results of the reconstruction with the true numerical phantom. The metric values lie between 0 and 1 (The reconstruction (A) is completely different than the Numerical Phantom (B) and the reconstruction (A) is identical to the Numerical Phantom (B), respectively) and represent different aspects of the similarity between the two images. The normalized sum squared difference (nSSD) metric is sensitive to large variation between two voxels [171], while the normalized sum absolute difference (nSAD) in each voxel value is less sensitive to those differences.

\[
nSSD(A, B) = 1 - \frac{1}{N} \sum_{i}^{x} \sum_{j}^{y} \sum_{k}^{z} [A(i, j, k) - B(i, j, k)]^2 \tag{6.1}
\]

\[
nSAD(A, B) = 1 - \frac{1}{N} \sum_{i}^{x} \sum_{j}^{y} \sum_{k}^{z} |A(i, j, k) - B(i, j, k)| \tag{6.2}
\]

where \( N = X \times Y \times Z \), \( X \) and \( Y \) are the imaging volume along the two axial axes and \( Z \) is the image size along the longitudinal axis. \( A \) and \( B \) are numerical model and reconstructed vessel respectively. \( i, j, \) and \( k \) are the coordinates in the imaging volume.

A similar trend in nSSD and nSAD indicates the absence of outlier voxel values in reconstruction. The normalized disparity (nD) is the similarity matrix after performing an XOR operation on two volumes: the numerical phantom and its reconstruction. A value of 0 indicates two completely different volumes; as the nD value gets closer to 1, similarity between the two increases.

\[
nD(A, B) = 1 - \frac{1}{N} \sum_{i}^{x} \sum_{j}^{y} \sum_{k}^{z} A(i, j, k) \oplus B(i, j, k) \tag{6.3}
\]

The correlation metric (R) indicates the strength of a linear relationship between the numerical phantom and its reconstruction. It produce values between
0 (for two different shape) and 1 (for two identical shape).

$$R(A, B) = \frac{\sum_i^x \sum_j^y \sum_k^z A(i, j, k) * B(i - m, j - n, k - o)}{\sum_i^x \sum_j^y \sum_k^z A(i, j, k)^2 * \sum_i^x \sum_j^y \sum_k^z B(i - m, j - n, k - o)^2}$$  \hspace{1cm} (6.4)$$

where the displacement with respect to the reference is expressed by $m$, $n$, and $o$.

6.1.1.2 1D vs. 2D Detectors

The evaluation metrics showed that 2D detector array delivered a better reconstruction performance than 1D detector array. Although the difference is small, practically it is difficult to employ a line of 49 detectors in an optical setup because the limited aperture size will only allow a certain number of radially aligned detectors. Thus, using 2D detector arrays offers simplicity in optical setup along with better performance (Fig.6.3)

Figure 6.3: Reconstruction performance of 1D and 2D detector arrays were compared for the same number of detectors (e.g. $1 \times 9$ vs. $3 \times 3$). (a1–e1) shows the 3D reconstruction results from the 1D detector array. (a2–e2) shows the reconstruction results from the 2D detector array. In the graph, evaluation metrics are shown: Black, Red, Purple and Blue are for correlation (R), disparity (nD), normalized Absolute Difference (nSAD), normalized Sum Square Difference (nSSD), respectively. The dotted-lines represent 1D detector array, and solid-line represents 2D detector array. Overall 2D detectors showed better performance than 1D detectors.
6.1.1.3 Number of Detectors

As shown in the previous case, an increase in the total number of detectors results in better reconstruction; however, increase in detector number brings a computational burden as the measurement data size increases. To highlight the optimal performance and complete characteristic of higher detector number, we carried the previous study one step ahead (to the maximum capacity of our personal computer). This simulation showed us that increasing the detector number beyond $7 \times 7$ does not provide a significant benefit, since most of the metrics reached a plateau, Fig. 6.4. Another restriction for the optical system is having a symmetrical layout of detectors. We reserved the middle detector for the source. The rest of the detectors had to be symmetric over the source point, which sets a constraint of having an odd number of detectors. As a result, we employed a $7 \times 7$ detector array for our optical system.

![Figure 6.4: The effect of detector number on reconstruction performance is shown for 2D detectors. Detector numbers were $3 \times 3$, $4 \times 4,...,8 \times 8$. Evaluation indicates an asymptotic performance with the increase in detector number.](image)

6.1.1.4 Number of Sources

The number of source positions is a critical parameter as it dictates both the size of the measurement and the acquisition time. Below we show that the effect of source number is stronger than the effect of the detector number. Similar to the detector number, the effect of source number showed an asymptotic behavior,
reaching a plateau at 26 × 26 source positions (Fig. 6.5). As we mentioned above, due to the symmetry constraint we chose a 7 × 7 detector array. To counterbalance the choice of a lower detector number, we chose a higher source number (31 × 31). The cooperative effect of both number of detectors and number of source positions is further investigated in the next section.

Figure 6.5: The effect of source number on reconstruction performance is shown for 2D detectors. Source numbers were 11 × 11, 16 × 16,..., 31 × 31. Evaluation indicates a steep asymptotic performance with the increase in source number.

6.1.1.5 Combined Effect of Detector and Source Number On Reconstruction

The total number of measurements is the product of the number of scan positions (source number) and the number of detectors. Mathematically, the number of equations is approximately the same for all cases. However, due to the different scale associated with detector sampling and source scanning, their respective density do not offer similar information. Here we tested 6 different measurement numbers and their reconstructions to show the data quality (Fig. 6.6). The results indicated that the source number has more impact on the reconstruction quality than the detector number. This confirmed our selection of source number and detector number above.
The combined effect of source and detector number was investigated by changing the source number and detector number, reciprocally. Metrics indicate a better reconstruction performance when a high number of source positions is used.

6.1.1.6 The Effect of Noise on Reconstruction

Sensitivity to noise is a crucial aspect of fluorescence imaging, even more so for fluorescence tomographic imaging. Fig. 6.7 shows the four evaluation metrics versus SNR at five different levels of noise. All four of the metrics showed similar response above an SNR value of 15dB, which indicates that reconstruction performance is also sensitive to noise. However, in comparison to our previous optical system with 1D detectors array which can reconstruct numerical models, with SNR > 40dB [114], the proposed 2D detector array still outperforms its former counterpart especially at low SNR levels.

Figure 6.6: The combined effect of source and detector number was investigated by changing the source number and detector number, reciprocally. Metrics indicate a better reconstruction performance when a high number of source positions is used.

Figure 6.7: Evaluation metrics for five different levels of noise SNR of 5-25dB were shown. The 2D detector array shows good performance down to an SNR level of 10dB.
6.1.1.7 Effect of Cooling and EM Gain on Sensitivity

In an optical detection system, the noise model is dependent on the contrast mechanism and on the type of detector used. The Noise Equivalent Signal (NES) for our EMCCD is formulated in the absence of high EM gain and deep cooling by the following equation [172]:

\[
NES = N_f^2 \sqrt{S + D} + \frac{\sigma_{\text{readout}}^2}{G^2}
\]  

(6.5)

where the noise factor \((N_f)\) has a strong contribution along with the mean number of generated electrons \((S)\), and the mean number of dark electrons \((D)\). \(N_f\) is inherent to all EMCCDs and stems from statistical variation during the electron multiplying process. The noise factor for EMCCDs has been well documented, and stated as \(\sqrt{2}\) [172]. Readout noise \((\sigma_{\text{readout}}^2)\) can be minimized by increasing gain \((G)\). With high enough gain and deep cooling, the effects of readout noise and dark current are minimized, resulting in a simplified noise model:

\[
NES = \sqrt{S} \star N_f
\]  

(6.6)

The parameters of Eqn.6.6 were estimated experimentally, using the data collection, described below. To test the performance under different conditions, we chose three different Electron Multiplying (EM) gains: No Gain, 50, and 200 where No Gain served as a control case. Then, we changed the cooling temperature gradually from room temperature \((21^\circ C)\) down to \(-30^\circ C\) and \(-75^\circ C\). Room temperature was used as a control case. We stopped at \(-75^\circ C\) because below that temperature the camera would require water cooling to stabilize in a short amount of time. Air cooling was sufficient to cool and operate the camera uninterrupted at \(-75^\circ C\) for a sufficient amount of time (< 1 min).

