IN VIVO FLUORESCENCE IMAGING AND TOMOGRAPHY METHODS TO QUANTIFY METASTATIC BURDEN IN LYMPH NODES

A Thesis

Submitted to the Faculty

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

by

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May 2016

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Abstract

Morbidity and complexity involved in lymph node staging via surgical resection and biopsy could ideally be overcome using node assay techniques that are non-invasive. Visible blue dyes, fluorophores and radio-tracers are often used to locate the sentinel lymph nodes from draining lymphatic vessels near a tumor, but they only rarely provide an in situ metric to evaluate the presence of cancer. As such, imaging systems that are quantitative and sensitive to surface- and subsurface-fluorescence could provide a radiation-free, less invasive alternative to existing approaches, and have the potential to perform both node mapping and metastasis sensing.

Issues of non-specific uptake, and high delivery variability complicate imaging of single tracers in a lymph node. To overcome these problems, ratiometric schemes using multiple fluorescent tracers along with modeling techniques to estimate cancer biomarkers have recently been demonstrated. In this approach, quantitative estimates of the cancer burden are attainable using micro-doses of fluorescence-labeled tracers targeted to cancer-specific receptors, thus providing high specificity in studying cancer progression and metastasis. In this work, an extensive review of commercial fluorescence imagers and their capabilities led to some unique directions in sensing of lymph nodes with alternative hardware and image processing methods. A previously designed high-frequency ultrasound-guided fluorescence tomography system was upgraded to enable multi-spectral capabilities with large source-detector spacing, to allow subsurface measurements, and the ability to spectrally decouple autofluorescence and signals from multiple fluorophores. Along with tomography, we used planar fluorescence imaging methods to image lymph nodes and lymphatic vessels to quantitatively study lymphatic uptake, flow variation, and lymphatic system changes with tumor progression. In particular, imaging the lymphatic flow in the lymph vessels relative to the uptake in the nodes provided a unique methodology to quantify
tumor burden. On the other hand, the value of targeted agents in lymphatic imaging appears to be sensitive to the location and method of delivery, and as such it was found that internal tissue normalization methods were more robust with non-specific fluorophores. Murine models of nodal involvement of metastases, and design of real-time fluorescence imaging tools were combined to propose the most constructive approach to non-invasive quantification of tumor burden in lymph nodes for future clinical implementation.
Dedication

To my parents Archibald and Freeda D’Souza, who have through their unconditional love, support, and guidance, enabled this and all my accomplishments.
Acknowledgements

This thesis work is the combined effort of several individuals, to whom I am truly grateful. Prof. Brian Pogue, my mentor and advisor at Thayer, has always been enthusiastic, and supportive of independent research whilst steering me towards new and exciting project directions. His farsighted vision, combined with his trustfulness of my pursuits and patience encouraged me to step out of my comfort zone, and undertake a variety of projects early on. He always made time to discuss even the smallest of problems, and silliest of question, without ever trivializing them.

I have had the good fortune of working in an intellectually rich and highly collaborative research environment at Dartmouth; the various leaders and members of the Optics in Medicine lab have always been a resource for solving any problems big or small. Drs. Jonathan Elliott, Kimberley Samkoe, Ken Tichauer, Brendan Flynn, Chad Kanick, and Scott Davis, were people I knew I could go to when I got into trouble, or needed advice on failing experiments, or simply needed to plan ahead, and they were always willing to spend time explaining things ranging from basic principles to abstract concepts. Special thanks to Jason and Kayla for holding my hand through biology experiments, and facilitating my explorations. I’d also like to thank the various postdocs and students in my group who helped me throughout my time here, through useful discussions mixed with a bit of fun, it’s been a pleasure working alongside all of you. Special thanks to Sally and Kathy, they work tirelessly to ensure that the rest of group can function productively.
Most importantly, I’d like to thank God for the countless blessings, and my family, without their support this work would not have been possible. I’d like to express my sincerest gratitude to my parents, who have selflessly supported my academic and creative endeavors all through the 27 years of my existence; they’ve been my inspiration, encouragement, and guiding light. To my siblings Adora, and Fayne, thank you for being you, and keeping the child in me young, I hope that this work will inspire you to follow your dreams and succeed in your own intellectual pursuits.

Finally, I’d like to thank my husband, my best friend, Shadab, whose boundless love continues to motivate all aspects of my being. Thank you for being with me through thick and thin.
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Figure 9.8 Phantoms in 1% intralipid and 1% whole porcine blood.

Figure 9.9 Fit coefficients are representative of relative contributions of the Cherenkov background, and the concentrations.

Figure 9.10 Spectra acquired from subcutaneous matrigel inclusion in a mouse, shown along with the fit results.
List of Acronyms

18F-FDG  2-deoxy-2-(18F)fluoro-D-glucose
2D      Two-Dimensional
3D      Three-Dimensional
A/D     Analog-to-digital
ALA     Aminolevulinic Acid
BSA     Bovine Serum Albumin
CBR     Contrast-to-background ratio
CCD     Charge-coupled device
CMOS    Complementary metal-oxide Semiconductor
CT     Computed Tomography
DOT     Diffuse Optical Tomography
EGF     Epidermal Growth Factor
EGFr    Epidermal Growth Factor receptor
EMCCD   Electron-multiplying Charge-coupled device
FDA     United States Food and Drug Administration
FEM     Finite-element Mesh
FNAC    Fine-needle Aspiration Cytology
FOV     Field-of-view
FT      Fluorescence Tomography
GPU     Graphics Processing Unit
H&E     Hematoxalin and Eosin
Hb      Deoxy-Hemoglobin
HbO₂    Oxy-hemoglobin
HDR     High-dynamic range
HIPAA   Health Insurance Portability and Accountability Act
HSA     Human Serum Albumin
ICCD    Intensified Charge-coupled device
ICG     Indocyanine Green
IR      Infrared
IRB     Institutional Review Board
LED     Light-emitting Diode
LINAC   Linear Accelerator
LN      Lymph Node
LN-MCI  Lymph Node Molecular Concentration Imaging
MB      Methylene Blue
MRI     Magnetic Resonance Imaging
NIH     National Institutes of Health
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NIR</td>
<td>Near Infrared</td>
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<tr>
<td>NIRF</td>
<td>Near-infrared Fluorescence</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
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<td>PET</td>
<td>Positron-Emission Tomography</td>
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<td>PpIX</td>
<td>Protoporphyrin IX</td>
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<tr>
<td>RGB</td>
<td>Red-Green-Blue</td>
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<tr>
<td>ROI</td>
<td>Region-of-interest</td>
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<tr>
<td>SBR</td>
<td>Signal-to-Background Ratio</td>
</tr>
<tr>
<td>sCMOS</td>
<td>Scientific Complementary metal-oxide Semiconductor</td>
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<tr>
<td>SLN</td>
<td>Sentinel Lymph Node</td>
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<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
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<tr>
<td>US</td>
<td>Ultrasound</td>
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<tr>
<td>USFT</td>
<td>Ultrasound-guided Fluorescence Tomography</td>
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<tr>
<td>USMFT</td>
<td>Ultrasound-guided Multispectral Fluorescence Tomography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WLS</td>
<td>White-light Spectroscopy</td>
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CHAPTER 1 Introduction

1.1 Rationale

Cancer is one of the leading causes of death worldwide. Overall, there were 14.1 million new cases and 8.2 million deaths in 2012\(^1\). Figure 1.1 shows the cancer incidence and mortality for the top 15 cancer types across both sexes, categorized into developed and developing regions of the world. Although effective screening and staging techniques, and improved treatments have lead to a reduction in cancer-related deaths, the current trends suggest that incidence of new cancer cases will increase to 22.2 million in 2030\(^2\).

Figure 1.1 Cancer incidence and mortality for both sexes in all world regions as surveyed by GLOBOCAN\(^3\).
Cancer survival rates are highly variable and dependent on type, stage at diagnosis, and available treatments. Figure 1.2 shows an example of five-year breast cancer survival rates in patients across the United States, categorized by cancer stage. A large difference exists between survival rates of patients with distant metastasis, and no metastasis or only regional nodal metastasis. Staging of a patient’s cancer and modifying treatment strategy accordingly can improve chances of survival if done early and reliably.

Figure 1.2 Breast cancer survival rates in the United States, based on data from SEER 18 2006-2012. Gray figures represent those who have died from female breast cancer. Green figures represent those who have survived 5 years or more^4.

Several cancer types, including melanomas^5, breast cancers^6, head and neck cancers^7, prostate cancer^8, reproductive cancers^9, and gastric cancers^10 have been shown to metastasize first to their sentinel (first tumor-draining) lymph nodes. Staging of regional lymph nodes is one of the most-important prognostic predictors of these cancers^11. Figure 1.3 shows an example of Stage III melanoma, where the regional lymph nodes
show presence of cancer detectable by histopathological testing. Surgical resection followed by pathological testing or biopsy of lymph nodes is commonly used to stage metastatic spread of cancer\textsuperscript{12}.

Figure 1.3 Melanoma metastasis to a sentinel lymph node\textsuperscript{13}. Main figure reproduced with permission from Terese Winslow LLC (original artwork produced for the National Cancer Institute © Terese Winslow, U.S. Govt. has certain rights). Adapted leftmost inset shows an H&E image of a lymph with metastasized melanoma detectible as brown melanin deposits\textsuperscript{a}.

Lymphoscintigraphy after administration of radio-colloids and visual localization following injection of blue dye are the two methods typically used for lymph node mapping and identification of nodes to be surgically resected for detecting metastasis\textsuperscript{15}. Although this is the current standard of care, neither of these methods conveys information about the recurrence risk of specific nodes. For this reason, lymph node dissections and biopsies can be associated with over-treatment, morbidity, and

\textsuperscript{a} Inset reproduced from Gabriel Caponetti with permission\textsuperscript{14}. https://en.wikipedia.org/wiki/Metastasis.
complexities such as lymphedema, potential nerve damage\textsuperscript{16}, and decreased mobility, resulting in an impact on patient quality of life\textsuperscript{17}. A mapping process that provides information about cancer presence in nodes is more ideal; this thesis examines some approaches to combining the clinically used paradigm with quantitative analysis of measured fluorescence-based signals.

1.2 Current state-of-the-art for node mapping and staging

1.2.1 Clinical gold-standard: Lymphoscintigraphy and sentinel node dissection

The most commonly employed approach to determining the presence of metastatic cancer cells in the sentinel lymph nodes of melanomas, breast cancers, and head and neck cancers involves node mapping followed by surgical resection. Radiotracer-based mapping is commonly performed prior to surgery, where a radiocolloid, typically Technitium-99m is administered near the primary tumor site. Radiocolloids are preferentially taken up by the lymphatic capillaries and drain into the nearby lymph nodes, enabling their identification using a gamma probe, gamma camera, or (rarely) single-photon emission computed tomography (SPECT). Figure 1.4b shows a gamma probe in use to detect sentinel lymph nodes. Figure 1.4a is an example of a lymphoscintigram, produced using a gamma camera, that shows the lymphatic tracks and node. Following node localization, the surgeon injects blue dye such as isosulfan blue or methylene blue near, or into the primary tumor. This dye is rapidly taken up by the lymphatic vessels and temporarily collects in the regional lymph nodes. The surgeon then surgically exposes the lymphatic vessels and lymph nodes, and resects nodes that are stained blue. Figure 1.4c–e show this process performed in sentinel node biopsy of a
melanoma patient. Vital blue dye or radiocolloids are sometimes also used on their own, although this is rare, and most established guidelines recommend they be used together\textsuperscript{18}. Once the node(s) is resected it is sectioned into slices <2 mm thick and embedded. Metastatic islands >2 mm in diameter, identified using H&E staining and pathologist evaluation, are called “macrometastases” and any smaller detectible islands are “micrometastasis”\textsuperscript{19}. Immunohistochemistry may also be performs for certain cancer types\textsuperscript{20}. In the case of breast cancer patients undergoing mastectomy, axillary lymph node dissection, where all fatty tissue including lymph nodes known to drain the tumor are removed and tested for presence of metastasis, is usually performed. However, several studies are now showing that sentinel node biopsy alone has similar prognostic outcomes, with reduced morbidity for surgery and fewer complications related to edema\textsuperscript{21}.

Figure 1.4 Surgical resection of an SLN has been summarized a-e represent the chronological order of events. a) Lymphoscintigram of radiotracer uptake from tumor injection site to SLN. b) Gamma probe used to locate SLN. c) Vital dye injection at tumor site, dye is taken up by lymphatics. d) Blue dye stain is used to track lymph vessels and locate the lymph node, shown in e). The node is then resected and sent for pathological testing to determine presence of metastasis. Images from http://www.melanomahopenetwork.org.
The high sensitivity of >80% for macrometastases, close to 100% specificity, and the lack of other mapping and metastasis detection methods with superior sensitivity have lead to the establishment of sentinel node biopsy using radiotracer-based mapping as the gold-standard of care. However, there are limitations in the ability of this approach to identify all sentinel nodes resulting in high false negative rates. These are highly subjective and dependent on various factors including surgeon’s experience, tumor location and patient age. Additional shortcomings include radiotracer half-life considerations, fast clearance of blue dyes owing to their small molecular size and their tissue staining properties, and most importantly overtreatment and morbidity from the surgical procedure. These issues have lead to research into more non-invasive structural and functional imaging approaches to detect nodal metastasis, which will be described next.

1.2.2 Ultrasound-guided fine-needle aspiration cytology (FNAC)

Ultrasound imaging is a useful tool to image SLNs, but as it lacks accuracy and specificity in identifying node-negative and node-positive patients, it has been combined with fine-needle aspiration cytology or surgical biopsy. Figure 1.5 shows ultrasound (top row) and \textit{ex vivo} H&E images (bottom row) of normal (A-D) and involved lymph nodes (E-F).  

![Figure 1.5 Ultrasound images (top row) of lymph nodes and their corresponding H&E images (bottom row). A-D are normal nodes where as E bears some metastasis and F is a fully involved node. Adapted from Bedi et al.29.](image)
This approach eliminates the need for sentinel node dissection, but does require sentinel node mapping, to localize tumor-draining nodes, which is usually performed using radiocolloid-based lymphoscintigraphy. Various configurations of ultrasound probes such as endoscopic, and endobronchial setups have also been explored in sentinel-node biopsies of cancers in the viscera. Ultrasound-FNAC has potential benefits of reduced hospital costs and patient morbidity due to surgery. However, it has a low sensitivity of \( \sim 57\% \)\(^{27} \), likely owing to sampling errors, and also the low accuracy of ultrasound imaging itself.

1.2.3 Structural imaging: Computed Tomography (CT) and Magnetic-resonance imaging (MRI)

Structural imaging modalities are often employed in predicting presence of metastasis in lymph nodes associated with tumors of the abdomen and pelvis, which are otherwise inaccessible without major surgical intervention. In the majority of cases, lymph nodes are categorized as suspicious based solely on the size criterion, using both contrast and non-contrast imaging techniques\(^ {31-33} \). These approaches are useful in non-invasive identification of nodal involvement, but suffer from poor sensitivity and specificity.

Iodinated contrast agents for CT imaging, and MR contrast agents based on iron oxide nanoparticles have been explored, as they have superior accuracy in detecting suspicious lymph nodes (See Figure 1.6).\(^ {34} \) Nevertheless, such techniques have high costs involved. The cost is still significantly lower than surgical node dissection, and is a viable option especially in the detection of metastasis in occult lymph nodes.
1.2.4 FDG-PET and FDG-PET/CT or MRI imaging

Positron-emission tomography (PET) using $^{18}$F-fluoro-2-deoxy-d-glucose (FDG) has been employed to study cancer growth and regional and distant metastases, with greater sensitivity (~90%) and specificity (~90%) than CT or MRI in several cancer types including lung cancers, head and neck cancers, pelvic cancers, and abdominal cancers. Being a glucose analog, FDG is marker for tissue uptake of glucose—which is upregulated in regions of high metabolic activity such as malignant tumors and their metastasis. FDG-PET does not exceed the specificity obtainable by sentinel node dissection, and hence has not replaced it in cases where the sentinel node can easily be excised through surgery such as in detecting regional metastasis in breast cancer and melanomas. FDG-PET on its own suffers from poor spatial resolution and difficulty in preoperative node localization owing to lack of visible anatomical landmarks on a PET.
scan, so PET imaging is often combined with an anatomical imaging modality such as CT or MRI. Figure 1.7 shows a CT image and an FDG-PET image overlaid to show a metastasized lymph node.

Figure 1.7 (Left) CT image of the thorax of a patient diagnosed with non-small cell lung cancer. No enlarged lymph nodes were seen. (Right) Coregistered FDG-PET/CT revealed presence of nodal metastasis. Reproduced with permission from Lardinois et al.39 Copyright Massachusetts Medical Society.

At present, FDG-PET imaging is the only approved non-invasive diagnostic test for detection of local and remote metastasis40. However, it is not done routinely, and generally performed only in cases where tumor-draining nodes are known to be occult and inaccessible during tumor surgery, as the accuracy of metastasis detection is lower than in histological analysis of resected nodes41. Such a preoperative imaging approach would nevertheless need intraoperative imaging and coregistration of preoperative imaging data, which is further complicated by the small size of lymph nodes and their abundance in the body42.

1.2.5 Fluorescence Imaging Methods

The issues related to use of radiotracers, their half-life considerations, and fast clearance of blue dyes owing to their small molecular size, and their tissue staining properties, that have encouraged research into the off-label use of approved, near-infrared fluorescent (NIRF) dyes specifically Indocyanine Green (ICG) for node mapping in staging of cancers. Several groups have published on the intraoperative
node-mapping efficacy of ICG stating that is equivalent to lymphoscintigraphy and potentially better than blue dye staining\textsuperscript{43,44}. Furthermore, fluorescence imaging has been shown to be feasible at micro-doses of fluorophore in human patients\textsuperscript{45} with a high degree of accuracy in locating sentinel lymph nodes\textsuperscript{46}. Although NIRF-based techniques for nodal staging are growing in use in node mapping, there is still need for surgical resection and pathological testing which is invasive and time consuming, and possibly requires follow-up surgeries. Studies using fluorescence mapping of lymph nodes\textsuperscript{47,48} have so far focused more on localization of nodes, than estimation of tumor-burden on identified nodes\textsuperscript{49}. The fluorescence rather than absorption or reflectance is advantageous as it allows use of trace amounts of contrast agent (fluorophore) with real-time capability for imaging, thus making it highly promising\textsuperscript{46,47,50}. Nevertheless, only a handful of studies have tried using fluorescence to quantitatively study lymphatic function\textsuperscript{51}. A large factor in fluorescence imaging of the lymphatics is the design and quality of the fluorescence imaging system. Device performance and sensitivity will ultimately decide the clinical impact of fluorescence imaging for node mapping and detection of nodal involvement. Several review papers have been published to elucidate the instrumentation and design aspects of these imagers; Chapter 3 discusses current devices for imaging, and their strengths and weaknesses, with the goal of lymphatic fluorescence imaging. This thesis builds on the existing fluorescence-based node mapping techniques to quantitatively study lymphatic function under normal and disease conditions and design metrics to estimate \textit{in vivo} metastatic burden non-invasively, and hence take fluorescence imaging methods from ‘nodal mapping’ to ‘metastasis sensing’.
Figure 1.8. White light image (left), white light + fluorescence overlay (right) with red pseudocolor for ~700nm emission and green for ~800nm emission. Here the mesenteric lymph nodes are highlighted after intravenous injection of Methylene blue (brackets) and a sentinel node (arrow) and lymphatic vessel show up after intraparenchymal injection of ICG. Reproduced from Troyan et al.\textsuperscript{50}, Copyright 2009, with permission from Springer.

1.2.6 Molecular Imaging approaches

Antibody-based cancer-targeted imaging has been investigated for several years and Sampath et al. provide a detailed review\textsuperscript{52} of antibodies conjugated to radio-colloids and (or) NIR fluorophores used in assessment of nodal involvement. Antibodies are beneficial as they are preferentially taken up by the lymphatics and have long lymphatic transit times enabling localization. While there exists a body of research on using antibodies labeled with radio-tracers and (or) fluorophores, translation to the clinic has been hindered by requirement for approval of such antibody conjugates and potential concerns about insufficient specificity.

Tichauer et al.\textsuperscript{53} showed that number of tumor cells in lymph nodes correlated with epidermal growth factor receptor concentration imaged using a dual-tracer combination of a targeted and an untargeted fluorescent tracers; but that a single targeted tracer imaging would not be a reliable metric of nodal involvement. It was shown that use of a single radio- of fluorescence-labeled cancer-targeted antibody tracer could have large non-specific uptake leading to low metastasis-detection specificity. While this method (lymph node molecular concentration imaging or LN-MCI) is attractive, it does require production of two tracers for eventual clinical use, so will have limited immediate
impact. In this work, we attempted to build on these established ideas, and improve the clinical translatability of techniques.

![Figure 1.9 Image demonstrating that the cancer targeted Erbitux tracer cannot reliably estimate metastatic burden 3h post injection, but when combined with the untargeted tracer uptake, the estimated EGFR concentration correlated with nodal involvement. Adapted by permission from Macmillan Publishers Ltd: Nature Medicine\textsuperscript{53}, copyright 2013.](image)

1.3 Thesis Outline

In this thesis, fluorescence-imaging methods are used to study metastasis progression and nodal involvement in preclinical models to provide a comprehensive set of fluorescence-based tools to non-invasively and quantitatively study lymph node tumor burden. A hybrid ultrasound-guided multispectral fluorescence tomography system is
also developed and tested with the goal of subsurface fluorescence quantification. The following is an outline of upcoming chapters and their content.

**Chapter 2** describes background material and theory to better aid the understanding of experiments and studies presented in this thesis. Basics of lymphatic physiology, light-tissue interaction, imaging and tomography are introduced.

**Chapter 3** discusses the instrumentation and design of various commercial and pre-clinical fluorescence imagers that are relevant to imaging the human lymphatic system non-invasively and during surgery. This work is awaiting review for publication in the Journal of Biomedical Optics.

**Chapter 4** details the work “Nodal lymph flow quantified with afferent vessel input function allows differentiation between normal and cancer-bearing nodes,” by DSouza AV, Elliott JT, Gunn JR, Barth RJ, Jr., Samkoe KS, Tichauer KM, and Pogue BW, published in *Biomedical Optics Express* in April 2015, where a single clinically approved fluorescent tracer was used to study differences between normal and cancer-bearing lymph nodes based on metrics derived from fluorescence imaging.

**Chapter 5** discusses the successes and failures of imaging combinations of cancer-targeting and non-specific fluorescent tracers of different sizes in rat lymph nodes bearing tumors.

**Chapter 6** describes the instrumentation, and validation of an ultrasound-guided multispectral fluorescence tomography device for subsurface fluorescence quantification. This chapter is based on the articles “White light-informed optical properties improve ultrasound-guided fluorescence tomography of photoactive protoporphyrin IX,” by Flynn BP, DSouza AV, Kanick SC, Davis SC and Pogue BW published in the *Journal of Biomedical Optics* in April 2013, and “ALA-PpIX variability quantitatively imaged in A431

Chapter 7 presents a snapshot imaging approach using a combination of cancer-targeted and non-specific fluorescent probes to simultaneously image lymph nodes and vessels, for real-time metastasis detection. This work will soon be submitted for review and publication in a peer-reviewed journal.

Chapter 8 deals with the quantitative analysis of active and passive lymphatic uptake components as visualized by fluorescence imaging approaches, which will soon be submitted for review and publication in a peer-reviewed journal.

Chapter 9 discusses various other imaging and visualization approaches related to imaging and quantification of metastatic burden on the lymphatic system. This chapter is based on the following published work: “Cherenkov-excited luminescence scanned imaging,” by Zhang R, DSouza A V, Gunn JR, Esipova TV, Vinogradov SA, Glaser AK, Jarvis LA, Gladstone DJ, and Pogue BW published in *Optics Express* in February 2015; and “Logarithmic intensity compression in fluorescence guided surgery applications,” by DSouza AV, Lin H, Gunn JR, and Pogue BW published in *Journal of Biomed Optics* in August 2015.

Chapter 10 concludes this thesis with a summary of accomplishments, limitations, and a discussion of future directions.
CHAPTER 2  Background

This chapter describes various concepts related to fluorescence imaging and tomography of the lymphatics starting from the basics of light interaction with tissue to models of light transport. Some background of lymphatic physiology is also provided.

2.1 Photon-Tissue Interaction

Electromagnetic radiation has been used in medical diagnosis over the past two centuries, based on its interaction with tissue. There is a range of interactions that can take place when photons travel through matter, and these depend largely on the photon energy level. The electromagnetic spectrum from $\gamma$-rays to radio waves is shown in Figure 2.1, energy of a photon is $E = h\nu$, where $h$ is the Planck’s constant, $\nu$ is the frequency of the wave, and $\nu = \frac{c}{\lambda}$, where $c$ is the velocity of light in vacuum and $\lambda$ is its wavelength.

![Electromagnetic spectrum](image_url)

**Figure 2.1 Electromagnetic spectrum**

b  English Wikipedia, source: Philip Ronan, reproduced with permission
X-rays have energy higher than the ionization energy level leading to complete removal of electrons resulting in ionization of the matter traversed. This is why all x-rays are called ionizing radiation. Lower energy photons in the UV – near-infrared range lead to electronic excitation of molecules, infrared (IR) photons result in molecular vibrations, and microwaves lead to molecular rotations resulting in heating\(^5\).

Figure 2.2 summarizes the various types of interactions of photons of increasing energies with matter. In this thesis, we focus on a narrow range of the electromagnetic spectrum from the visible range to short-wavelength infrared range (also called near-IR), i.e. \(\sim400 – 850\) nm. Within this wavelength range, the two fundamental interactions are absorption and scattering. In some materials the absorbed light is re-emitted almost instantaneously after absorption at a different energy (wavelength); this phenomenon is called fluorescence, and is the basis of this thesis research. We will describe tissue absorption and scattering, and fluorescence in some detail, and also discuss Cherenkov emission, as it is relevant to a portion of this thesis.

\[\text{http://ef.engr.utk.edu/hyperphysics/hbase/mod3.html},\text{ reproduced with permission from Dr. Rod Nave.}\]
2.1.1 Tissue Absorption

Photons are electromagnetic waves with a particular frequency and molecules are a system with charge separation (negative electron field and positive nucleus). Absorption of a photon occurs in a quantized fashion, that is, only when the photon energy matches the molecule’s energy transition levels. This makes absorption spectrally dependent, resulting in an array of applications, such as therapy by damaging diseased tissue, and spectroscopic diagnosis of chemical composition. Visible light absorbers, also called chromophores are abundant in the body, and both endogenous and exogenous chromophores can serve as a basis for optical contrast in tissue differentiation and delineation. Some of the most common endogenous biological chromophores and their relative absorption spectra are shown in Figure 2.3. Hemoglobin and melanin are the primary chromophores that absorb light in the visible range of wavelengths. The low absorption of water in the visible — NIR makes this window diagnostically valuable. Furthermore, overlap of the water minimum with the Hemoglobin minimum in the 650 – 950 nm range provides a narrow yet highly useful window for delivering therapeutic light to subsurface depths, and also enables subsurface imaging and tomography, with >700 nm lasers able to penetrate down to a few centimeters.

![Absorption coefficients of primary biological chromophores. Adapted from Vogel et al.](image)

Figure 2.3 Absorption coefficients of primary biological chromophores. Adapted from Vogel et al.\(^55\) with permission, Copyright 2003 American Chemical Society.
Absorption of light as a function of wavelength is quantified by an absorption coefficient \( \mu_a \). It is related to the probability that a photon will survive after traveling unit distance through the medium, and can be defined as follows. According to Beer-Lambert Law, transmitted intensity \( I_T(\lambda) \) decreases exponentially with distance traversed \( d \).

\[
I_T(\lambda) = I_0(\lambda) \times e^{-\mu_a(\lambda)d}
\]

Here \( I_0(\lambda) \) is the incident intensity, and the absorption coefficient is a summation of all chromophore contributions. By using multiple excitation wavelengths and measuring the resulting spectrally resolved intensity, relative chromophore contributions can be estimated; this is the basis of tissue-spectroscopy. However, scattering dominates absorption in tissue and complex models need to be utilized to account for it.