For each test, the raw images of the capillary with fluorescence \((s)\), capillary with DI water \((b)\), and with the shutter closed \((r)\), were taken. Then, signal to noise ratio was calculated. The data acquired from the MFMT system was analyzed to compute the SNR (dB) following the formulation:
\[
SNR = 10 \log \left[ \frac{\sum_{n_x=1}^{n_{x}} \sum_{n_y=1}^{n_{y}} s(n_x, n_y)^2}{\sum_{n_x=1}^{n_{x}} \sum_{n_y=1}^{n_{y}} n(n_x, n_y)^2} \right] \tag{6.7}
\]

where \( n_{x,y} \) are the \( x \) and \( y \) indices of the output frame of the system. The main fluorescence signal (middle) and the backscattered shadow signal (right) are typical to MFMT data (c.f. Fig.6.8) and are essential for the reconstruction process. The raw images (\( a \)) were generated after the scanning was completed.

The signal was calculated by subtracting the background (\( b \)) from the raw signal (\( s = a - b \)). Similarly, we calculated the noise by subtracting the reference from the background signal (\( n = b - r \)). Both signal and background were then linearly scaled so that the minimum pixel intensity was set to zero. This was done to mitigate any negative pixel intensities from the subtraction process.

Finally, the significance of the two factors, Cooling Temperature and EM Gain, was estimated by two-sample t-test. Results show that both EM gain and deep cooling had a significant effect on overall system SNR. Applying EM gain and deep cooling resulted in a general trend of increasing SNR for each frame. There is a large difference between the control (no gain or deep cooling) SNR and the tested SNR (with EM Gain and Cooled Temperature).

Typical examples of preprocessed images and SNR are provided in Fig.6.8, which correspond to a detector, \( \sim 2 \) mm away from the source.
Figure 6.8: (a-c) Images for detector 10 (∼ 2mm source-detector separation) at all source positions for a 10nM sample. (a) no gain and no deep cooling. (b) 50X gain and deep cooling of −30°C. (c) 200X gain and deep cooling of −75°C. The center bright region of each image is where the capillary is located, while the dimmer region (right) is where the backscattered shadow is located. (d) The average SNR values for detector 10 at various parameters for the corresponding images (b-c).

More importantly, EM gain and deep cooling had a profound effect on the SNR of detectors further away from the source. Fig.6.9 shows that both 50 and 200 gain increased the SNR by at least 3 fold for various source-detector separations. Furthermore, EM gain allowed the SNR for the furthest detector, a distance of ∼ 3.8 mm from the center, to become positive, resulting in the signal levels surpassing the noise. Note that larger source-detector separations result in lower signal levels. Here, EM gain enables capturing of signals that are otherwise impossible to detect.
Figure 6.9: The average SNR values for detectors at a distance of ∼1.3 mm, ∼2.6 mm, and ∼3.8 mm away from the source (center pixel) is presented. These SNR values were taken for a 1nM sample at −30°C. SNR exhibited a visible difference when gain is applied.

The signal intensity exponentially decays as the contrast molecules go deeper in the tissue. The following experiment showed the effect of EM gain during deep cooling (−75°C) while the fluorescence target was placed at different depths: at the surface and 1mm, 2mm, 3mm below the surface. To show the effect of EM gain and Deep cooling we chose a detector that is ∼1.2 mm away from the source position.
Figure 6.10: Effect of gain on SNR values at different depths for a glass capillary tube of Alexa590 with a concentration of 10nm is shown. For all cases, EM gain (100) showed an increase on SNR level even in the most challenging conditions of 3mm depth.

The result shows that even at 3\textit{mm} deep, signal can be improved by $\sim 2$ fold by using EM gain. It is expected to see a decrease in the SNR level by applying too much EM gain on the signal, Fig.6.10.

The most important benefit of EM gain is to be able to increase the SNR level without increasing the exposure time or increasing the injected power on the sample which is the procedure traditionally. However that puts a limit on acquisition time. EM gain enables collection of a signal with reasonable SNR without compromising the acquisition time. This feature will be valuable especially for imaging live cells, as fluorescing molecules are sensitive to power density of light and photobleach quickly under prolonged illumination.

6.1.2 Summary

We optimized the source and the detector number for our second generation MFMT system (2GMFMT). Due to the dominant effect of source number, we selected a higher source number ($31 \times 31$) than detector number ($7 \times 7$). Then we
characterized the sensitivity of the system under air cooling and electron multiplying gain (EM). The sensitivity study showed that the 2GMFMT system is capable of detecting 1nM fluorophore at 2mm deep in scattering tissue with an SNR value of \( \sim 5dB \) at the furthest detector. As a result, we conclude that the system is capable of detecting concentrations \( \sim 3 \) times lower in same conditions \( (\sim 300pM \text{ at } 2mm) \) as we adapted the similar extrapolation methods similar to those in the literature [173].

6.1.3 3D Reconstruction

6.1.3.1 Forward Problem

Eq.4.1 is the basis of the optical inverse problem in MFMT. We can form a linear system of equations that link the acquired measurements with the unknown distribution of the fluorescent probe. The image space is discretized in elements of unit volumes (voxel) and then the linear system can be expressed as:

\[
F \equiv \begin{bmatrix}
F(r_{s1}, r_{d1}) \\
F(r_{s2}, r_{d1}) \\
\vdots \\
F(r_{sp}, r_{d1}) \\
F(r_{s1}, r_{d2}) \\
F(r_{s2}, r_{d2}) \\
\vdots \\
F(r_{sp}, r_{dq})
\end{bmatrix} = \begin{bmatrix}
U_{11} & \cdots & U_{1N} \\
\vdots & \ddots & \vdots \\
U_{M1} & \cdots & U_{MN}
\end{bmatrix} \begin{bmatrix}
C_{r1} \\
C_{r2} \\
\vdots \\
C_{rN}
\end{bmatrix}
\]

(6.8)

where \( F \) is the measurement vector from each source-detector pair and for each source position \( (r_{si}) \). \( M \) different measurements \( (M = p \times q) \) are acquired to solve \( C(r) \), which is represented by \( N \) discrete voxels and \( p \) and \( q \) are source and detector numbers, respectively. \( U = [U]_{M \times N} \) is referred to as the weight matrix or
sensitivity (Jacobian) matrix. The optical inverse problem aims to solve this set of linear equations to retrieve $C(r)$. This is done by first constructing the Jacobian matrix using a light-propagation model and *a priori* knowledge of the sample geometry, endogenous optical properties (absorption and scattering coefficients), and fluorophore characteristics (extinction coefficient and quantum yield) as well as the relative positions of the source-detector pairs. Then, since the linear system cannot be directly inverted, an appropriate solver is employed to form the tomographic reconstructions.

We utilized Monte Carlo (MC) simulation to generate our sensitivity matrix. Our studies enabled us to use a planar boundary and our relative source-detector positions have symmetry among themselves. Incorporating all of these aspects, we used the voxel-based MC (*tMCing.exe*). Inherently, voxel-based MC is computationally expensive. However, due to the symmetry of the source-detector pairs we only simulate unique pairs (49 pairs), then populate the rest of the source-detector locations over the entire field of view.