### 2.1.2 Tissue Scattering

Scattering of light occurs whenever there is a change in refractive index of media. We will describe elastic scatter, that is, direction change without energy loss; inelastic scattering is outside of the scope of this thesis. Tissues are composed of intracellular and extracellular structures that govern their shapes and anatomies. All structural components such as lipid bilayer membranes, collagen fibers, and muscle fiber striations provide the necessary refractive index changes that result in scattering. The size of these components affects intensity of scattering. In biological tissue, based on the range of particle sizes (nm to \( \mu m \)) scattering is composed of Rayleigh scattering – where particle size is much smaller than the wavelength of light and is strongly dependent on wavelength, as well as Mie scatter – where scatter is greatly weighted by the particle size. Mie scatter dominates interactions in the far-red and NIR region as shown in Figure 2.4.
Magnitude of scattering as a function of wavelength is expressed as the scattering coefficient $\mu_s$, and is related to the probability of transmission of a photon with a change in direction. Scattering of light in the Mie regime is rarely an isotropic event, and anisotropy $g$ is used to describe the amount of forward directionality retained after a scatter event; $g$ is defined as the average cosine of the scattering angle, $\theta$.

\[ g = \langle \cos \theta \rangle \] \hspace{1cm} \text{Equation 2.2}

In biological tissue, scattering events are more numerous than absorption events, i.e. $\mu_a \ll \mu_s$, and under such conditions, the scatter characteristics can be simplified to the reduced scattering coefficient, $\mu_s'$, defined as the inverse of the mean free path.

\[ \mu_s' = \mu_s \cdot (1 - g) \] \hspace{1cm} \text{Equation 2.3}

Estimation of reduced scattering coefficient of tissues, enables characterization, and also may be essential in estimating chromophore and fluorophore distributions\textsuperscript{57}.

![Image](image.jpg)

Figure 2.4 Mie and Rayleigh scatter shown in typical skin and breast tissue. Adapted from Jacques\textsuperscript{58}.

### 2.1.3 Fluorescence

Fluorescence is a form of luminescence that is emitted after a molecule (fluorophore) has absorbed light. The molecule, which is at its excited state due to
absorption, returns to the ground state after light emission, as such the emitted light is always of lower energy than absorbed light. Figure 2.5a shows a Jablonski diagram representing elevation of fluorophore from ground state \( S_0 \) to excited state \( S_1 \) through photon absorption. The excited state exists for a finite time (typically 1–10 nanoseconds) during which, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. This non-radiative energy transition, yields a singlet excited state \( S_1^0 \) from which de-excitation to the ground state \( S_0 \) results in emission of a longer wavelength photon (fluorescence). Both endogenous and exogenous fluorophores provide a means of tissue contrast, especially when they are tagged structural, immunologic, metabolic, or immunological markers\(^59\).

![Jablonski diagram](image)

Figure 2.5 a) Jablonski diagram depicting energy level diagram of a molecule during photon absorption and fluorescence emission. b-c) Molecular structure, and absorption and emission spectra of PpIX (endogenous fluorophore) and ICG (exogenous fluorophore).

Fluorophores are characterized by their wavelength-dependent absorption and emission spectra (see Figure 2.5b-c), and their wavelength-independent fluorescence quantum yield \( \eta \), where
\[ \eta = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}} \] ………………Equation 2.4

The fluorophore absorption is linear with its concentration and can be used in estimation of distribution when appropriate models of light transport are applied\(^6\).

Fluorescence emissions are more likely when excitation light is of short wavelength as compared to long wavelength excitation light, and several biological tissue components such as collagen, NADH, tryptophan, porphyrins, and chlorophyll are excitable using UV – blue light. These often contribute to the undesirable background signal during imaging and measurement of fluorescence where signal from tissue needs to be significantly higher than that from the background. Excitation of fluorophores using long wavelength light in the far-red and NIR wavelength bands takes advantage of both low autofluorescence, and improved tissue penetration depth, enabling imaging deep into tissues with high sensitivity.

### 2.1.4 Cherenkov Emission

Cherenkov emissions are produced when a charged particle traverses a medium at a velocity greater than the speed of light (in the medium). The movement of the charged particle causes polarization of molecules along its path, and at high speeds, the polarization of the medium becomes asymmetric resulting in a radiation pulse in the form of Cherenkov light.

In the context of radiation therapy, energetic charged particles with energies greater than the threshold energy for Cherenkov light emission are produced either directly using MeV Figure 2.6 Schematic showing the direction of Cherenkov light emission relative to the direction of travel of the charged particle.
electron beams, or indirectly through the various photon interactions in matter using MeV X-ray photon beams. Cherenkov emission is inversely related to $\lambda^2$, and is hence dominated by blue and UV photons, however, due to the high attenuation of these short wavelength photons, by tissue, most photons of interest are closer to the red region.\textsuperscript{61} Cherenkov radiation has been used to excite fluorophores and phosphors, enabling measurements at much longer wavelengths than the Cherenkov radiation itself\textsuperscript{62,63}. This type of imaging setup also enables probing deeper tissue than visible or NIR light, as MeV photons travel through tissue with minimum scattering as compared to the scatter dominated transport of visible photons.

### 2.1.5 Modeling fluorescence and light transport in biological tissue

The transport of light in biological tissue is most accurately modeled by the radiation transport equation (RTE):

$$
\frac{1}{v} \frac{\partial L(\vec{r}, \vec{s}, t)}{\partial t} = -\vec{s} \cdot \nabla L(\vec{r}, \vec{s}, t) - \mu_t L(\vec{r}, \vec{s}, t) + \mu_s \int_{4\pi} L(\vec{r}, \vec{s}', t) P(\vec{s}' \cdot \vec{s}) d\Omega' + S(\vec{r}, \vec{s}, t),
$$

Equation 2.5

where $L(\vec{r}, \vec{s}, t)$ is the irradiance of light at point $\vec{r}$, $S(\vec{r}, \vec{s}, t)$ is the source, $v$ is the velocity of light in the medium, $P(\vec{s}' \cdot \vec{s})$ is the scattering phase function from direction $\vec{s}'$ to $\vec{s}$, and $\mu_t = \mu_a + \mu_s$ is the extinction coefficient, and $\mu_a$ and $\mu_s$ are the absorption and scattering coefficients respectively. The RTE being a complex integro-differential equation is difficult to solve analytically, however analytical solutions can be derived for simple geometries. A commonly employed simplification is possible within the diffuse regime where the diffusion-approximation to RTE is given by,

$$
\frac{1}{v} \frac{\partial \phi(\vec{r}, t)}{\partial t} + \mu_a \phi(\vec{r}, t) - \nabla \cdot [D \nabla \phi(\vec{r}, t)] = S(\vec{r}, t), \quad \text{Equation 2.6}
$$
Here \( D = \frac{1}{3(\mu_a + \mu_s')} \) is the diffusion coefficient, \( \mu_s' = \mu_s(1 - g) \) is the reduced scattering coefficient, and \( g \) is the scattering anisotropy parameter. In the context of this thesis, models of light transport (far-red and NIR wavelengths) in tissue were used in fluorescence tomographic estimations of fluorophore distributions within turbid biological and bio-mimicking media where light transport is dominated by scattering events, and is hence diffuse. The diffusion-approximation to the radiation transport equation forms the basis of light-transport models used in this thesis.

### 2.1.5.1 Fluorescence diffusion equation

When a fluorescent inclusion is present in the medium, some of the light interacts with the fluorophore and there is additional light fluence due to fluorescence emissions. If the fluorescence yield is \( \eta \mu_{af} \), where \( \mu_{af} \) is the absorption coefficient of the fluorophore at the excitation wavelength and \( \eta \) is the fluorophore quantum efficiency, then the coupled system of transport equations derived from the diffusion-approximation of radiation transport at steady state will be\(^{64,65}\):

\[
\begin{align*}
\left( \mu_a + \mu_{af} \right) \phi_x(\vec{r}, t) - \nabla \cdot [ D_x \nabla \phi_x(\vec{r}, t) ] &= S(\vec{r}, t) \\
\mu_{am} \phi_m(\vec{r}, t) - \nabla \cdot [ D_m \nabla \phi_m(\vec{r}, t) ] &= \eta \mu_{af} \phi_x(\vec{r}, t)
\end{align*}
\]

Equation 2.7

where subscripts \( x \) and \( m \) represent excitation and emission wavelengths respectively, and \( \mu_{a,x,m} \) is the absorption coefficient of the medium at the emission or excitation wavelength. \( \phi_x(\vec{r}, t) \) is the fluence due to the excitation source and \( \phi_m(\vec{r}, t) \) is the fluence due the fluorophore at \( \vec{r} \).
2.1.5.2 Computational models: Forward and Inverse Problems

All computational models for this thesis were designed using NIRFAST software framework developed at Dartmouth\textsuperscript{66}, based on a finite-element implementation of the fluorescence diffusion-approximation of light transport under a mixed boundary condition, i.e. type III index mismatched air-tissue boundary, where fluence at the tissue edge exits and does not return. We do not provide a mathematical description in this thesis, and interested readers are directed to review the vast literature available on this topic\textsuperscript{66,65, 67}. In brief, the volume was discretized into finite elements, and light propagation was modeled after fixing the known source and detector locations, and the estimates (if available) of distribution of optical properties. The light fluence in each voxel of the finite element mesh could be measured and estimate of light measured at each detector is available through the forward model. The forward model is used to simulate data in the inverse reconstruction process.

The inverse solution of light transport, where optical properties (fluorescence distribution in this thesis) of the tissue/phantom are estimated from the detected boundary fluence, is an ill-posed problem. The fluorescence concentration can be calculated using an iterative error minimization algorithm with the objective function,

\[ \chi^2 = \| \phi_{\text{meas}} - \phi_{\text{sim}} \|^2 \], ..........................Equation 2.8

where \( \phi_{\text{meas}} \) is the measured light fluence at the emission wavelength, and \( \phi_{\text{sim}} \) is fluence simulated using the forward model. This can be reduced to the form

\[ \delta \eta \mu_{af} = (J^T J + \lambda I)^{-1} J^T \delta \Phi \], ..........................Equation 2.9

where \( J \) is the Jacobian sensitivity matrix, which conveys the change in the boundary fluence with respect to changes in optical properties, \( \eta \mu_{af} \) is the fluorescence yield, \( \delta \Phi \) is
the error between $\phi_{meas}$ and $\phi_{sim}$, and $\lambda$ is the regularization parameter. This equation can be solved in a least squares sense, to estimate the fluorophore distribution in the measured phantom/tissue, with Tikhonov regularization (most popular). In Chapter 6, we describe an implementation of the above models in tomographic estimation of subsurface fluorescence quantification using prior spatial information from ultrasound imaging.

### 2.2 In Vivo Fluorescence Imaging and Tomography

There are a variety of imaging techniques that can be employed to detect *in vivo* fluorescence emissions from tissues *in vivo* including microscopic and macroscopic methods. The work in this thesis focuses on macroscopic imaging and tomography methods, and hence a description of fluorescence microscopy is beyond the scope of this thesis. Planar fluorescence imaging is commonly employed to detect and visualize surface and subsurface fluorescence emissions. The setup usually involves a broad-beam light source and a filtered detector such as a camera\(^6\). This section will briefly describe available types of systems for human and small animal imaging, and Chapter 3 will focus on the instrumentation, design and comparison of various commercial and preclinical fluorescence imagers for patient imaging.

Planar imaging methods can further be classified into epi-illumination and trans-illumination setups (see Figure 2.7)\(^7\). In the epi-illumination setup, excitation light is shone on the tissue from the same side as the detector. Owing to the simplicity of this setup and the potential for high-throughput imaging, it is very common among commercial small-animal imagers such as LI-COR Pearl Impulse, and Perkin Elmer IVIS series. Most broad-beam imagers designed for human or large animal also use an epi-
illumination geometry. For all animal-imaging studies in this thesis, planar small-animal fluorescence imagers were used. Trans-illumination, on the other hand involves illumination of the imaging target from a direction opposite the source, and hence through the tissue, like an X-ray scanner. This setup is very uncommon but is seen in some tomography instruments for imaging breasts\textsuperscript{70}, and small animal imagers such as Perkin Elmer IVIS.

Tomographic imaging and measurements using both epi- and trans-illumination type setups are also being developed and used. Here the source and detector positions vary in time and space to sample the tissue volume repeatedly. The resulting images are back-projected using forward and inverse modeling of light transport to reconstruct optical properties. Tomographic imaging may involve contact or free-space technologies. In this thesis a contact reflectance tomographic setup using fiber-probe based illumination and detection is explored for use in subsurface fluorescence quantification. Chapter 6 describes the instrumentation and methods in detail.

Figure 2.7 Planar fluorescence imaging approaches: epi-illumination and trans-illumination
2.3 Physiology of the Lymphatic System

The lymphatic system is the drainage system of the body. It acts in conjunction with the blood circulatory system and maintains fluid flow, and recycles excess nutrients and proteins. It is involved in the transport of fats from the intestine to the body cells, and also plays a critical role in the transport and distribution of immune cells. The lymphatic system lacks its own pump, but relies on bodily movements, small muscular contractions within the walls of some lymphatic vessels, and the presence of valves in the vessels to provide unidirectional flow of lymph from extracellular spaces into the subclavian vein\(^71\). Blood travels to tissues via arteries, and at the smallest capillary-level, fluids, nutrients and proteins are transported out of the vessels into the interstitial space.

Figure 2.8 Schematic showing the lymphatic capillaries and lymph vessels\(^d\)

Cells exchange material and proteins with the fluid in the interstitial space, and some of these materials are recycled by capillary uptake. However, the capillary wall is not permeable to macromolecules and cells, resulting in an osmotic pressure in the interstitial

\(^d\) http://cnx.org/contents/xEZkXdm8@4/Anatomy-of-the-Lymphatic-and-I
space, causing net fluid flux out of the capillaries. The excess fluid and macromolecules are taken up by the highly permeable lymphatic capillaries (fluid is now called lymph), resulting in maintenance of osmotic and hydrostatic pressure in the interstitial spaces (see Figure 2.8). The net fluid flow rate affects the lymph formation and thus the lymphatic flow, which is 100-500 times less than blood flow. Owing to the low shear stresses, the lymphatic system enables dissemination of immune cells and tumor cells. Furthermore, lymph nodes along the vessels act as reservoirs of nutrients as they are junctions between the blood and lymph systems. Wiig et al., Swartz, and Schmidt-Schonbein provide very detailed descriptions of lymphatic structure and physiology, and we recommend that they be read for a thorough understanding of the principles. We provide a brief summary of the physiology of the lymphatic system as relevant to this thesis.

2.3.1 Organizational constituents of the Lymphatic System and their functions

The lymphatic system is comprised of the lymphatic capillaries, collector lymph vessels, lymph nodes, trunks and ducts. We describe the capillaries, lymph vessels and nodes here.