A representative example of different sensitivity matrices is shown in Fig.6.11. Since the 2D detector array scans through the field of view on a point by point basis, the sensitivity matrix has to model that behavior. Thus, the model would include $m$ number of equations and $n$ number of voxels for each source-detector. The total size of the matrix will be governed by the following formula (6.9 and 6.10):
Figure 6.11: Population of sensitivity matrices is shown. Due to the symmetry in photon propagation, we generated only one source-detector pair set (typically 1 source and 49 detectors). The Black dashed area depicts effective field of view, and red lines depict the raster scanning points. As a demonstration we presented the extreme corners: (a) Source position 1 and Detector 1, (b)Source position 931 and Detector 43, (c) Source position 31 and Detector 7, (d) Source position 961 and Detector 49. That enabled us to model high number of source locations in less than 30min.

\[ m = d_x * d_y * scan_x * scan_y \]  \hspace{1cm} (6.9)

\[ n = v_x * v_y * v_z \]  \hspace{1cm} (6.10)

where \( d_{x,y} \) is the number of detectors in the \( x, y \) directions, \( scan_{x,y} \) is the individual source location that the light dwells on, and \( v \) is the voxel number with which the
interrogation volume is discretized (Appendix B.1). With this configuration, we generated the sensitivity matrix with the MC code (tMCimg.m). For the optimized values of $31 \times 31$ source positions and $7 \times 7$ detectors, it took 1.5 hours for $10^6$ photons, and 15 minutes for $10^5$ photons to complete the matrix generation on a personal computer (Intel® Core™ i7-3820 CPU, 3.60 GHz with 64 GB Memory).

![Diagram of raster scanning](image)

**Figure 6.12:** A schematic of 1D and 2D raster scanning is shown. As can be seen 2D has an increased effective field of view for the same amount of scanning time as well as increased number of collected data.

### 6.1.3.2 Inverse Problem

#### 6.1.3.2.1 $l_2$ - norm Regularization

After forming the forward problem, the 3D distribution of the fluorophore can be estimated using an inverse solver [174]. The diffuse optical inverse problem is well-known as one of the most difficult ones to solve. It is typically ill-posed (less measurements than unknowns) and always ill-conditioned due to the diffuse nature of light propagation. Moreover, reflectance geometry provides a more challenging inverse problem than transmittance geometry due to limited angular sampling [175].

The standard approach to solving inverse problems is to minimize $\|Ax - b\|$ using an iterative solver where $A$ stands for the Jacobian, $x$ is the unknown fluorophore distribution, and $b$ is the measurement. With such solvers, the estimate (image space) is updated iteratively to minimize the norm between the experimental measurements and the estimated measurements as obtained by the product of the
Jacobian and the image space. Typically the iterative process is terminated either when a preset number of iterations is reached and/or when the residual of the norm is below a set value (tolerance). The maximum number of iterations and tolerance are chosen *ad hoc* and may vary based on the solver used. The most common iterative solvers employed in the field are the conjugate gradient (*CG*) method [27,38,39,176], the least squares (*LSQR*) method [36], and algebraic techniques [33,177].

However, solving the linear system using these iterative solvers is still difficult since the system is ill-conditioned and thus very sensitive to noise propagation [178]. Hence, a regularization term is typically introduced to reach a balance between accuracy and high-frequency noise mitigation in the estimate. This regularization parameter is used to effectively control the influence of the model mismatch, noise, and systemic error during reconstruction [22]. Even in the case of over-determined systems such as MFMT systems in the de-scanned configuration [29,38], regularization is still required. A typical formulation of the inverse problem in MFMT is:

\[ x' = (A^T A + \lambda D)^{-1} A^T b \]  

(6.11)

where \( b \) is the measurement vector, \( x' \) is the estimated spatial distribution of fluorophores, \( A \) is the Jacobian matrix, and \( \lambda D \) is a regularization parameter. If \( D \) is the identity matrix, then it forms the Tikhonov regularization that has been used successfully in MFMT [22, 60]. However, for optimal performance in reflectance geometry, \( \lambda D \) should be a depth-dependent regularization term [129]. In this case, \( D \) is a diagonal matrix whose elements are the square-root of the corresponding diagonal elements of \( A^T A \) [38,179], and \( \lambda \) is a scaling factor selected via L-curve analysis [158]. One caveat of these approaches is that the classical L2-norm employed in conjunction with regularization smooths out the reconstructions, degrading the resolution.

To enhance the resolution, a hybrid scheme of L2-norm Tikhonov regularization and simultaneous iterative reconstruction technique (SIRT) was proposed [33,34]. Theoretically, if the iterative process in SIRT leads to a regularized solution, it is a semi-convergent technique that produces better resolution than the Tikhonov regularization in the case of sparse solutions. Better resolution is obtained
for high iteration numbers at which the SIRT is over-reconstructing the results. Alternatively, there has been considerable development in the retrieval of sparse signals in the last decade, leading to the blossoming field of compressive sensing [180]. Among all the different approaches, sparsity constraints implemented as regularization terms have been successfully applied to DOT to improve resolution [65, 66]. These techniques are extremely well-suited for MFMT due to their inherently sparse fluorescence signals (by design) [176].

An example of improvement in MFMT performances when using sparsity constraints ($L_1 - norm$) over CG/LSQR is demonstrated in [114]. This example focuses on retrieving labeled vasculature in murine brain tissue. Compared to CG and LSQR methods, the $L_1 - norm$ approach retrieves the vascular beds at all depths with high accuracy. Note that these continuous structures are the most difficult to image and even better results are expected for sparse cell imaging. Ultimately, the combination of dense spatial data sets with compressive sensing-based methods should push MFMT resolution close to $100\mu m$ or beyond even at depths of several millimeters.

6.1.3.2.2 $l_1 - norm$ Regularization

A reconstruction algorithm is presented to resolve distribution of the fluorescence inclusion in an homogenous media, based on the linear model of the form with noise perturbations $v$,

$$ b = Ax + v $$ (6.12)

where $x \in R^n$ is the vector of unknowns, $b \in R^m$ is the vector of measurements, $v \in R^m$ is the noise, and $A \in R^{mxn}$ is the data matrix. When $m$, the number of observations, is smaller than $n$, simple least-squares regression can lead to overfitting. The $l_p - norm$ is a quantitative index to measure the sparsity of a signal. An $l_p - norm$ solution is sparser and more localized than the minimum norm least square solution ($MNLSQ$). If the Lp-norm of the solution is taken as a constraint, we have a Lagrange multiplier expression of the inverse problem, Eqn.6.13

$$ \min \|Ax - b\|^2 + \lambda \|x\|_p $$ (6.13)
where, \( \|x\|_2 = \left( \sum_i x_i^2 \right)^{1/2} \) denotes the norm of \( x \) and \( \|x\|_p = \left( \sum_i |x_i|^p \right)^{1/p} \) denotes the \( l_p \) norm of \( x \) and \( \lambda \) is the regularization parameter. This problem always has a solution, but it need not be unique. By introducing a regularization parameter \( \lambda \) in the standard \( l_p \) norm, the objective function becomes differentiable and its convex region is shown to be controllable by the regularization parameter. Moreover, by working in the null space of the measurement matrix, the optimization problem becomes unconstrained and hence it can be solved by using efficient quasi-Newton methods, a detailed comparison can be found in elsewhere [114].

In summary, we showed the benefit of utilizing a 2D detector array (EMCCD camera) in optical reconstruction. It is a well known phenomenon that an increase in the number of projections over the sample delivers better performance. In reflectance geometry, we proposed to increase the number of projections by using a 2D detector array. Our \textit{in silico} study showed that 2D detectors yield better performance. Our findings led us to another issue: optimizing the detector and source number. Based on our quantitative evaluation metrics, we selected \( 7 \times 7 \) detector array, and \( 31 \times 31 \) source locations on the sample surface \((x-y)\). Then, we ran our simulation to evaluate the sensitivity of 2GMFMT. The 2D detector array was able to reconstruct the signal level \( > 10 dB \) while 1D detectors were able to reconstruct the signal level \( > 40 dB \) [114]. Resolution was another parameter that we characterized \textit{in-silico}, which will be addressed in scattering solid phantom experiment in the next section, Section 6.2.1. As a result, we were able to resolve \( 200 \mu m \) separation between the small branches of the artificial vasculature. However, the resolution is ultimately limited by the discretization of the image space. Thus if we were to use smaller voxel size, we would be able to achieve higher resolution.