2.3.1.1 Lymphatic Capillaries: lymph formation

The lymphatics originate in the interstitial space; at this level, fluid and materials from the interstitium are drawn up by the lymphatic capillaries (also called initial lymphatics or terminal lymphatics), which are single endothelial-cell thick, non-fenestrated, basement membrane-free, 10 – 60 μm-diameter blind-ended structures. These capillaries lack smooth muscles and valves, and are anchored to the extra-cellular
matrix (ECM) by means of anchoring filaments\textsuperscript{76} that facilitate lymph formation by exerting tension on the capillary wall causing the luminal volume to increase in response to interstitial stress, producing a pressure gradient and causing fluid influx\textsuperscript{77}. Evidence also suggests that the overlap junctions on the capillary walls act as a valve system that prevents efflux of lymph from the capillary lumen\textsuperscript{78}.

Lymph formation at the capillaries is driven by hydrostatic and osmotic pressure gradients between the capillary lumen and interstitium, and net fluid (solvent) flux $J_V$ can be described using Starling’s Law\textsuperscript{71-73},

$$J_V = L_p \frac{s}{V} (\Delta P - \sigma \Delta \pi),$$

Equation 2-10

where $L_p$ is the permeability, $s/V$ is the surface-area-volume ratio, $\Delta P$ is the hydrostatic pressure-difference, $\Delta \pi$ is the colloidal osmotic-pressure difference, and $\sigma$ is the capillary osmotic reflection coefficient. Here $\sigma \Delta \pi$ arises from the assumption that the membrane is impermeable to proteins, however not true in the case of lymphatic capillaries, and their (solute) flux $J_S$ across the capillary wall can be modeled as the sum of diffusive and convective fluxes\textsuperscript{79} occurring simultaneously by the Patlak equation\textsuperscript{80},

$$J_S = J_V (1 - \sigma) C_p + PS (C_p - C_{if}) \cdot \left[ \frac{x}{e^x - 1} \right],$$

Equation 2-11

Here $C_p$ and $C_{if}$ are the protein concentrations in the plasma and interstitium respectively, $PS$ is the permeability-surface-area product, which is a measure of the diffusive capacity of the capillary membrane, and $x$ is the Peclet number, which describes the convective flux relative to diffusive capacity of the membrane\textsuperscript{73}. The first term of Equation 2-11 is the convective flux term, and the second term is the diffusive flux term.
Resting

Figure 2.9 Lymphatic capillaries are shown with their ECM anchors at resting and lymph formation states. Interstitial stress causes radial tension on the anchoring filaments, locally increasing the luminal volume of the lymphatic capillary, resulting in fluid inflow. Reproduced from Swartz\(^72\), copyright 2003, with permission from Elsevier.

2.3.1.2 Collector Lymph Vessels: lymph propulsion and unidirectional flow

The lymphatic capillaries drain the lymph into the collecting lymphatics (lymph vessels), which are larger vessels without ECM anchors, but have a smooth muscle lining. They also have one-way valves that prevent retrograde lymph flow\(^81,74\), as shown in Figure 2.10.

The narrow part of each valve is held to the vessel wall by buttresses that prevent inversion. There are no muscles in the valve and they operate only by pressure changes in fluid. P1 pressure in front of valve, P2 is downstream pressure, and P3 is pressure in sinuses. The valve opens when pressure P1 > P3 and closes when P2 = P3, valve opposes opposite flow i.e when P2 > P3 it remains closed\(^74\). Valves are mostly bicuspid, and are sail, funnel or pocket shaped. Larger vessels can have several layers and have elastic fibers as well\(^82\). The valves divide the lymph vessels into segments or units called ‘lymphangions’. Smooth muscle contractions along the walls of the lymphangions raise the pressure P1, and cause the valve to open resulting in forward peristaltic propulsion of lymph. From here, the collector lymph vessels may carry lymph to a lymph node, and
is referred to as an ‘afferent lymph vessel’. Lymph vessels also arise from lymph node and are called ‘efferent lymph vessels’

Figure 2.10 A-B) 160× magnified images showing bicuspid valves in the lymph vessels are shown during smooth muscle relaxation (left) and contraction (right) in cat mesentery. Reproduced from Zweifach et al81 with permission from The American Physiological Society. C Schematic showing the valve structure and cross-sections54. D Shows valve function. C and D reproduced from54, no permission was required.

2.3.1.3 Lymph Nodes

Lymph nodes are spheroidal organs 1 to 10 mm in diameter along the lymphatic system. There are hundreds of lymph nodes all over the body and they receive a rich supply of blood and act as filters and reservoirs, where immune cells and cancer cells can take residence in and proliferate with access to nutrients.

Lymph node structure includes a capsule enclosing the sinuses, which are separated from each other by fibrous inward extensions of the capsule called trabeculae. Incoming
afferent lymph vessels bring proteins, cells and lymph into the node and drain them into the sinuses. Here the lymph flows, and is also filtered by the presence of macrophages that trap foreign particles. At the sinuses, exchange of material can also take place between the blood and lymph, and the lymph exiting the node undergoes changes in protein concentration. Small molecules such as dyes may exit the lymphatic system here and enter the blood stream, whereas large proteins and macromolecules can either stay in the node or move further downstream along the lymphatic system via efferent vessels. In this thesis, our studies and analyses are restricted to the initial lymphatics draining a tumor and the first-tumor draining lymph nodes, as these are the primary structures involved in cancer metastasis.

Figure 2.11 Lymph node cross-section. Reproduced from Mosby, with permission from Elsevier, copyright 2009.
CHAPTER 3  Fluorescence Imaging Systems
for open-field human imaging

This chapter describes various planar fluorescence imaging systems, designed for open-field imaging use during surgical but with potential off-label applications in imaging lymphatics and lymph nodes in small and large animals (humans). We describe the design, instrumentation of features of the various commercial FDA-approved and preclinical systems and compare the systems to each other based on these, to enable a reader to not only understand the various design aspects but also in choosing the right imager for lymphatic imaging. The chapter is adapted from the manuscript, “Review of Fluorescence Guidance Systems for Open Surgery: Identification of Key Performance Goals,” by Alisha V. DSouza, Huiyun Lin, Jonathan T. Elliot, Scott C. Davis, Eric R. Henderson, Kimberley S. Samkoe, and Brian W. Pogue, submitted to the Journal of Biomedical Optics, and under review as of May 2016.

3.1 Introduction
In recent years, there has been an explosion of interest in fluorescence-guided surgery, which has led to a steady demand for new commercially developed and approved fluorescence imaging devices. For any system to make the greatest clinical impact, it needs to not only provide a solution to the immediate clinical goal, but also do so in a way that seamlessly blends into current clinical workflow, or provides important new information that affects the patient outcome. There are several new fluorescence imagers that have been cleared for market by the 510(k) process at the US Food and Drug
Administration (FDA) for open surgical use. Beyond this, new commercial research systems with important features are regularly emerging. However, there often is a disconnect between this emergent technology, and the clinicians’ needs and flexibility.  

Several reviews discuss the design, applications, and need for such imagers and tabulate the specifications of available imagers in the market. However, none of them provide direct guidance on how to choose the right imager based on objective criteria. System selection can be subjective and dependent on the end user’s preferences; however, defining basic criteria for comparing imagers for specific applications can help the field of imaging medicine mature in an organized manner.

<table>
<thead>
<tr>
<th>Company</th>
<th>Fluorescence imaging system</th>
<th>Year approved/510(k) cleared</th>
<th>FDA 510(k) number</th>
<th>Indication Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Novadaq Technologies, Inc.</strong></td>
<td><strong>SPY Imaging system</strong></td>
<td>2005</td>
<td>K042961</td>
<td>Blood flow</td>
</tr>
<tr>
<td><strong>Novadaq Technologies, Inc.</strong></td>
<td><strong>SPY Imaging system SP2000</strong></td>
<td>2007</td>
<td>K063345</td>
<td>Tissue perfusion &amp; transfer circulation free-flaps, plastic &amp; reconstructive surgery</td>
</tr>
<tr>
<td><strong>Novadaq Technologies, Inc.</strong></td>
<td><strong>SPY Fluorescent Imaging system SP2001</strong></td>
<td>2008</td>
<td>K073088</td>
<td>510(k) with SPY SP2000</td>
</tr>
<tr>
<td><strong>Novadaq Technologies, Inc.</strong></td>
<td><strong>SPY Fluorescent Imaging system SP2001</strong></td>
<td>2008</td>
<td>K073130</td>
<td>510(k) for modified device</td>
</tr>
<tr>
<td><strong>Novadaq Technologies, Inc.</strong></td>
<td><strong>SPY Intra-operative Imaging system</strong></td>
<td>2011</td>
<td>K100371</td>
<td>Additional gastrointestinal imaging</td>
</tr>
<tr>
<td><strong>Hamamatsu Photonics K.K.</strong></td>
<td><strong>PDE Photodynamic Eye</strong></td>
<td>2012</td>
<td>K110480</td>
<td>510(k) with SPY K063345 &amp; K073130</td>
</tr>
<tr>
<td><strong>Hamamatsu Photonics K.K.</strong></td>
<td><strong>PDE Neo</strong></td>
<td>2014</td>
<td>K133719</td>
<td>510(k) with PDE K110480 for modified device</td>
</tr>
<tr>
<td><strong>Fluoptics</strong></td>
<td><strong>Fluobeam 800 Clinical Imaging Device</strong></td>
<td>2014</td>
<td>K132475</td>
<td>510(k) with PDE</td>
</tr>
<tr>
<td><strong>Quest Medical Imaging</strong></td>
<td><strong>Artemis Light Engine</strong></td>
<td>2015</td>
<td>K141164</td>
<td>510(k) with Karl Storz &amp; Olympus Winter</td>
</tr>
<tr>
<td><strong>Quest Medical</strong></td>
<td><strong>Artemis Handheld Imaging Systems</strong></td>
<td>2015</td>
<td>K143474</td>
<td>510(k) with PDE &amp; Fluobeam 800</td>
</tr>
<tr>
<td><strong>Visionsense Ltd.</strong></td>
<td><strong>VS3-IR-MMS system</strong></td>
<td>2015</td>
<td>K150018</td>
<td>510(k) with SPY 063345</td>
</tr>
</tbody>
</table>

Table 3.1 FDA clearances through the 510(k) process based on the device being safe and effective, with substantial equivalence to a predicate device. These are all ICG fluorescence imaging systems shown along with their year of pre-market approval by FDA, case number, and the indications that they are approved for.
The exponential growth in the field is evidenced by the total number of published articles in the area of fluorescence guidance in surgery, which has grown from under 50/year in 1995, to 100/year in 2005 to nearly 500/year in 2015. This growth is almost equally divided between papers discussing the growing clinical imaging approaches with indocyanine green (ICG)\textsuperscript{91}, and the growing development of targeted molecular contrast agents for specific vascular, metabolic or immunologic features of tissue, by the research community\textsuperscript{97,92}. As seen in Table 3.1, while most ICG imaging has been performed by use of the Novadaq SPY system since 2005, several new systems with improved imaging performance have gained 510(k) clearance in the last two years. These are all approved for procedures involving imaging blood flow, tissue perfusion and transfer circulation in free flaps, plastic surgery and reconstructive surgery. Despite the availability of these technologies, most surgeons still rely largely on visual and tactile cues combined with pre-surgical radiologic imaging to distinguish tissues in the operating room. The potential benefit to surgical patients of improving tissue identification based on molecular differences, particularly to those undergoing removal of cancers, is a compelling force driving the research and development in this field. Using molecular signals in the surgical field is a natural progression that follows the development of molecular pathology to identify lesion phenotype, in conjunction with image-guidance from MRI or CT, as a decision tool for patient management, and there is compelling evidence that these phenotypes might be imaged \textit{in vivo} to allow better real time definition of the surgical margin\textsuperscript{93-99}.

In addition to ICG, there is extensive ongoing research using fluorescein and 5-aminolevulinic acid (5-ALA)-induced protoporphyrin IX (PpIX) imaging for neurosurgery\textsuperscript{100-104}, a procedure that has gained clinical approval in a handful of
countries†. Additionally, PpIX fluorescence imaging using blue light illumination for subspecialty use such as bladder cancer detection\textsuperscript{105-107}, and gynecologic oncology\textsuperscript{108-110} been used in clinical trials, and has local approvals in some countries. These fluorophores are FDA-approved for certain indications and emit fluorescence within the visible light range, so that existing surgical microscopes can be modified with some basic filters to allow surgeons to switch back and forth between white light and fluorescence modes. However, while there is apparently good sensitivity, the lack of high specificity of PpIX in some indications has limited widespread adoption of this endogenous metabolic fluorophore. Moreover, these emission bands correspond to the visible light window, and hence suffer from high background autofluorescence and limited depth penetration, allowed only surface imaging. Near-infrared (NIR) fluorophores, on the other hand, emit in a window with very low tissue autofluorescence and also have greater penetration depth due to the reduced hemoglobin absorption in the 650-900 nm range\textsuperscript{111}. Available within this range, methylene blue is a weakly fluorescent visible blue dye that is currently approved for use as a visible stain for lymph node mapping\textsuperscript{85}. While some research groups have investigated its use as a far-red fluorophore\textsuperscript{112}, the low fluorescence yield and lack of any functional groups for addition of ligands have limited its use in clinical applications\textsuperscript{112}. ICG is the only approved fluorophore in the NIR window and several imagers have been designed and commercially launched to allow ICG-guidance in surgery for blood flow\textsuperscript{113-115}, hepatic function assessment\textsuperscript{116, 117} and vessel patency and perfusion evaluation especially in reconstructive\textsuperscript{118, 119} and bypass surgeries\textsuperscript{120, 121}. The lower tissue autofluorescence in the NIR wavelengths further simplifies the task of filtering out background signals, and since ICG is the primary reimbursable agent today,

\footnote{Approved by the European Medicines Agency (EMA) in September 2007 and is approved for use in the all EU, EEA, and EFTA states.}
almost all imaging device companies have built systems specifically for ICG imaging. Currently, there is growing use of ICG in off-label and investigational applications such as lymphatic imaging\(^\text{122}\), and surgical procedures such as sentinel lymph node identification and mapping\(^\text{50, 123, 124}\). Yet a strong motivation for future development in this field is to achieve the potential for imaging molecular tracers that report on new vascular, structural, metabolic, immunologic or genetic features of the tissue\(^\text{125}\).