### 6.2 Solid Phantom Studies for Resolution Characterization

The 2GMFMT acquires densely sampled data sets which lead to high performance reconstruction. Herein, we will demonstrate the resolving power of 2GMFMT in a scattering environment.
6.2.1 Phantom Preparation

First, we prepared wetting mixture for red fluorescent polystyrene beads, UVPMS-BR-0.995 335 – 425µm, according to the recipe provided by the company (Cospheric, Santa Barbara CA, USA). To wet the spheres, we dispensed 0.1% Tween solution (Tween20 Surfactant, Cospheric, Santa Barbara, CA, USA) and centrifuged it for 5-10 minutes [181].

We used Agarose powder (Sigma-Aldrich, USA) to construct the solid phantom and Intralipid, 20% emulsion (Sigma-Aldrich, USA) to introduce scattering into the phantom. For the resolution study, we did not introduce any absorber to the media.

We stirred 1% Agar solution at 60 – 80°C for 5-10 minutes. Then we added 20% Intralipid solution for 0.5% concentration and stirred it for 15-20 minutes. The mixture was left stirring while the heat was turned off. We waited for the solution to get cooled down slowly. When the solution reached 40 – 50°C, we poured the solution into our predesigned mold.

To form the resolution experiment sample, we designed a plexiglass sample holder in which a sandwiched agar phantom resides. Polystyrene spheres were placed between the bottom layer (≈ 1mm) and the top layer (≈ 1mm). Then we sealed our sample holder with plastic screws. In the end we placed plexiglass (transparent) imaging windows on both sides which enables both inverted and epi-configuration imaging, and the all-plastic sample holder made our sample compatible with MRI imaging, Fig.6.13
Figure 6.13: Plastic sample holder is shown. (a) Side view shows the plexiglass thickness. Imaging was conducted through 2.75mm thick plexiglass. (b) Size of the sample holder is shown. The agar phantom dwells in the plexiglass.

6.2.2 Multimodal Raw Data Acquisition and 3D Reconstruction

A multimodal approach was taken to validate the 2GMFMT reconstruction. A similar approach will be used for the tissue engineering study as well. First, a Wide Field Fluorescence Microscopy (WFFM) image was taken, 6.14(a), prior to covering the top slice of the agar phantom to avoid scattering and hence to get the bead location on axial plane, accurately. Second, LSCM and MR images (MRI) were collected to get the 3D location of beads within the scattering agar phantom, 6.14(b-c). Lastly, we acquired the image with 2GMFMT, 6.14(d), and conducted the reconstruction for all three imaging modalities (LSCM, MRI and 2GMFMT).
A commercial software was used to merge all of the modalities (Amira 5.6, FEI, USA). For comparison purposes, a numerical replica of the fluorescent beads was placed within the reconstruction volume to show the location of the beads and to serve as a ground truth for the reconstruction, 6.15(a). The size of the beads was given based on WFFM image, and the depth is determined by the predetermined agar phantom thickness and validated by MRI (Appendix B.2).

We used an MRI imaging protocol for Agar phantom as follows: Echo time is 72msec., Rare Factor is 12, and the Repetition time is 1,300msec. The isotropic cubic voxel was 100 $\times$ 100 $\times$ 100$\mu m^3$ with a total volume of 2 $\times$ 2 $\times$ 2cm$^3$ and data acquisition took $>2$ hours.

LSCM was operated on Zeiss LSM 510 laser scanning confocal microscope, with Plan-Neofluar 10x/0.3 objective ($WD = 5.5mm$) and 7.5$mW$ laser power ($514nm$). The pinhole diameter was adjusted to 280$\mu m$. The standard excitation and emission filter set was used, HFT 458/514 and BP565 – 615IR, respectively, along with a beamsplitter ($NFT545$). The voxel size was 7$\times$7$\times$20$\mu m$ and total data acquisition took $\sim3$ minutes.

2GMFMT imaging volume was discretized by 100 $\times$ 100 $\times$ 100$\mu m^3$ isotropic voxels. Two spectral channels were acquired sequentially and each channel data was
completed in < 2min. The iterations for reconstruction was completed in ∼ 5min.

Figure 6.15: 3D Reconstruction of all three imaging modalities. The yellow bounding box indicates the MFMT interrogation agar phantom volume. (a) Numerical replica of beads (Purple) shows the depth in z direction, (i) 1.25mm and the separation between them,(ii) ∼ 175μm. (b) 3D reconstructed MRI beads (white) overlaid with the ground truth. (c) LSCM reconstructed 3D image (red) overlaid with the ground truth. Grid lines for LSCM shows the field of view for LSCM. (d) MFMT reconstruction (green) overlaid with the ground truth.

Absolute volume estimations were compared with the numerical phantom, by using a commercial software. Both absolute volumes and percentage error values are reported in Table 6.1. Our results indicates that the 2GMFMT delivered the smallest volume error.
Table 6.1: Volume comparison table. The ground truth spherical volume and estimated sphere volumes are reported for MRI, LSCM and MFMT. Their volume errors reported in percentage.

<table>
<thead>
<tr>
<th></th>
<th>Ground Truth</th>
<th>MRI [mm^3]</th>
<th>LSCM [mm^3]</th>
<th>MFMT [mm^3]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol.1</td>
<td>0.027 mm^3</td>
<td>0.040 mm^3</td>
<td>0.031 mm^3</td>
<td>0.039 mm^3</td>
</tr>
<tr>
<td>Vol.2</td>
<td>0.022 mm^3</td>
<td>0.037 mm^3</td>
<td>0.015 mm^3</td>
<td>0.021 mm^3</td>
</tr>
<tr>
<td>Vol.1 Err. (%)</td>
<td>-</td>
<td>47.3%</td>
<td>49.5%</td>
<td>21.5%</td>
</tr>
<tr>
<td>Vol.1 Err. (%)</td>
<td>-</td>
<td>62.3%</td>
<td>45.8%</td>
<td>33.0%</td>
</tr>
<tr>
<td>Avg. Err. (%)</td>
<td>-</td>
<td>54.8%</td>
<td>47.7%</td>
<td>27.3%</td>
</tr>
</tbody>
</table>

One other performance metric was the sphericity, indicating the accuracy of the reconstructed shape. We used the sphericity equation, similar the one used in image processing literature [182,183]:

\[
Sphericity = \frac{36 \times \pi \times Volume^2}{Area^3}
\]  

(6.14)

We conducted our experiment in a reasonably low scattering phantom. LSCM failed to deliver accurate shape (sphericity) despite the highest sampling among the three modalities, Table 6.2. Due to strong refractive index difference between the polystyrene spheres and agar phantom, MRI was able to yield good reconstruction shape. 2GMFMT yield the best performance in terms of shape and volume. On top of that, 2GMFMT data acquisition took much less (<2min) than MRI (~2hrs.) and LSCM (~3min).

Table 6.2: Sphericity comparison between modalities.

<table>
<thead>
<tr>
<th></th>
<th>Ground Truth</th>
<th>MRI</th>
<th>LSCM</th>
<th>MFMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Sphericity</td>
<td>100%</td>
<td>90.1%</td>
<td>14.1%</td>
<td>82.6%</td>
</tr>
</tbody>
</table>
6.3 Laser Direct Write Printed Sample and Sensitivity Characterization

Gelatin-based Laser Direct-Write (LDW) is a biologic, forward transfer technique that utilizes a pulsed laser to precisely pattern cells and other biologic materials onto a receiving substrate [184]. Recently, LDW has been extended to create and pattern microbeads in a single step through the \textit{in situ} crosslinking of alginate, encapsulating the desired biologic payload in an immobilized 3D microenvironment [185]. LDW has the capacity to fabricate thick, multilayered cellular constructs with micron-scale spatial precision and complex composition (e.g. different cell types and microenvironment). Although LDW may allow for micron-scale precision, a chief difficulty for LDW is the ability to quickly assess the deposition process on thick samples due to turbidity. Classical microscopic modalities do not provide enough depth sensitivity and have limited field of view, making them unsuitable for this task.

2GMFMT may be a good candidate to assist monitoring LDW deposited multilayered cellular constructs. Herein, we report on the synergy of these technologies. We utilized LDW to create a thick multilayered cellular construct by printing mats of interconnected 3D microbeads, in a layer-by-layer fashion. We then employed a second generation Mesoscopic Fluorescence Molecular Tomography (2GMFMT) technique to accurately localize the constructs in 3D, at depths relevant for \textit{in-vivo} monitoring.

6.3.1 Cell Culture and Sample Preparation

GFP-labeled MDA-MB-231-gfp (M231) human breast cancer cells were cultured in standard conditions for 3 days until confluent and then resuspended in a 2% alginate hydrogel. A UV-transparent, quartz print ribbon was spin-coated with a thin layer of 10% gelatin/2%alginate, and pipetted with 1ml of the 2% alginate, cell-suspension. The ribbon was incubated and then wicked to an even thickness. A previously described technique [184, 185] was adapted to print a \(9 \times 9\) overlapping microbead mat in layer-by-layer fashion, Fig.6.16(a). This created a three-layer, cellular construct onto a thick scaffold. The scaffold was created from an agar solution.
mixed with Intralipid. This scaffold had an estimated reduced scattering coefficient ($\mu'_s$) of 1$mm^{-1}$ and an absorption coefficient of 0.002$mm^{-1}$. Each agar substrate was thinly spin-coated with 10%gelatin /2%CaCl2, placed within the center of a similarly prepared petri dish, and then incubated. After printing, a second layer of agar was added to create a 2$mm$ thick sample. Immediately after the LDW transfer, we images the 3D tissue construct with our MFMT system. For visualization purposes, a single layer mat, Fig.6.16(b) and a 3$\times$3 unstacked microbead pattern, Fig.6.16(c), are shown.

Three-layered microbead mats were successfully printed to the agar phantom. The mats measured approximately 1.2$mm$ $\times$ 1.2$mm$ using wide-field microscopy with approximately 7,000 cells. The 3 $\times$ 3 microbead array, Fig.6.16(c) was used to measure and estimate the average diameter and number of cells per microbead (250$\mu m$ diameter; 29$cells/\text{bead}$; 1.86M$cells/ml$). The estimated thickness (0.32$mm$) of the construct was measured through a 1$mm$ thick agar phantom substrate using the MFMT system. The reconstruction algorithm accurately located the printed cellular construct as depicted in Fig.6.17(a). Measurements of the cellular construct using this system were found to be 1.2$mm$ in length, 1.2$mm$ in width and a thickness of 0.32$mm$. Our result performed with a 2% location error comparing the location from wide-field microscopy measurements.
Figure 6.17: (a) The GFP-expressing M231 cells were reconstructed. (b) Reconstruction depth is 1mm, matching the phantom design. (c) Diameter of the reconstructed figure ∼ 1.68mm. (d) Location of the GFP-labeled cells were estimated as (4.74, 4.47) by the fluorescence wide field image of the sample. (e) The reconstruction had a 98% accuracy for identifying cellular locations.

6.4 Ink-Jet BioPrinted Tumor Microenvironment with Patient Derived Glioblastoma for a Longitudinal Study

Glioblastoma multiforme (GBM) is the most prevalent and lethal primary brain tumor. More than half of all brain tumors are GBM in the United States, alone. Despite its prevalence and lethality, there is no effective treatment for GBM, mostly due to inter/intra tumor heterogeneity. Since 2005, the standard-of-care has changed to concomitant/adjuvant therapy with Temolozomide (TMZ). Although the new therapy regimen increased the median survival time from 12.1 to 14.6 months [186], the GBM still shows high recurrence (37%) [187] and the 5 year survival rate is less than 10%. Targeted therapy is another emerging approach to the issue; however, more than half of the patients failed to respond and many develop resistance to the therapy [188].

These statistics highlight a pressing need for a high throughput drug evaluation platform regardless of the drug type and whether it is an untargeted or targeted
drug [188,189]. A platform that utilizes current image-driven endpoints [190] can potentially transform the drug efficacy assessment without requiring patient administration. Intact tumors are clinically characterized with volumetric assessment along with other parameters [191]. Increased anatomic volume of tumors was shown to be an indication of worsened overall survival (OS) time. Similarly, a steady or decreasing tumor volume usually indicates a progression free survival (PFS) [192].

Two challenges arise for such a task: (i) building and maintaining a 3D tissue-like environment and (ii) assessing the volumetric change without perturbing the media. The first task is to mimic the original tissue environment in 3D [193], which requires a well-controlled environment (i.e. temperature, humidity, nutrition transport etc.). The second task requires a fast, high resolution, high sensitivity imaging modality.

LSCM is a standard method to visualize structures in 3D as it operates with all available fluorophores for \textit{in-vitro} [194] and \textit{in-vivo} [195,196] tumor imaging applications. Recent progress with Swept confocally-aligned planar excitation (SCAPE) microscopy has shown greatly reduced acquisition time. However, limited imaging depth hinders the use of the technique for tissue engineering applications. Multi-photon microscopy surpasses the imaging depth limit to a certain extent (Hwang et al., 2011) but non-linear optical phenomena disqualifies this technique for long wavelength fluorophores. These techniques provide critical information for cellular signaling, cell differentiation or cell migration [197] in $\sim 30\mu m$ thick microenvironment; however it is a challenge for these microscopic techniques to provide a complete picture of the tissue due to small field of view.

There is a possibility of mosaicking, but that would increase the acquisition time proportionally and brings the risk of missing low intensity signals due to the finite dynamic range. On top of that, densely packed tumor spheroids block the view of those techniques and the ability to image beyond center plane [198].

Photoacoustic (PA) imaging regained interest in the last decade, due to its wide span of resolution and imaging volume [199]; depending on the configuration and desired resolution, acquisition time can vary from minutes to hours [199,200]. Regardless, PA imaging is an emerging modality that can provide structural and
functional information in the presence of endogenous high-contrast agents (HbO2, HbO, and melanin etc.) or exogenous contrast agents (i.e. ICG) for tumor imaging [201].

The aforementioned imaging modalities offer great benefits to tissue engineering studies and tumor microenvironment studies. However, they are lacking in combining critical features namely, large field of view, fast data acquisition, and high sensitivity to molecular reporters. Those features are essential for monitoring *in-vitro* models in order to minimize the distress on cells and the time they spend outside incubation conditions.

To address those issues, we propose to monitor GBM tumor microenvironment with our second generation Mesoscopic Fluorescence Molecular Tomography (2GMFMT) system.

### 6.4.1 Cell Culture and Hydrogel Preparation

Human umbilical vein endothelial cells (HUVECs) were cultured at 37°C in 5% CO₂ in *EGM*-2 Endothelial Cell Growth Medium-2 (Lonza). Patient-derived glioblastoma multiform (GBM) cells (SD01 and SD02) were cultured on laminin-coated tissue culture flask in *NeuroCult*TM NS-A proliferation media for human (STEMCELL Technologies). We changed the culture media every other day to maintain the cell viability and integrity of the construct. HUVECs and GBMs were transfected with lentivirus expressing EGFP (green) or mCherry (red). For the cell seeding on bio-printed channels, HUVECs were harvested using 0.25% Trypsin-EDTA, and kept as cell suspensions on ice until seeding process. To create the GBM spheroid, we harvested GBM cells by accutase cell detachment solution, and then 1,000 5,000 cells were placed into 96-well low-attachment plates (Corning). The GBM cells were cultured on the low-attachment plates for 7-21 days until the spheroids reached the desired diameter (> 400µm).