Surgical oncology and in situ metastasis detection is in a position to benefit greatly from fluorescence imaging technology, and in particular, targeted molecular surgical guidance is poised to follow the widespread adoption of molecular pathology phenotyping\(^\text{87}\). Several companies are now producing NIR emitting agents which can be imaged in systems designed for ICG, but with substantially higher fluorescence yields than ICG, and availability of molecules which can be accurately conjugated to targeting moieties for future indications which have molecular specificity: IRDye 800CW\(^\text{126}\) (LI-COR Biosciences, Lincoln, NE), ZW800-1\(^\text{127}\) (Curadel ResVet Imaging, Worchester, MA), VivoTag 800 (Perkin Elmer, Hopkinton, MA). As applications emerge with specific agents designed for molecular diagnostic imaging, the sensitivity and dynamic range requirements will vary greatly from the current paradigm of ICG flow imaging—typical ICG studies were performed with mg/mL level concentrations in the blood, whereas molecular probes are typically imaged at µg/mL to ng/mL concentration levels. The sensitivity along with the interplay of various factors that affect system performance such as ambient room lighting, image threshold and visualization, ultimately decides the apparent signal-to-background (SBR) performance of a system within the surgical environment\(^\text{128}\). Using systems in a ‘real-time’ mode that display the fluorescence signal in a video feed imposes restrictions on the system sensitivity. This is particularly true in
open surgery where the operation is conducted with bright light; this is less of a
challenge in minimally invasive surgery where the procedure can take place in a darkened
room. The lack of professional society guidance or standards documents on system
quality and accuracy assessment further complicates matters, as there is no single test to
compare systems under standard conditions as yet, and hence any signal-to-background
performance limitations are established based on clinical trials. Several research groups
have been discussing the challenges in defining standardized testing platforms, and have
been working towards designing appropriate phantoms\textsuperscript{129}; these advancements will pave
the way for establishment of sensitivity limits of systems, and improve our understanding
of their utilities in studies involving microdoses of tracer administration.

While system sensitivity is critical for microdose-level tracer administration, this
requirement is not as important in ICG imaging. Trade-offs that sacrifice SBR in favor
of functional utility result in a more usable and flexible device. However, several FDA-
approved commercial imagers do not yet have the ability to simultaneously capture, and
register white light and fluorescence images, or operate reliably under operating room
light conditions. This limitation requires the surgical team to switch between viewing the
imager display and the surgical field, making fluorescence imaging cumbersome, and
limited in its ability to accurately register fluorescence-labeled tissues with what is seen
with visible light; the surgeon now needs to mentally register the while light illuminated
field in front of him, with the fluorescence intensity image devoid of major anatomical
landmarks. Furthermore, providing information in a useful manner is often an
understated requirement for the successful design and use of such imagers. While the
currently approved systems such as Novadaq’s SPY system have been instrumental in
launching fluorescence-guided surgery into growing clinical use, we believe that
identifying the most important basic requirements from imager must be re-evaluated to address the unmet needs in future systems. Figure 3.1 shows a panel of figures demonstrating the use of fluorescence imaging, a typical setup using the market-leading Novadaq SPY-Elite imager, and progression of imaging devices with custom devices leading the way and FDA approved following along.

Figure 3.1 a Demonstrates the progression of systems in terms of their regulatory approval status along with parallel technologies. b-g Show examples of surgical fields paired with white light (left) and fluorescence images (right) shown side by side in various applications. b and c, and d and e are reproduced from Van Dam et al.93, and Whitney et al.130 respectively with permissions from Macmillan Publisher Ltd. f and g are
reproduced from Matsui et al.\textsuperscript{131} with permission from Elsevier. h A typical surgery setup with Novadaq’s SPY Elite system being used for perfusion assessment.\textsuperscript{\textregistered}

In this chapter, we describe the key engineering design criteria and the desirable features of an imager; we also supplement the discussion of desirable features with a description of system instrumentation in the sections that will follow. We finally compare current available commercial imagers on the basis of functionality, usability, and technical specifications.

3.2 System Uses & Feature Goals

The overall goal of every fluorescence imager is to provide information on the distribution of the weak (relative to white-light reflectance) fluorescence signal within the surgical field. Clinical compatibility is improved by providing the information in a user-friendly, distraction-free manner, whilst minimizing background signals and noise. This can be simplified to the singular goal of maximizing the signal-to-background ratio (SBR) to distinguish between diseased versus disease-free tissue, or to identify tissue to save, such as nerves and blood vessels, amongst tissue being resected. SBR on its own however does not make a system description complete, and does not mean much without the right means to convey information to a user. Achieving high SBR in a surgical environment is a complicated problem influenced greatly by ambient lights. Surgeons rely on visual information to identify landmarks, and are trained to identify tissues in their native pink-red hues; any additional fluorescence information, especially that from outside the visible range of the light spectrum, must therefore be co-registered to the white-light field. This calls for a need to overlay white-light RGB and fluorescence

\textsuperscript{\textregistered} Image from http://www.lymph.org/new-page/, reproduced with permission from The Friedman Center for Lymphedema Research and Treatment.
images in real-time. Furthermore, operation rooms are well-lit spaces, and imagers that operate only in low-light will have limited impact in most clinical setups.

As the field of fluorescence imaging for open-field imaging moves towards adoption of targeted fluorescent probes\textsuperscript{92} and the quantification of disease biomarkers, the reliance on fluorescence imagers for image data acquisition will increase. While reliable absolute quantification in an open setup has not yet been fully realized, there have been significant advances in ratiometric methods, and tracer combinations can be used to quantify receptor and molecular concentrations\textsuperscript{53,132}. There is also recent growth in the development of diagnostic agents that would be administered at microdoses as compared to therapeutic agents, to match the receptor concentrations available in tissue. This would demand reliable operation from imagers in the nanomolar concentration range. Based on these needs, a listing of key desirable features of an open fluorescence imager is as follows.

i. **Real-time overlay** of white light and fluorescence images

ii. Fluorescence-mode operation with **ambient room lighting present**

iii. **High sensitivity** to tracer of interest

iv. Ability to **quantify** tracers *in situ*

v. Ability to image **multiple fluorophores** simultaneously

vi. **Maximized ergonomic use**

In order to fully appreciate the complexity of enabling these features, a discussion of current instrumentation is necessary; this is provided in **Section 3.3**. We then discuss each of the above listed features in detail in the following **Section 4**.
3.3 Imaging System Components And Instrumentation

The basic components of a fluorescence imager are (i) a spectrally-resolved light source(s), (ii) light-collection optics and filters (iii) camera(s), (iv) instrument control, acquisition and display software, and (v) computing, input and display hardware.

3.3.1 Excitation light source

Choice of excitation light source is based on the spectral bandwidth, solid angle of output beam, output efficiency, and regulatory considerations. Commonly used excitation-sources are (i) filtered broadband lamps, (ii) laser diodes, and light-emitting diodes (LEDs). Ease of filtering at detection, illumination of large field-of-view (FOV), output fluence rate, mounting requirement, and cost are the main factors that influence the choice of excitation source type. Among available options, filtered lamps have the lowest efficiency, largest spectral bandwidth and largest solid angle, and hence least spatial and spectral confinement. Furthermore, a large fraction of the output photons are rejected at the excitation filter resulting in high heat dissipation, thus making their use cumbersome and suboptimal. Such excitation source setups are seen currently in surgical microscopes.

Laser diodes have the highest spatial and spectral confinement of all sources. The low spectral bandwidth allows maximum excitation light filtering at the fluorescence detection camera. High power options are available and deliver the best light fluence rate for low fluorophore concentrations, but safety concerns related to maximum permissible exposure limits for skin and eyes complicate the regulatory approval of systems that use these. Moreover, a beam expander would be necessary to ensure illumination of large enough FOV. Laser diodes also require precise temperature and current control to ensure fidelity of the output spectrum and power, thus necessitating additional hardware,
and remote mounting away from the patient. Such systems would use fiber coupling from the light source to the illumination head. These are seen in the Fluobeam and Curadel LAB-Flare systems.

LEDs provide a trade-off between output power, efficiency, cost, and spectral bandwidth. With the growth of the LED market it is becoming increasingly economical to produce high power LEDs. To ensure homogeneity of the excitation field, LEDs would need to be combined into an array. However, one of the major drawbacks of using LEDs is that when fluorophores with small Stokes shifts are used, there will be leakage of excitation-light past the emission filter leading to reduced SBR. An excitation filter would thus need to be used to confine the output of the light source. Some of the newer imagers such as Perkin Elmer's Solaris utilizes LED-based excitation, and we should expect to see further increase in their use.

### 3.3.2 Collection optical components and emission filters

Multiple tradeoffs exist when discussing collection optics. These are field-of-view size, depth of field, lens F-number, and operating distance. Collection optics may be designed for fixed magnification or variable magnification depending on the need to have variable field size and operating distance. Most common imagers have a working distance of 10 – 30 cm with a maximum FOV size of about 10×10 cm², but the tolerance for focus errors varies among manufacturers and end users.

Emission filter design and choice is critical in maximizing detection sensitivity by limiting background light. Filter choice is influenced by spectral overlap between the reflected excitation light, ambient lighting, and the Stokes shift of fluorophore of interest. Fluorophores with large stokes shift such as PpIX, which can be excited in the blue range (~405 nm), and detected in the red range (~635 nm) pose few problems,
whereas those that have significant overlap between absorption and emission spectrum such as ICG, require more careful selection (See Figure 3.3). Long pass, band pass, and notch filters are broadly the categories for selection. Interference filters generally provide superior out of band rejection and transmission in the pass band as compared to absorption-based filters. Spectral characteristics of filters also vary with incidence angle of light and there will be transmission of undesirable excitation light incident at steep incident angles. This along with the excitation leakage through the rejection band determines the noise floor of a device and hence affects its sensitivity.

### 3.3.3 Imaging Sensor

The factors that influence detection performance of the camera are dynamic range, read-out rates, pixel resolution, and on-chip gain. For quantitative imaging bit-depths of 10-bit or more are necessary to provide signal detection of 2-3 orders of magnitude while maintaining a low noise floor. Charge-coupled device (CCD)-based cameras are used in fluorescence imagers almost ubiquitously but suffer from low quantum efficiency in the far-red and NIR wavelength range, and slow read-out time (<30 Hz). They can achieve low readout noise when cooled, and generally have high resolutions and can be used with 16-bitA/D converters. Further improvements in sensitivity are achievable with electron multiplied-CCDs (EMCCD) and intensified-CCDs (ICCD); these can provide analog gains of over 1000×. The scientific-CMOS (sCMOS) cameras are now a contender against CCD cameras as they provide high readout rates, high bit-depth and the specific advantage of many more pixels per frame. The compact-size, lightweight and low readout noise in addition to the high-readout rate, and high bit-depth makes sCMOS cameras the sensor of next-gen fluorescence imagers. One of the drawbacks however is the higher cost as compared to standard CMOS technology. Among the commercial
systems available, the Perkin-Elmer Solaris is the only system that uses an sCMOS camera. More sophisticated camera configurations such as hyper spectral and multispectral cameras, though rarely used in most systems, will be discussed briefly in Section 3.4.1.

3.3.4 Software control, computing, data storage, and display hardware

Software designs vary in the degree of user customizability; those that target clinical use generally have the least flexibility while investigational systems and research-oriented systems allow a good deal of user customization. With growing bit-depths, high-speed data transfer and data storage become important considerations. Imaging and storing large video sequences, potentially from multiple cameras, can result in several gigabytes of imaging data per hour – storage one hour of single channel 1024×1024 pixel² 8-bit fluorescence data at 30 fps for an hour would need >100 GB of space – posing a significant data management challenge. One approach to tackle this is to store compressed video files only, and save data into an 8-bit format, even if the camera provides >10-bits per pixel. Alternative strategies include saving user-prompted snapshots from a continuous video stream. The ability to customize storage and export on to external drives, or servers may be a solution as well, but local protected storage is likely the best candidate for clinical systems to maintain HIPAA compliance. Data storage is critical in research settings, to allow post processing, and image analysis post-acquisition. Systems such as the PDE Neo, lack on-board storage options and provide only screen captures, which is non-ideal and non-quantitative.

As devices grow in sophistication, software control of instruments becomes more complex especially when multi-camera systems are used. On-board GPUs are often necessary for simultaneous overlay and streaming. It is important that systems be
customizable yet easy to use for clinical staff and surgeons. The software functionality also directly ties in with data visualization and display optimization. Need for ROI intensity measurement tools and on-screen window-level, and compression options will increase as systems are used for quantitative or semi-quantitative imaging, and as image bit depths exceed the display bit depths. Software design is the most understated aspect of fluorescence imagers, but since their use during surgery would need seamless integration with the surgical protocol, robust, intuitive design is key.

3.4 Design Goals And Analysis of Key Features

3.4.1 Real-time overlay of white light and fluorescence images

Most FDA approved imagers, such as Novadaq SPY, Hamamatsu PDE Neo, and Fluoptics Fluobeam 800 are single channel fluorescence video/image display systems. However, imagers that have the ability to provide the fluorescence image overlaid on a white light illuminated RGB image in a real-time video stream would provide richer and more complete information to a surgeon. To produce such images in real time is significantly more complex than a single channel fluorescence video stream. While there are several approaches to achieving overlaid data, wavelength-based separation of fluorescence (>650 nm) and visible white light (<650 nm) is the main principle upon which imager designs are based.

The most commonly employed technological approaches to achieving simultaneous white-light and fluorescence imaging are use of beam-splitters and multiple cameras, or multispectral cameras that separate visible, and far-red and/or near-infrared (NIR) wavelengths within the camera itself using prisms and multiple charge-couple device (CCD or CMOS) sensors. The Flare intraoperative prototypes from the Frangioni lab use
three cameras\textsuperscript{50}, and Curadel’s LAB-Flare imager, based on the Flare prototype, uses three CCD sensors within a single camera body to simultaneously image two fluorescence channels and a white-light RGB channel, by first removing the $\sim$800 nm (NIR) fluorescence component, then the $\sim$700 nm far-red fluorescence component, and using the remainder to produce the RGB image, as shown in Figure 3.2b and Figure 3.2d. The Quest Spectrum system (previously called Artemis) achieves similar wavelength-based separation using prisms within a single camera, and can simultaneously image and overlay two NIR channels ($700 – 830$ nm and $830 – 1100$ nm) on the white-light RGB image stream. However, expansion to more channels requires additional cameras/sensors for simultaneous acquisition. Visionsense Iridium also uses two CCD sensors to produce simultaneous white-light RGB and NIR fluorescence images from the $\sim$800 nm emission channel, and merges the two in real-time. These approaches works well in practice, allows independent gain adjustments for each channel, and does not require sequential pulsing of excitation lights. Perkin Elmer Solaris system on the other hand features two cameras, one for white light image acquisition and one for fluorescence acquisition with overlay capability. A screenshot of the Solaris display in Figure 3.2a shows ‘white light RGB’ and ‘white light RGB + fluorescence overlay’ images showing fluorophore uptake in murine lymph nodes\textsuperscript{6}. Additional considerations for multi-camera setups include coregistration of the various video streams, and magnification corrections. For example, Novadaq SPY, uses two cameras, that are not coregistered and have different pixel dimensions and zoom, as such no overlay functionality has been implemented, and white light images are available only in snapshot mode.