### 6.4.2 Bio-Printing of Vascular-GBM Model

3D collagen I matrix (3 mg/mL) was used as a base material for vascular channels. After printing layers of collagen matrix, a thermoreversible hydrogel, 10% gelatin laid on the surface of the collagen as a sacrificial material (Fig.6.18). Once
the vascular channel locations were defined a GBM spheroid was placed between these two vascular channels (Fig.6.18). The collagen precursor was polymerized by \(\text{NaHCO}_3\) nebulization on the plexiglass bio-chamber. That process filled the biochamber half way to its inner space (Fig.6.18, step 1). We then printed 10% gelatin, a thermo-reversible hydrogel while keeping heat at 37°C (Fig.6.18, step 2). In room temperature, the gelatin solidified within 1-2 minutes.

A GBM spheroid was carefully picked up from the low attachment plate by gentle pipetting. The spheroid was placed on the printed collagen layer in between the two gelatin channels (Fig.6.18, step 3). Excess media around the spheroid was removed and a small amount of collagen I was added to fix the spheroid location. After 10 minutes of waiting time for collagen gelation, more collagen layers were printed on top of the gelatin channels and GBM spheroid (Fig.6.18, step 4).

To form a hollow lumen whole structure was incubated for 20-30 minutes. We then injected the HUVEC suspension into the hollow channels. Then HUVECs (seeding density: 8 million cells/mL) in suspension were injected into the channels to enable monolayer cell lining on the top and the bottom of the channels (Fig.6.18, step 5). The entire construct was printed in a plexiglass sample holder. The plexiglass chamber hosts a flow chamber for stable, long-term perfusion. Four plastic needles (2 for inlet, 2 for outlet) were installed on the flow chamber for the perfusion system. The construct was cultured with EGM-2 media for up to 70 days at (37°C) in 5% \(CO_2\). The culture media was changed 3-4 times a day through the vascular channel.

### 6.4.3 Drug Treatment

GBM cells from the embedded GBM spheroid infiltrated into the collagen matrix over the culture period. When the invasion distance became 1-2 mm, drug treatment was begun by adding Temozolomide (final concentration = 100 µM, Sigma Aldrich) to the culture media.

### 6.4.4 Patient Derived GBM Longitudinal Monitoring

Four GBM tumors from two different cell lines were monitored up to 72 days. The longitudinal process includes two segments: a growth period and a drug regimen
Figure 6.18: The collagen tissue construct printing method is depicted. (1) Collagen base was printed and then (2) gelatin was printed to form the vascular channel shape. (3) Patient derived GBM was embedded between gelatins and (4) they covered with another collagen layer. (5) Gelatin was liquified to enable cell seeding through perfusion. (6) Finally monolayer of HUVECs was laid on the inner surface of the lumen.

for the GBMs. In this study, we used a naming convention to identify the GBMs, used in our experiments. An example name is as follows: $X\diamond - SDY$. $X$ represents the batch number (1, 2, 3, ...). It is followed by a letter which indicates the specific GBM in that batch (A, B, C, ...) and finally $SDY$ indicates the cell line (SD1 or SD2). Throughout this study, we worked on 5 batches and each batch has 3-4 tissue constructs with an embedded GBM.

Here, we showed the results from Batches 2, 3, 4, 5 with H, G, C, E GBMs, respectively. Those GBM spheroids, embedded in collagen samples, are from two different cell lines: SD1 (G and E) and SD2 (H and C). We present the data from 2H-SD2, 3G-SD1, 4C-SD2, and 5E-SD1. These four tumors were imaged up to 72 days depending on the survival of the cells and the integrity of the tissue construct. The amount of data available per each tumor for each imaging modality varied due to the similar reasons. The number of data points presented in Table 6.3.
Table 6.3: The number of data points are shown in the table. Experiment data may be acquired at different dates for each modality.

<table>
<thead>
<tr>
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<th>2H-SD2</th>
<th>3G-SD1</th>
<th>4C-SD2</th>
<th>5E-SD1</th>
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</table>

The state of the GBMs was assessed by non-concurrent imaging with Widefield Fluorescence Microscopy (WFFM), LSCM, MRI and 2GMFMT. Our goal is to monitor the cellular invasion and volumetric change of the GBM spheroids. The imaging orientation is shown for LSCM and 2GMFMT in Fig.6.19. The main reason we chose this orientation was to conduct our validation study. We placed the GBM and vascular channels close to bottom plexiglass (< 500µm) so that LSCM could work in its best condition while 2GMFMT collected the data from the top through ∼ 3mm thick plexiglass and a few millimeters of collagen.

Figure 6.19: (a) The actual collagen matrix in custom designed plexiglass chamber is depicted. The red rectangular shows the collagen matrix. (b) LSCM imaging was conducted from the bottom side and 2GMFMT raster scanned the sample from the top of the plexiglass.

The longitudinal images were acquired pre- and post-administration of a clinically approved drug (Temozolomide TMZ 50-100µM) to replicate the progression
of disease and the clinical protocol where imaging carried out before initiating the treatment and after treatment ends. Typical acquisition times varied across the imaging modalities: $\sim 2$ minutes for WFFM, $\sim 50$ minutes for LSCM, $\sim 2$ hours for MRI, and $< 2$ minutes for 2GMFMT.

6.4.5 **Image Driven Tumor Analysis**

Clinical process tries to standardize the tumor response against treatment with image-based endpoints. Two widely-used methods focus on the tumor diameter(s) (i.e. cross sectional area) and tumor volume for the drug response assessment [202, 203]. Here we will investigate the change in mean intensity of the spheroid, invasion area and the volume of tumors before and after drug treatment while comparing the imaging modalities with their common information.

WFFM and 2GMFMT were used to compare the mean intensity and invasion area of tumor profile; LSCM, MRI and 2GMFMT were used to compare the volume change in tumor. For visual comparison of different modalities, especially for WFFM and 2GMFMT comparison, we normalized all data points to the first 2GMFMT date. So each modality normalized within itself.

6.4.5.1 **Intensity Based Comparison**

We used WFFM and 2GMFMT images to make an intensity-based comparison. We compared the 2GMFMT center detector with the WFFM image. These are equivalent to each other because zero source detector separation would yield surface image same as epi-fluorescence microscopy. The tumor spheroid exhibited a strong fluorescence intensity with a gaussian-like distribution, peaking at the center of the core area, over the field of view, Fig.6.20. The mean intensity ($z$-axis) plot profile ($x$-axis) over a selected region for each tumor spheroid (white dashed line rectangle) for each experiment day was presented. The y-axis enumerates the data points. In Fig.6.20 we observed a strong variation in profile for the four tumors over the course of experiment days even for the same tumor. Only 2H showed a correlation between WFFM and 2GMFMT. This difference may be associated to different sensitivity of imaging systems, fluctuation in the source, etc.
6.4.5.2 Area Based Comparison

Tumor invasion was investigated in two segments: tumor core expansion and stellar cell invasion. For the purpose of image analysis, we defined the tumor core radius as where the intensity drops to 50% of its maximum (full width half maximum, FWHM), the invasion area as where the intensity falls between 50 – 10% of its maximum, and the background signal area as where the intensity goes below 10% of its maximum. The 2H-SD2 tumor is depicted for each segment with full field of view (I), core area (II), invasion area (stellar cells) (III) and the background area (IV) in Fig.6.21. To demonstrate the tumor invasion, we selected the first experiment data.
(Day 14) and the last experiment data (Day 69).

Figure 6.21: The first experiment and the last experiment for 2H-SD2 was shown. Column I shows the full field of view of 2GMFMT center detector raw data (WFFM equivalent), Column II and III depicts core area and cell invasion area, respectively. Column IV shows the background signal area.

Comparison of the mean intensity of tumor core area and stellar invasion area provides an idea about tumor invasiveness. It is an accepted phenomenon that GBM exhibit strong inter/intra-tumor heterogeneity. Hence, it is highly expected to see this phenomenon in our study.

In Fig.6.22, core area and invasion areas of the 2H-SD2 tumor were compared. The first closest experiment day was Day 14 for WFFM and 2GMFMT and the last closest day was Day 69. We provided the 2D images for those days and other data points are shown in scatter plots. WFFM and 2GMFMT shared the a similar trend in stellar cell invasion in that both increased their coverage area and core area as high as $\sim 50\%$ for 2GMFMT and invasion area at least $200\%$ for each. Another expected outcome and an observation was that the 2GMFMT had more continuous invasion area than WFFM. The WFFM has higher resolution than 2GMFMT, it can resolve individual cells or small cell clusters.

Tumor core area followed an increasing trend in both modalities but the par-
allel trend diverged after Day 60 (Appendix A.1).

Figure 6.22: The figures shows the spatial distribution of tumor 2H-SD2 over the course of 68 days. Black dashed line represents the first day of drug administration (Day 26). (a) The change in core area of tumor 2H-SD2 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). (b) The change in invasion area of tumor 2H-SD2 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). Each data series is normalized within each data set to the first experiment day.
Fig. 6.23 shows the tumor growth and shrinkage trend for 3G-SD1. 3G-SD1 demonstrated \( \sim 50\% \) increase in its core area up until Day 30 while its invasion area increased almost 300\%. Both 2GMFMT and WFFM showed similar trends in tumor growth and tumor shrinkage (Appendix A.2).
Figure 6.23: The figures shows the spatial distribution of tumor 3G-SD1 over the course of 57 days. Black dashed line represents the first day of drug administration (Day 26). (a) The change in core area of tumor 3G-SD1 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). (b) The change in invasion area of tumor 3G-SD1 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). Each data series is normalized within each data set to the first experiment day.
Tumor 4C-SD2 invasion characteristics were depicted in Fig. 6.24. 4C-SD2 showed the highest volume increase rate among all four tumors and both modalities, WFFM and 2GMFMT, confirmed that behavior (Appendix A.3).
Figure 6.24: The figures show the spatial distribution of tumor 4C-SD2 over the course of 71 days. Black dashed line represents the first day of drug administration (Day 47). (a) The change in core area of tumor 4C-SD2 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). (b) The change in invasion area of tumor 4C-SD2 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). Each data series is normalized within each data set to the first experiment day.

The 5E-SD1 tumor showed the most steady behavior out of all four tumors. Both the core and invasion area showed steadily fluctuating behavior over the course
Almost all of the points were within the one standard deviation over mean (Appendix A.4).

Figure 6.25: The figures shows the spatial distribution of tumor 5E-SD1 over the course of 58 days. Black dashed line represents the first day of drug administration (Day 34). (a) The change in core area of tumor 5E-SD1 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). (b) The change in invasion area of tumor 5E-SD1 is depicted. The left 2D image is the raw data from 2GMFMT (top) and WFFM (bottom), presented for the first and last coinciding experiment day (circled). Each data series is normalized within each data set to the first experiment day.
6.4.5.3 Volume Based Comparison

We examined the volume change of the tumors and compared the results across different modalities. Our preliminary experiments indicated that MRI is only sensitive to the core of the spheroid. In some of the cases spheroid density was not high enough to create a contrast between the collagen media and the tumor. We reported the MRI volume values and quantitative volume comparison was conducted between LSCM and 2GMFMT. We also compared the 2GMFMT center detector image, WFFM image, and sum of z-stacks of LSCM slices. This comparison helped us to shed some light on the root cause of differences between 2GMFMT 3D reconstruction and LSCM 3D reconstruction.

2H-SD2 tumor 3D reconstruction by LSCM and 2GMFMT are shown in Fig.6.26. The difference between LSCM and 2GMFMT may be due to the optical sectioning and depth limitation of LSCM. Our observation and previous reports showed that LSCM was not able to visualize the upper hemisphere of spheroids. This difference can be seen in Fig.6.26(c). Since LSCM cannot capture the fluorescence signal deeper in the construct, reconstruction revealed a decreased volume in LSCM where 2GMFMT kept increasing due to the preservation of core signal.

Below, we also incorporated volume values across three modalities (MRI, LSCM and 2GMFMT) for each data point. For the 2H-SD2 tumor, 2GMFMT volume estimation showed a gradual increase (up to 50%) even after more than 1 month of drug regimen. LSCM volume estimation on the other hand showed a slight drop at the end of the drug regimen (< 20%). Similarly, MRI volume also followed a similar trend in decreased volume (~ 50%), Fig.6.26(d), (Appendix B.2).

The difference between LSCM and 2GMFMT was seen in 3G-SD1, similar to the one seen for 2H-SD2. The exact root cause for this difference could be found if an invasive analysis such as cryo-sectioning is performed. Our hypothesis is that the signal captured by both WFFM and MFMT was beyond the depth that can be achieved by LSCM. 3G-SD1 volumetric changes followed a similar trend across the modalities but with different rates. From the first experiment until Day 20 all followed a decreasing volume trend, 6.27(a-c). From Day 20 to around Day 35 all of the modalities demonstrated an increase in volume. For the remaining portion of the
Figure 6.26: (a) 2H-SD2 3D reconstruction of (a) LSCM images for pre and post drug administration is shown. (b) 3D reconstruction of 2GMFMT was shown for pre and post drug administration. (c) Pre and post drug administration 2D images from 2GMFMT raw data, WFFM and z-projection of LSCM slices were shown. (d) Volume estimation of 2H-SD2 tumor (normalized to 100). MFMT volume estimations are depicted with (blue) circle, MRI with (green) triangular, and LSCM with (brown) star. Dashed line indicates the start of drug regimen.
experiments all of the modalities showed a decreasing trend. One distinct feature was the slow but gradual volume decrease after drug administration, Fig.6.27(d).

The 4C-SD2 tumor exhibited the largest volume change among all four tumors. Although LSCM showed an increase in the invasion area, Fig.6.28, LSCM volume showed a shrinkage. That mismatch may be associated with cellular resolution of LSCM. Because, in contrast to 2GMFMT, LSCM acquires signal from individual cells and clusters. The overall volume reconstructed by 2GMFMT may have decreased due to sparsity of signals. Another possibility is that LSCM was not able to resolve deep enough to the tumor and it delivered only the volume that it can resolve. The latter explanation can be supported by matching the WFFM and 2GMFMT images. Both WFFM and 2GMFMT reported strong signal over the core area where the LSCM core area signal diminished significantly.

Similar to 3G-SD1, 4C-SD2 tumor volume estimations from MFMT and LSCM followed a similar pattern but in different rates of change. LSCM showed a dramatic increase at the beginning and a strong volume drop at the end of drug regimen. MFMT volume slowly increased and stayed steady until the beginning of drug regimen and slowly decreased. MRI on the other hand showed completely different characteristics, Fig.6.28(d).

As reported by the core area and invasion area changes, 5E-SD1 showed a steady trend over the course of 58 days. The steady behavior from MFMT reconstruction was confirmed by both WFFM and LSCM.

The last tumor, 5E-SD1 consistently showed the most steady characteristics for intensity, area (Fig.6.25), and volume change across all modalities, Fig.6.29(d).