\textsuperscript{1} Image from Perkin Elmer
Other related approaches employ Foveon X3 sensors (HyperEye Medical System, Mizuho Medical, Japan) and detect unabsorbed NIR light or modify the Bayer filter pattern and filter the NIF signal at the entrance to the sensor, thus separation happens within the camera itself (See Figure 3.2c). All of these approaches, while overcoming the problem of merging multiple streams, can be limited in their sensitivity to weak fluorescence emissions, which is most significant for targeted tracers at low concentrations in tissues.

Figure 3.2 Shows various white light and fluorescence overlay schemes. a Shows a screenshot from the Perkin Elmer Solaris imager during lymphatic imaging. The imaging windows displays white light and fluorescence overlaid white light images simultaneously. User processing controls such as ROI measurements and display gain adjustments are also available. b Shows the commonly used wavelength-based separation of collected light using dichroic mirrors and filters as seen in the Flare prototype system. Curadel Lab-Flare uses a similar setup with slightly different wavelength specifications on beam-splitters and emission filters. c Modified bayer filter is an alternative approach to perform simultaneous NIR detection, though this approach reduces the active area for the fluorescence channel, reducing sensitivity. d Shows an example of simultaneous imaging and display of 700 nm (red) and 800 nm (green) fluorescence channels from the Flare prototype from the Frangioni lab. Here the mesenteric lymph nodes are highlighted after intravenous injection of Methylene blue (brackets) and a sentinel node (arrow) and lymphatic vessel show up after...
intraparenchymal injection of ICG. Figures b and d have been reproduced from Troyan et al. with permission from Springer. Figure c has been reproduced from Chen et al. with permission from the OSA.

The optimization of fluorescence visualization and displays is often under-reported as systems are only now beginning to exploit overlay-based displays. With the growth in use of 10–16 bit acquisition cameras, proper scaling and mapping of display on to traditional display monitors is an additional concern. Application of appropriate transparency functions to the fluorescence overlay, choice of appropriate colormaps, and need for compression techniques to display high bit-depth images are important areas with only limited discussion in the literature. The field of high dynamic range (HDR) imaging has allowed optimal display of high dynamic range data, yet this approach has not penetrated into medicine much as of today. As fluorescence imagers incorporate simultaneous multiple channel imaging, this will gain greater importance. Elliott et al. provide a set of guidelines for effective visualization of fluorescence during surgery using surgical microscopes, that are applicable to open surgery as well.

3.4.2 Fluorescence-mode operation with ambient room lighting present

For an imaging system to be easily translatable into a surgical suite, or clinical environment, it is desirable that it operates under room lights and provides reasonable SBR. This issue is critical, especially when working with low fluorophore concentrations and when quantitation is necessary. Figure 3.3e shows a plot of most common room light sources, such as tungsten bulbs, halogen bulbs, compact fluorescence lights, and the newer white light LED lights. It can be seen that tungsten and halogen lamps have significant output in the 600–850 nm range and thus may contribute a major portion of the detected signal during any red to near infrared fluorescence imaging. Use of these
lamps in rooms is seeing a declining trend, to the ultimate benefit of fluorescence imagers.

As a general guideline for researchers and other users of such systems, use of tungsten and halogen lamps should be avoided completely. Both LEDs and CFL lights have minimal signal contribution over 780 nm and thus imaging of ICG and similar NIR fluorophores in rooms lit with these sources should be attainable with simple filtering techniques. However, it should be noted that CFL lights can often emit in the 700–800 nm wavelength range during the warm-up phase, which can last 5 to 10 minutes (data not shown), and thus contamination of detected fluorescence can occur at these times. For imaging fluorophores in the visible to far-red window, that is, 500 nm to 750 nm, normal room lighting would contribute to the detected signal, and sophisticated background removal methods are necessary. Pulsing an LED or laser diode excitation light source synchronized to a gated- or shuttered-detector system such as CMOS or ICCD camera is one technique that may be employed to address background contamination. Similar background mitigation can be achieved using frequency modulation and lock-in detection. Finally, additional considerations, such as operating in a sunlit room, would require further mitigation to reduce background contamination of the signal for best performance.

Given the above information about spectral contribution, the ideal room (surgical) light would use white light LEDs. The Perkin Elmer Solaris system performs background correction by pulsing the excitation sources to sequentially image fluorescence emission and background light leakage to make on-line corrections. It has been shown that systems that perform some kind of background correction tend to
perform better than those without;\textsuperscript{39} this improvement in performance can also be seen from our sensitivity and linearity tests (upcoming section).

Figure 3.3 a-d Absorption and emission spectra of FDA approved fluorescent dyes Fluorescein, Protoporphyrin IX, Methylene Blue, and Indocyanine Green are shown. e Normalized Emission spectra of common room light sources.

3.4.3 High sensitivity to tracer of interest and ability to quantify in situ

The concentrations of fluorescent tracers in tissue vary greatly depending on their distribution, and targeting to specific disease biomarkers. Non-specific tracers such as ICG are usually administered intravenously and generally remain in the tissue at concentrations in the low micromolar range. Target-specific tracers, on the other hand, are generally given time to clear normal tissue, thus will usually be present in mid-low nanomolar concentrations in tissue. This poses a challenge when devices designed for ICG imaging are used to image novel targeted probes, as the sensitivity limits are not always optimized for low concentration probes.

As the majority of the devices in the market are intended for ICG imaging, as it is the only FDA approved NIR fluorophore, we evaluated all available systems in their
ability to detect and quantify signal from 3 pM to 25 µM of IRDye 800CW (LI-COR Biosciences, Lincoln, NE) in phosphate-buffered saline. The samples were imaged individually to allow the user to modify any available gain and exposure settings, and maximize the ability to detect fluorescence emission. We grouped the systems into 8-bit imagers (Figure 3.4a) and >8-bit imagers (Figure 3.4b). Plots showing $\log_{10}(\text{Fluorophore concentration})$ versus $\log_{10}(\text{Normalized fluorescence signal})$ are shown in Figure 3.4. A handful of imagers also performed imaging in the 700 nm channel, so IRDye 680RD samples were used to evaluate them. As a reference, the performance was compared to the LI-COR Pearl Impulse preclinical imager, which provides over 20-bits of dynamic range, and performs imaging in an ambient light-free chamber. Slopes of linear fits to the log-log data (ideal slope = 1 for linearity) and the lowest detectable concentrations are shown in Figure 3.4d.

Figure 3.4 Plots of $\log_{10}(\text{Fluorophore concentration})$ versus $\log_{10}(\text{Measured signal})$ are shown for 8-bit imagers for measurements of IRDye 800CW are shown in a. b Shows
imagers with $\geq 10$-bits of camera bit-depth for IRDye 800CW measurements. Measurements from the LI-COR Pearl Impulse pre-clinical imager are shown for comparison. c Shows similar plots for all systems with far-red emission imaging capability when IRDye 680RD samples were tested. A handful of imagers also performed imaging in the 700 nm channel, so IRDye®680RD was tested on these. As a reference, performance was compared against the LI-COR Pearl Impulse preclinical imager. d Fit slopes and the lower limit of detection are shown. *Fluoptics has two distinct imagers Fluobeam700 and Fluobeam800 for imaging in the 700 nm and 800 nm emission bands respectively. ** The Li-COR Pearl imager was included as a standard of linearity and sensitivity achievable using a light-tight imager.

All imagers, under ideal conditions and dimmed lighting were able to detect down to a surface concentration of $\sim 10$ nM of both IRDye 800CW and IRDye 680RD (for those systems that have this channel). Thus, per this criterion, all systems seem suitable for imaging high concentrations of ICG, as intended. For imaging lower concentrations of fluorophores, it is observed that systems with high bit depths, variable electronic gain settings, and/or background-light correction during acquisition have the best sensitivity. The Visionsense Iridium system outperforms all other instruments in terms of sensitivity to low concentrations owing to its high camera bit-depth and gain adjustment ($1 – 200\times$) capability. The Solaris comes in as a close second in terms of sensitivity, again due to the high bit-depth and background correction functionality based on pulsing excitation light; but the lack of gain adjustment likely limits its sensitivity beyond $\sim 1$ nM. The Fluobeam800 system with the ability to manually vary exposure time, can achieve sensitivity down to $\sim 5$ nM, but this comes at the expense of long-exposure times on the order of seconds, which may not be feasible within a clinical setting. Similar sensitivity is obtained with the Novadaq SPY system in real-time video mode. The Quest Spectrum though equipped with a 14-bit camera, compresses the image data to 8-bits at the camera output resulting in reduced sensitivity and dynamic range, which severely affect the overall system performance. In terms of quantitative ability, the closer a fitted slope is to
1, the more reliable a system can be for quantitation, which we see well with the Solaris system, owing to background correction. The Quest spectrum and Visionsense Iridium devices device utilize 14-bit and 12-bit cameras respectively, but ultimately map their data to 8-bit thus resulting in non-linear compression, and a fit slope of <1. While Visionsense uses smart image processing algorithms to produce a wide dynamic range, Spectrum lacks such features, thus the range of detection suffers, and sensitivity is about \(~10\) nM. It should be noted that for data from Visionsense and Fluobeam fluorescence signal measurements were scaled by the gain settings and exposure times respectively. Curadel’s Lab-Flare R1 was excluded from Figure 3.4 as a final commercial system was unavailable for testing at the time of publishing this thesis.

3.4.4 Ability to image multiple fluorophores simultaneously

Barring excitation sources, and emission filters, a large part of the optics and instrumentation of an imager is more or less independent of the fluorophore being imaged. Some systems have been designed to house excitation sources for multiple excitation wavelength bands along with emission filter sets, to allow for multi-fluorophore imaging, either simultaneously or by switching between channels using a filter wheel. This multi-channel functionality certainly adds to the cost of the device, but such a system can be a worthy investment for a research group working with multiple imaging agents, and combinations of targeted and untargeted tracers for quantification of disease biomarkers for surgical margin assessment in cancer surgery. A total of 3 out of the 7 imagers we compared are capable of multiple fluorophore imaging; the Curadel Lab-Flare R1 and the Quest Spectrum can image in the \(~700\) nm and \(~800\) nm channels simultaneously, while the Solaris is capable of imaging \(~470, ~660, ~750, \) and \(~800\) nm channels independently (non-simultaneous). Figure 2d shows an example of
simultaneous white light, 700nm, and 800nm fluorescence imaging with the merged display available on the Flare imagers.

3.4.5 Maximized ergonomic use

As a general principle, compact, portable units are easier to deploy in a surgical suite than large roll-in systems. However, the computer, display monitors, and illumination unit contribute significantly to the size and usability of a system. Studies have shown that choice of display, their location, and setup can significantly affect surgical tasks and their outcomes. Currently fluorescence-imaging systems are either compact, handheld systems such as Visionsence Iridium, Fluobeam and PDE Neo, and or larger, overhead, wheel-based systems with significant footprints. The former do provide mounting arms and carts to users who need them while the latter can be large enough to need a 10×10 ft² room for storage. Figure 3.5 shows photographs of the various commercial systems. Though not to scale, the systems can be compared relatively for size and footprint.

Hand-held system can provide better access to complex tissue geometries, such as around the head and neck or inside limbs, and are also highly mobile, whereas the larger systems generally have a wide range of functionalities such as multiple fluorophore capability, large field-of-view (FOV) size, and large working distance. Both the Solaris and Curadel Lab-Flare system have a large range of FOV sizes over which focus errors are minimal; the Lab-Flare in particular has been optimized to maintain parfocality from 0.9×0.9 cm² to 25×25 cm² over working distances from 12” to 18”, enabling it’s usage in a wide range of surgical applications. The appropriate working distance depends highly on intended usage, but it is useful to note that the Solaris is the only system with a working fixed working distance of 75 cm, which keeps the imaging head well out of the way of a surgeons workspace, and along with the use of multiple excitation angles, this
system attempts to provide a highly ergonomic solution to imaging fluorophores during surgery. Nevertheless, as surgical applications are highly varied ranging from the inside abdominal cavity to under the armpit, there is hence no single optimal design, and selecting a system will require consideration of its intended use.

Figure 3.5 Images of the leading fluorescence guidance systems evaluated here, targeted for open surgery use. The Perkin-Elmer Solaris and the Curadel ResVet are not approved for human use, while the others are. All have capability to image ICG in surgical trials, with differing levels of sensitivity and features.

3.5 Discussion

The very limited set of approved fluorophores and approved procedures, and the lack of medical reimbursement codes in the United States for fluorescence-guided surgery procedures has limited the market for imagers in clinical settings; however, owing to the direct surgical impact of these systems on surgical workflow, their overall demand continues to grow. Due to the simplicity of design, most systems are specified for operation only in the NIR-range to capture ICG emission in vessel flow, since this use remains the only approved NIR fluorescence procedure. Table 3.2 presents all commercially available open surgery fluorescence imagers compared on the basis of the
‘key desirable features’ described in 3.4. A listing of specifications is also provided in. In reviewing the systems presented here, we found that the Solaris is the only openly marketed system with specifications well laid out, with the capacity to image a wide range of fluorescence emission channels. While other instruments predominantly target the ICG market and cost at about half of that of the Solaris, they will be important devices for imaging the other ~800-nm agents that are in various stages of preclinical and investigational new drug (IND) development. Meanwhile, off-label use of approved fluorophores has become increasingly common in sentinel lymph node mapping142, tumor resection surgeries143, and perfusion assessment, identification of disease in situ demands the use of target specific fluorophores128. This is especially true in oncological applications such as margin assessment and metastasis detection.