6.5 Summary

Here, we reported our second generation Mesoscopic Fluorescence Molecular Tomography system. We introduced the design characteristics such as detector formation, number of detectors and number of sources. Our in-silico study outcomes led us to choose a 2D detector array (EMCCD camera), which enabled dense sampling over the sample surface. The data acquisition parameters were optimized to $31 \times 31$ source positions and $7 \times 7$ detector array. One of the merits of using
Figure 6.27: 3G-SD1 (a) 3D reconstruction of LSCM images for pre and post drug administration is shown. (b) 3D reconstruction of 2GMFMT image was shown for pre and post drug administration. (c) 2D images from 2GMFMT raw data, WFFM and z-projection sum of LSCM slices were shown for the first day measurement for closest possible days, before and after drug treatment. (d) Volume distribution of 3G-SD1 tumor (normalized to 100). MFMT volume estimations are depicted with (blue) circle, MRI with (green) triangular, and LSCM with (brown) star. Dashed line indicates the start of drug regimen.
Figure 6.28: 4C-SD2 tumor (a) 3D reconstruction of LSCM for pre and post drug administration is shown. (b) 3D reconstruction of 2GMFMT image was shown for pre and post drug administration. (c) 2D images from 2GMFMT raw data, WFFM and z-projection sum of LSCM slices were shown for the first day measurement for closest possible days, before and after drug treatment. (d) Volume distribution of 4C-SD2 tumor (normalized to 100). MFMT volume estimations are depicted with (blue) circle, MRI with (green) triangular, and LSCM with (brown) star. Dashed line indicates the start of drug regimen.
Figure 6.29: (a) 3D reconstruction of LSCM images for pre and post drug administration is shown. (b) 3D reconstruction of 2GMFMT image was shown for pre and post drug administration. (c) 2D images from 2GMFMT raw data, WFFM and z-projection sum of LSCM slices were shown for the first day measurement for closest possible days, before and after drug treatment. (d) Volume distribution of 5E-SD1 tumor (normalized to 100). MFMT volume estimations are depicted with (blue) circle, MRI with (green) triangular, and LSCM with (brown) star. Dashed line indicates the start of drug regimen.
EMCCD camera was the Electron Multiplying Gain, which allows for SNR values otherwise was below the detection limit of the camera. With that feature, our system was able to minimize the need for higher power and longer acquisition time to attain higher SNR.

The highly-density data are then carried into the reconstruction process where we adapted $l_p$-norm regularization due to the sparse nature of our collected data. Our in-silico reconstruction comparison delivered that $l_p$-norm reconstruction outperformed $l_2$-norm in terms of the shape integrity and robustness against noise.

After the above mentioned characterization studies, we conducted a series of experiments demonstrate the resolution, sensitivity, and acquisition speed. To the best of our knowledge, for the first time, we have reported a resolution characterization for a mesoscopic tomography system. This study showed that our 2GMFMT system has a resolution power of at least 175 $\mu m$ at 1.25mm deep in scattering phantom. Laser Direct Write, a high precision tissue printing technique, enabled us to characterize the sensitivity of our system in terms of minimum detectable cell count and cell concentration, $\sim 7,000$ cell or $\sim 1.8 \times 10^6$ cells/mL. Finally, we reported a longitudinal study for patient-derived GBM tumor spheroids. In this study, we introduced a novel in-vitro platform for drug response evaluation. In our preliminary study we monitored 4 GBM brain tumors from 2 different cell lines for up to 71 days and we reported the volumetric change of the tumor spheroids.

Our studies indicates that the 2GMFMT is a powerful, high resolution mesoscopic tomography system that swiftly acquires data and delivers high accuracy in reconstruction. We believe that the 2GMFMT system and its counterparts have huge potential to further investigate tissue engineering applications (in-vitro) as well as small animal imaging studies (in-vivo).
Chapter 7
Summary

In this work, we showed the potential applications for the synergy of Mesoscopic Fluorescence Molecular Tomography and tissue printing technologies. 3D tissue engineering by bioprinting has the potential to generate large structures that may be difficult to image with superficial imaging modalities. However this potential brings challenges with it. The main challenge is depth resolved imaging for thick and highly scattering samples. Since these samples have to reside in a bioreactor, the imaging modality has to be fast and sensitive. Our studies demonstrated that our MFMT system is capable of accomplishing this task.

As a demonstration, we applied Mesoscopic Fluorescence Molecular Tomography (MFMT) on reporter gene imaging. Since MFMT does not require a tight focus on the imaging spot, it relieves the excessive excitation on an imaging area. At the same time, MFMT was shown to be effective up to 3-mm depth in scattering media with multiple markers. Therefore, this imaging modality may be useful to assess large, 3D printed structures. Using multiple markers yields both functional information, from different cell types, and structural information. Despite having lower resolution than multiphoton imaging, MFMT may be useful for rapid, close to real-time imaging and analysis of tissue engineered constructs in-vitro. We then pursued another experiment on an in-vivo animal model to retrieve the biodistribution of a Photodynamic Therapy agent (HPPH). The result of this study revealed a heterogeneous distribution of agents, which was an important finding that can potentially be utilized be utilized to inform treatment planning and manage drug administration.

In the second phase of our study, we built a second generation MFMT system.
(2GMFMT) and demonstrated its resolution power, sensitivity, and compatibility to longitudinal studies. 2GMFMT system is capable of dense sampling of the target surface which yields a high resolution performance (at least 175\(\mu m\)). Integrating an EMCCD camera into the second-generation MFMT brought the benefit of increased sensitivity without compromising the acquisition time. On the contrary due to the parallel data acquisition, 2GMFMT surpassed the data acquisition speed of the first generation MFMT by 3 fold. Our experiment results indicate that the 2GMFMT is a powerful, high resolution mesoscopic tomography system that swiftly acquires data and delivers high reconstruction accuracy. We believe that the 2GMFMT system has shown its potential to close the gap between microscopy and macroscopy for tissue engineering applications (\textit{in-vitro}) as well as small animal imaging studies (\textit{in-vivo}).

The 2GMFMT has proven itself as a powerful imaging modality for high resolution, fast, thick tissue imaging in mesoscopic scale. By combining other imaging modalities, especially the ones offer structural information, 2GMFMT can provide 3D reconstruction images for molecular contrasts (i.e. reporter genes, biological markers etc.) from \textit{in-vitro} to \textit{in-vivo} level.
REFERENCES


Appendix A
Image Analysis Data

A.1 Volume Percentage Changes for Tumor

The volume estimation through 2GMFMT reconstruction are reported in below tables.
Table A.1: 2H-SD2 Volume Percentage Changes with respect to the first experiment day

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Table A.2: 3G-SD1 Volume Percentage Changes with respect to the first experiment day

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Table A.3: 4C-SD2 Volume Percentage Changes with respect to the first experiment day

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Table A.4: 5E-SD1 Volume Percentage Changes with respect to the first experiment day

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Appendix B
Supplementary Data File

B.1 Data Collection Movie

Description: The accompanying movie file demonstrates the data collection configuration. The EMCCD camera raster scans over the surface of a sample and detector frames are formed simultaneously.

B.2 3D Reconstruction Movies

Five different movie shows the 3D reconstruction of 2GMFMT and validation modalities such as MRI, and LSCM. Movie 1 shows the resolution phantom study results by MRI, LSCM, and 2GMFMT. Movies 2-5 shows the longitudinal reconstructions of GBM within a perfused chamber. Animations includes reconstructions of 2H-SD2 and 4C-SD2 tumors by LSCM and 2GMFMT, as the file names indicates.

1. Movie: Resolution4Modality
2. Movie: 2H – SD2 – LSCM
3. Movie: 2H – SD2 – MFMT
4. Movie: 4C – SD2 – LSCM
5. Movie: 4C – SD2 – MFMT