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<th>Curadel-LAB Flare</th>
<th>Quest-Spectrum</th>
<th>Novadaq-SPY Elite</th>
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<td>Compact, good sensitivity</td>
<td>Excellent optics, large range of FOV</td>
<td>Similar FOV, multi-channel fluorescence overlay</td>
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<td>Non-linear, low sensitivity</td>
<td>No white light overlay, large scale</td>
<td>No white light imaging, non-linear</td>
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Table 3.2 Comparison of the various commercial fluorescence imagers for open surgical applications based on the key operational factors. *Vision sense Iridium has an FDA-approved unit with 805 nm excitation laser. For the sensitivity test a 785 nm laser was used, which Vision sense sells as a research-only option. **Quest offers a research-only
custom-designed option on the Spectrum system that has 12-bit raw data export capability, such a system designed to our specifications was unavailable at the time of writing this article, and all tests were performed using the FDA-approved version of the system.

The development of new agents is driving technological advances by increasing the demand for new systems with expanded capabilities. The adoption of fluorescence-guidance for surgery within research settings is well underway, and several imaging instrument companies have identified them as their initial target customers. This has led to emergence of several systems such as Curadel ResVet Lab-Flare, and Perkin Elmer Solaris that are not immediately seeking FDA approvals, as the imaging agents being used need approvals as well, but present feature-rich systems with significant benefits for imaging. As such, there is a small commercial market developing in some areas for these research units, which will pave the way for future indications and potential FDA clinical approvals. There are also a few companies such as Quest medical imaging and Surgvision that are designing high-end systems customizable to the specific requirements of their users (specific research labs) through industry-academic partnerships. The number of non-approved systems will likely grow as the field of fluorescence guided surgical research develops.

In the near term, it should be expected that several new systems will be launched in the upcoming months, including the SurgVision imager\(^9^3\), which is similar in design to the LAB-Flare instrument but uses a single EMCCD-based fluorescence camera and customizable emission filters. Commercial fluorescence imagers are still in a developmental phase and the right developmental trajectory for these systems is not yet clear. This new trend in development of customizable systems built to user specifications with flexibility in choice of excitation and emission wavelengths will likely have its own
trajectory, with leading research users seeking local institutional review board (IRB) approvals for research use, allowing the use of more fully featured systems in a trial, rather than waiting for an FDA-cleared versions with fixed specifications. This will certainly impact the drug discovery and development processes. In the long term, it should be expected that a fair amount of reorganization and consolidation could occur, as the industry converges on what the eventual demands will be from a clinical point of view, and what is needed for further research and development. To date, with the exception of Perkin Elmer, the larger imaging companies have remained on the sideline in the open surgery area. Notable exceptions are the advances of Olympus, Leica and Zeiss in niche surgical areas such as endoscopy and neurosurgery. However, as more and more clinical indications in open surgery are approved and the issue of reimbursement is sorted out, we can expect larger amounts of research capital to flow into the industry, ensuring a continued positive feedback loop on the market.

3.6 Conclusions

In summary, a proposed a set of ‘desirable features’ has been described, in descending order of importance; these are suggested to be the right judgement criteria for evaluating a fluorescence-imaging device for open-field use. These criteria and the results of the analysis are based on extensive testing and evaluation of each FDA-approved and pre-clinical imager presented here. *Real-time fluorescence overlay on RGB white-light images* and *fluorescence-mode operation under ambient room lighting* are proposed as the most important requirements because these aspects limit the utility of a system if not present, irrespective of its sensitivity. *Sensitivity to low fluorophore concentrations* and the *ability to linearly quantify* relative fluorophore concentrations are next most important in rank, as these will
ultimately determine the clinical impact of the imaging device. Furthermore, as the adoption of fluorescence imaging, to guide surgery and for lymphatic imaging, continues to grow, the quantitative ability will play an important role in comparing data in multicenter-trials, in and comparing results spatially and longitudinally both within and among patients. Next, *simultaneous multi-fluorophore imaging capability* is an “extra” feature to most users, but can be critical for research labs developing next generation imaging agents, and novel methods to improve cancer extent and margin assessment using combinations of imaging agents. Lastly, we discussed *ergonomics* as the final important criterion for selecting a system, as this is again greatly tied to system utility during surgery. While we have proposed this set of desirable features, each system does comes with its own set of positive and negative aspects and there is no single “best” system in the market, and we hope that this article can simplify the task of selecting the right system to invest in for both clinicians and researchers.
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<th>Perkin Elmer</th>
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Table 3.3: Technical Specifications
CHAPTER 4  Fluorescence Imaging of Lymphatics and Lymph Nodes in Small Animals using clinically relevant Blue Dyes

This chapter has been adapted from the submitted paper entitled “Nodal lymph flow quantified with afferent vessel input function allows differentiation between normal and cancer-bearing nodes” authored by Alisha V. DSouza, Jonathan T. Elliott, Jason R. Gunn, Kimberley S. Samkoe, Kenneth M. Tichauer, and Brian W. Pogue published in Biomedical Optics Express in November 2015.

4.1 Introduction & Background

Studies using fluorescence mapping of lymph nodes have focused more on localization of nodes, than estimation of tumor-burden on identified nodes. The approach of using fluorescence rather than absorption or reflectance allows use of trace amounts of contrast agent (fluorophore) with real-time capability for imaging. Tichauer et al. showed that number of tumor cells in lymph nodes correlated with epidermal growth factor receptor concentration imaged using a dual-tracer combination of a targeted and an untargeted fluorescent tracers; but that a single targeted tracer imaging would not be a reliable metric of nodal involvement. While this method is attractive, it does require production of two dyes for eventual clinical use, so will have limited immediate impact.

In this study, low dose methylene blue (MB), a dye clinically approved for use in sentinel lymph node procedures, was used to locate tumor-draining nodes with
fluorescence imaging\textsuperscript{145}, to quantify lymph flow through the nodes, and test the hypothesis that kinetic parameters could be used to discriminate normal from cancer-bearing nodes. MB has been shown to have a reasonable near-infrared (NIR) fluorescence quantum yield of \(~4.4\%\) with peak absorbance at 668 nm, extinction coefficient of 69,100 mol L\textsuperscript{-1} cm\textsuperscript{-1} and peak emission at 688 nm\textsuperscript{131, 146} at low concentrations. This provides the opportunity to image methylene blue at very low doses, much lower than those used in the clinic, thus avoiding problems of dye toxicity and skin staining.

![Figure 4.1](image1.png)

Figure 4.1 Quenching (concentration versus fluorescence) curve for MB in PBS at pH 7.4 (solid circles) or swine urine (open circles). Reproduced from Matsui et al.\textsuperscript{131}, copyright 2010, with permission from Elsevier.

![Figure 4.2](image2.png)

Figure 4.2 Chemical structures and optical properties of MB. Reproduced from Matsui et al.\textsuperscript{131}, copyright 2010, with permission from Elsevier.
It has previously been shown that lymphatic vessels that supply the axillary and brachial lymph nodes drain the cutaneous regions of the forepaw\textsuperscript{147} thus providing a simple, consistent route for fluorophore administration (see Figure 4.3). The heterogeneity known to be present in fluorophore delivery to draining lymph nodes was studied together with a model of lymphatic flow, analogous to well-studied methods of deconvolution-based cerebral blood flow in dynamic contrast-enhanced computed tomography\textsuperscript{148, 149}. While there is evidence that supports lymphatic remodeling and lymphangiogenesis associated with metastasis\textsuperscript{6}, we do not attempt to study the changes in uptake patterns with progression in metastasis, but instead offer a method to identify cancer-bearing from normal lymph nodes that can be used as a preclinical test model and translated to the clinic with relative ease. To the best of our knowledge, this is the first paper to attempt to quantitatively correct non-cancer specific lymphatic uptake differences in lymph nodes using fluorescence imaging of afferent lymph vessels.

Figure 4.3 Cutaneous lymphatic drainage of the nodes in the axilla. Adapted from Tilney \textit{et al.}\textsuperscript{147}.

4.2 Preliminary Tests on Methylene Blue tests using the Pearl Impulse Fluorescence Imager

In order to establish sensitivity and quenching limits on methylene blue imaged using the LI-COR Pearl® Imager, we performed the following set of experiments. First, serial dilutions of Methylene Blue in PBS and 1% Bovine Serum Albumin (BSA) were prepared and filled into a 96-well plate (400 μL per well), see Figure 4.4. Concentrations
used ranged from 0.1 mM to 10 nM MB. Results shown in Figure 4.5 show signal saturation at ~10 µM (likely dye self-absorption) and sensitivity down to 0.1 µM for MB in Albumin. Methylene blue spontaneously\textsuperscript{150} yet weakly binds (Association constant, $K_a = 4.012 \times 10^4$ M$^{-1}$ at 298K\textsuperscript{151}) with albumin through electrostatic interaction. It should be noted that although MB was mixed into 1% BSA, this corresponds to a molar ratio of MB:BSA much less than 1, and it is highly likely that most MB exists as free unassociated dye. On the other hand several studies report that MB has no affinity or much lower affinity for albumin than other dyes such as Evans blue and Patent Blue owing to the absence of sulphonic acid groups in MB\textsuperscript{152,153}.

Figure 4.4 700nm channel image of 96-well plate containing serial dilutions of MB. Top row shows decreasing concentration of MB from left to right with 1% BSA. Bottom row contained MB diluted in PBS only.

Figure 4.5 Fluorescence intensity versus concentration of MB diluted in only PBS (red) and MB in 1% BSA (blue). A power fit was performed on the log-log plot and a slope of
~1 was identified in the MB in BSA fluorescence emission data to identify a linear range from 0.1 µM to 10 µM.

Next, various MB concentrations along with fixed IRDye 800CW concentration were injected subcutaneously into mouse skin to form fluorescent inclusions. This is shown in Figure 4.6. The ratios of fluorescence intensities versus methylene blue concentrations are plotted in Figure 4.6, 0.05 µM IRDye 800CW was used in all inclusions, and MB concentration was varied from 200 µM, serially diluted down by factor of 2 to 0.39 µM. This ratio-metric approach eliminated effects of dilution, inclusion and skin thickness etcetera. By fitting a power function to the log-log data and identifying regions with slope of fit ~1, we confirmed that concentrations of Methylene blue from ~0.8 µM to 6.25 µM in a lymph node of interest would provide a reasonable linear region for contrast.

Figure 4.6 Mouse with subcutaneous dye injections shown on the left. Plot on the right shows ratio of MB Fluorescence intensity : IRDye 800CW fluorescence versus methylene blue concentration, when IRDye 800CW concentration was constant. Power Fit slope of ~1 on the log-log plot indicates that a region of linearity exists from ~0.8 µM to 6 µM of Methylene blue.
The overall plan of this study was that an intra-nodal cancer-cell injection model was used to implant lymph nodes of rats with cancer. Then, low concentration of methylene blue was injected into the lymphatic system via footpad tissue, and imaged *in vivo* on a planar fluorescence scanner. Its uptake through lymph vessels and lymph nodes was measured and analyzed to compare cancer-bearing, healthy and control lymph nodes.

### 4.2.1 Cancer Cell Model and Methylene Blue Preparation

Bioluminescent human breast cancer cells from MDA-MB-231-luc-D3H2LN (PerkinElmer, Waltham, MA) were cultured at 37°C in high-glucose Dulbecco’s Modified Eagle Medium (HyClone® SH30243.01, Fisher Scientific, Pittsburg, PA) supplemented with 10% fetal bovine serum (HyClone® SH30910.03, Fisher Scientific), and penicillin-streptomycin (#30-002-CI, Cellgro, Mediatech Inc., Manassas, VA). MB was mixed into 5 mM bovine serum albumin (BSA) before use for injection.

### 4.2.2 Animal Lymph Node Implantation Procedures

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at Dartmouth College under approved protocols. A total of 16, 8 – 10 week old athymic nude female rats (Charles River, Wilmington, MA) were used in this study. Animals were grouped into 3 cohorts – normal (6), control (4) and cancer-bearing (6). Rats in the normal group did not undergo any surgery prior to imaging; animals in the cancer-bearing group received cancer cell injections by the procedure described below; and animals in the control group received sham phosphate-buffered saline (PBS) injections in place of cancer-cell injections.

All animals were maintained on a fluorescence-free diet for a week prior to imaging. Animals were prepped by shaving fur, and by application of Nair hair removal cream.
over shoulders, forelimbs, and armpits one day prior to imaging; this was done to minimize autofluorescence from fur. In order to deliver cancer-cells to the lymph node that could be visualized by fluorescence imaging in the desired imaging orientation, the animals were imaged in a preparatory procedure (as described below) using fluorescence visible in an axillary (or brachial) lymph node to guide implantation.\textsuperscript{147} This fluorescent node was exposed surgically while causing minimal damage to the surrounding tissue. 1 Million cancer cells in a phenol red-free and growth factor-reduced matrigel medium ($\#356230$, Corning) in a total volume of 20 $\mu l$ were injected into the node using a 27-gauge syringe needle. The area was then sutured and the surgical site was allowed to heal for 5 days, after which fluorescence imaging was carried out to study dye uptake (as described in the next section). Control animals received 20 $\mu L$ of PBS instead of cancer-cells.

Figure 4.7 1 (a-b & d-e) Images of a single rat post MB administration are shown. White light images are overlaid with pseudo-colored red fluorescence images. Yellow arrows point to lymph nodes. Top row shows animal during implantation, bottom row shows animal 5 days after cancer-cell implantation. The middle column shows exposed nodes. Scale bar for (a, b, d, e) is shown on (a). (c) Photograph of node implantation. (f) In vivo bioluminescence imaging; (g) Ex vivo bioluminescence imaging of excised axillary and brachial lymph nodes. Color overlay shows bioluminescence from cancer-bearing nodes.
The cancer bioluminescence was imaged to confirm cancer presence in nodes, using Luciferin (#88294 Thermo Scientific) administration, prior to fluorescence imaging using the Xenogen VivoVision IVIS bioluminescence system (Perkin Elmer, Waltham, MA). A total of 6 animals were found to exhibit tumor presence in at least one lymph node at 5 days post tumor injection. Cancer cells remained confined to the inoculated nodes and no spreading to nearby nodes was seen.

### 4.2.3 In vivo fluorescence lymphatic imaging

Prior to imaging, each animal was anaesthetized using 1.5 - 3% isofluorane (Piramal, Bethlehem, PA) in 1.5 L/min oxygen. It was then placed on its side with the forelimb on one side of the body stretched out so as to expose lymph nodes of the axilla; shown in Figure 4.7. Next, 25 nmoles of Methylene Blue (Sigma-Aldrich, St. Louis, MO) in 5 mM bovine serum albumin (Sigma-Aldrich, St.Louis, MO) in a 20 µl volume of PBS, were injected intra-dermally using a 27-gauge syringe-needle into the base of a forepaw of each animal. Care had to be taken to ensure the injection was not deeper than ~0.5 mm; deep injections lead to delayed uptake of dye into the lymph system, as it is the dermis that is richly supplied with lymph capillaries. 3 out of 16 animals required their paws to be massaged for 30 seconds, to ensure fluorophore uptake by lymph capillaries. Some of the animals required their paw to be massaged for a few seconds, to improve fluorophore uptake by lymph capillaries. It has previously been shown that lymphatic vessels supplying the axillary and brachial lymph nodes drain the cutaneous regions of the forepaw.\(^{147}\) Additionally, the large injection volume produced pressure that caused the lymphatic capillaries to take up dye.

All fluorescence imaging was performed using a Pearl® Impulse system (LI-COR Biosciences, Lincoln, NE) that provides planar surface images in the near infrared
band\textsuperscript{53}. The system was set up to acquire grayscale white light and 700 nm channel images at 2 frames per minute each for 40 – 60 minutes. The 700 nm channel setting uses a 685 nm laser excitation and a 720 ± 20 nm bandpass emission filter. The timing allowed capture of the dynamics of lymph flow through paws, lymph vessels, and lymph nodes.

4.2.4 Modeling based Estimation of Nodal Lymph Flow

Interpretation of the flow dynamics of lymph nodes is complicated by the large heterogeneity seen in the supplying afferent lymph delivery, and the known complexity of pseudo-compartments present in the nodes, which are incompletely separated by thin trabeculae. Compartment kinetic modeling of the flow requires identification of the minimum number of pertinent regions affecting the observed flow, which can be difficult.

In this study, a nonparametric deconvolution method was developed to mathematically estimate the lymph flow across the node, based on dynamic MB fluorescence data – an approach analogous to methods used in cerebral blood flow characterization in CT perfusion\textsuperscript{148,149} and near-infrared spectroscopy\textsuperscript{154,155}. To described this model, we assume $L(t)$ is the uptake (or concentration) of dye into lymph node represented by the fluorescence signal from the node, and $C_a(t)$ is the delivery by the afferent lymphatic vessel represented by its fluorescence. $R(t)$ is the impulse residue function of the lymph node in question, and by definition, is the fraction of dye remaining in the lymph node after an idealized bolus injection (Dirac-delta or “impulse” function) into the afferent vessel. $F_{LN}$ is the nodal lymph flow, defined as a constant rate of flow of dye molecules through the node and had conventional units of flow, such as
ml/g/min. The following equation describes the convolution governing the tissue kinetics.

\[ L(t) = F_{LN} \cdot R(t) \ast C_a(t) \]  

Equation 4-1

Here, “\( \ast \)” represents the convolution operator. \( L(t) \) and \( C_a(t) \) are measurable quantities and deconvolution of \( C_a(t) \) from \( L(t) \) produces a scaled version of \( R(t) \) whose peak can be used to estimate nodal lymph flow (\( F_{LN} \)). \( L(t) \) and \( C_a(t) \) were measured over the duration of imaging by manually selecting regions-of-interest over the lymph node and afferent lymph vessel respectively, followed by data smoothing which preserved peak intensities and peak positions. Equation 4-1 was solved in the least squared sense to recover \( F_{LN} \cdot R(t) \), using a matrix form:

\[ L = F A R \]  

Equation 4-2

and minimizing the objective,

\[ \arg \min_{F_{LN}R} \| C_A \cdot FR - L \|^2 \]  

Equation 4-3

To stabilize this inversion, physiological constraints were applied, which include, (i) tracer inflow via lymph vessel require a minimum amount of time, \( \ell \) to enter the node, (ii) \( R(t) \) represents the residue function of a Dirac delta function, (iii) the first dye molecule spends a finite amount of time, \( M \), in the node before exit, and (iv) that no retrograde flow occurs, and lymph flow is unidirectional. Both \( M \) and \( \ell \) are on the order of minutes. These provide the following constraints for \( R(t) \).

\[ R(t) = \begin{cases} 0 & 0 \leq t < \ell \\ 1 & \ell \leq t \leq M \end{cases} \]  

Equation 4-4

\[ \frac{dR(t)}{dt} \leq 0, \quad t \geq \ell + M \]  

Equation 4-5
The use of this physiologically constrained deconvolution-based approach was performed in collaboration with Dr. Jonathan Elliott, and has been described in complete detail elsewhere\textsuperscript{156-158}. The above enables the recovery of $F_{LN}$, since it is equal to the maximum of $F_{LN} \cdot R(t)$, which is the parameter recovered by inversion of Equation 4-1. Additionally, nodal lymph volume $V_{LN}$ was calculated by integrating $F_{LN} \cdot R(t)$ over time and mean transit time, $\bar{t}_{LN}$ of lymph through the node was calculated as $\frac{V_{LN}}{F_{LN}}$, according to the central volume principle. It should be further noted that $L(t)$ and $C_{a}(t)$ were in units of fluorescence, but were measured on the same series of images; therefore, they are both linearly related to concentration of methylene blue by approximately the same factors (for example, quantum efficiency, absorption, scatter, and detector efficiency) as long as the tissue optical properties at both locations are not significantly different. For this reason we present the values in the conventional units of blood flow: millilitre of lymph fluid, per gram of tissue, per minute (ml/g/min).

4.2.5 Image data Analysis

The 22-bit images from the Pearl imager were read using Bio-Formats plug-in (LOCI, University of Wisconsin-Madison) and analyzed using a combination of ImageJ and Matlab (Mathworks, Natick, MA) software. Fluorescence measurements were made on temporal 700 nm channel images by calculating the mean signal intensity from regions-of-interest over lymph nodes, lymphatic vessels, and skin free from fur and melanin. Pre-injection measurements were subtracted from all post-injection measurements to eliminate autofluorescence. Additionally, build-up of fluorescence in the skin over the duration of imaging necessitated background subtraction, for which mean signal from the selected skin region was used. Choice of ROI size for lymph
vessels was based on lymphangion length measured from one animal whose exposed lymph vessels clearly showed segmentation on a lymph vessel indicating lengths separated by valves. This is shown in Figure 4.8h. Measurements on normal, sham, and cancer-bearing nodes were tested for statistically significant differences using two-tailed Welch’s t-tests, using Microsoft Excel.

Figure 4.8 Early time-point frames (white light image overlaid with red pseudo-colored fluorescence image) for various normal, sham control and cancer-bearing rats are shown. Yellow arrows point to nodes and green arrows point to afferent lymph vessels. (a-d) Normal rats imaged, images are tagged with “N” to indicate cohort. e-g) Rats from sham control cohort, indicated by letter “S”. (i-l) Rats from the cancer-bearing nodes cohort, images are tagged with letter “C”, also asterisks indicate cancer-bearing nodes. Fluorescence from injection site was blocked off manually in the overlay. (h) shows fluorescence from an exposed lymph vessel on which segments separated by valves within the vessel can be clearly demarcated. This is the only image in this panel showing exposed lymphatics, lymph node is not visible here. Lymphangions are visible and their lengths were used to inform ROI selection in all animals.
4.3 Results

Figure 4.7 shows an example of fluorescence imaging of a lymph node in a rat during and post implantation. Corresponding *in vivo* and *ex vivo* bioluminescence images are shown; these confirmed cancer presence in the node(s) imaged post implantation. A comparison of Figure 4.7 a-b and Figure 4.7 d-e qualitatively confirms that the direct-injection tumor model successfully produces a tumor-bearing lymph node in a very short time frame of 5 days with minimal disruption of lymphatics while ensuring that this node drained the paw and could be imaged using methylene blue. Animals that went through surgeries had porphyrin-rich surgical scars near the axilla, which had to be covered with shrouds to minimize autofluorescence from them.

Figure 4.8 shows single frames at early time-points post methylene blue injection for various animals. Dye delivered to the paws was successfully taken up by axillary lymph nodes. However, lymphatic routes taken by the dye varied across animals. Among the normal and sham control animals, a few rats showed uptake into two lymph nodes (see Figure 4.8 e, and i), while all others showed uptake into one node each. Among the cancer group, at least one rat showed dye uptake into two nodes (see Figure 4.8l): both these nodes were visible at the time of cancer-cell implantation and both were accordingly inoculated with cancer cells. Figure 4.8h demonstrates the ability to visualize segments (lymphangions) along a lymph vessel when the vessel is exposed. Fluorescence from lymph vessels appeared to fluctuate in intensity spatially and temporally along the vessel length. Temporal variation was attributable to the pulse-like flow of lymph fluid through the lymph system, while spatial variation was attributable to differences in lymph vessel depth at various locations. Furthermore, in several animals, dye took routes through ventrally located vessels, as opposed to the dorsal lymphatic routes seen
in the majority of animals. All this indicates significant delivery differences, leading to differences in uptake of dye into the corresponding node(s).

Figure 4.9 Lymph node fluorescence, afferent vessel fluorescence and impulse residue functions are shown for all animals. Animals are color and marker-coded across columns. Each curve represents an animal, and these are color-coded across each row. Solid lines are used to plot lymph node signal, and dotted lines represent afferent vessel signal.

Figure 4.9 (a,b,d,e) demonstrates a temporal plot of fluorescence intensity in nodes and afferent lymph vessels; fluorophore injection was performed at $t = 0$ minutes. Various measurements were used to study variation within each cohort, and compare normal, control and cancer-bearing nodes. Average node and vessel fluorescence was calculated on measured fluorescence data. 74% (standard deviation from mean) variability in average fluorescence signal was seen among normal healthy nodes, and a
variability of 59% was seen in the average fluorescence signal from their feeder vessels. Control nodes showed 60% and 118% variability in average fluorescence in nodes and afferent vessels respectively. Cancer-bearing nodes (indicated on Figure 4.8 with asterisks) exhibited 105% and 61% variability in average fluorescence from nodes and lymph vessels, respectively. Peak fluorescence from nodes showed slightly lower variation – of 58%, 51% and 102% in normal, control, and cancer-bearing nodes, respectively – than average node fluorescence. This likely arose from large differences in retention at late time points among all nodes (88%, 77%, and 152% respectively for normal, control, and cancer nodes), contributing to the increased variability in average fluorescence.

Figure 4.10 Boxplots comparing parameters measured from normal (A), control (B), and cancer-bearing animals (C) as shown. *Indicates significant differences ($p < 0.05$) between groups B & C; †Indicates significant differences ($p < 0.01$) between groups A & C; ††Indicates significant differences ($p < 0.005$) between groups A∪B & C.

From Figure 4.9, one can also observe that the time at which peak-MB-fluorescence appeared varied among nodes. This arrival time had 62%, 63% and 78% variation in
cohorts respectively, with no significant differences between the groups. We looked at time-of-arrival of peak-fluorescence intensity, relative to injection time, and peak fluorescence signal from lymphatic vessel. In an attempt to eliminate the subjectivity of these measurements to injection administration and time delay between injection and image acquisition, time delay between peak fluorescence in nodes and vessels was calculated as the difference in their peak arrival times respectively. However, owing to the large heterogeneity in flow through lymph vessels, this quantity showed large variability (>100%) in all groups.

Figure 4.11 Boxplots comparing modeled parameters measured from normal (A), control (B), and cancer-bearing animals (C) as shown. *Indicates significant differences (p < 0.05) between groups B & C; ‡Indicates significant differences (p < 0.01) between groups A & C; †Indicates significant differences (p < 0.005) between groups AUB & C.

The peak fluorescence intensities from afferent lymph vessels ($P_a$) were used to normalize the fluorescence signal in the lymph nodes downstream from them. This normalization process reduced variations in average node fluorescence to 75%, 37% and 65% in each cohort respectively, which appeared to be an improvement over the raw intensity analysis. Of all the quantities tested, the measurement with lowest variability (39% in normal, 23% in control, and 44% in cancer-cell injected nodes) was the peak-normalized node fluorescence (can also be called ratio of peak node fluorescence to peak vessel fluorescence, $\frac{P_N}{P_A}$). Moreover, both normalized node fluorescence measurements
showed statistically significant differences ($p < 0.01$) between the cancer-bearing and cancer-free cohorts.

Table 4.1 Various parameters used to compare normal (A), control (B), and cancer-injected (C) nodes. *Indicates strongly significant differences ($p < 0.01$); † indicates significant differences ($p < 0.05$).

Table 4.1 reports mean ± standard deviation and intra-group variation for the various measurements, and p-values from Welch’s two-tailed t-tests between cancer-free and cancer-bearing cohorts. Rows 1 through 11 show parameters measured directly from

<table>
<thead>
<tr>
<th>#</th>
<th>Parameter</th>
<th>Normal Animal Cohort (A)</th>
<th>Sham Surgery Cohort (B)</th>
<th>Cancer-bearing Cohort (C)</th>
<th>p-value from Welch’s T-test between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Average Node Fluorescence ($A_N$)</td>
<td>0.04 ± 0.03 (74%)</td>
<td>0.02 ± 0.01 (60%)</td>
<td>0.03 ± 0.03 (118%)</td>
<td>0.27, 0.63, 0.70, 0.94</td>
</tr>
<tr>
<td>2</td>
<td>Average Afferent Vessel Fluorescence ($A_A$)</td>
<td>0.005 ± 0.003 (59%)</td>
<td>0.0025 ± 0.003 (118%)</td>
<td>0.009 ± 0.007 (77%)</td>
<td>0.30, 0.08, 0.18, 0.12</td>
</tr>
<tr>
<td>3</td>
<td>Peak Node Fluorescence ($P_N$)</td>
<td>0.10 ± 0.06 (58%)</td>
<td>0.05 ± 0.03 (51%)</td>
<td>0.09 ± 0.09 (102%)</td>
<td>0.11, 0.35, 0.86, 0.79</td>
</tr>
<tr>
<td>4</td>
<td>Peak Vessel Fluorescence ($P_A$)</td>
<td>0.04 ± 0.02 (54%)</td>
<td>0.02 ± 0.013 (65%)</td>
<td>0.07 ± 0.05 (67%)</td>
<td>0.13, 0.05, 0.17, 0.10</td>
</tr>
<tr>
<td>5</td>
<td>Retained Node Fluorescence (at 40 minutes post injection)</td>
<td>0.03 ± 0.02 (88%)</td>
<td>0.013 ± 0.01 (77%)</td>
<td>0.023 ± 0.0235 (152%)</td>
<td>0.25, 0.54, 0.86, 0.90</td>
</tr>
<tr>
<td>6</td>
<td>Time of Peak Node Fluorescence (mins)</td>
<td>2.7 ± 1.7 (62%)</td>
<td>2.9 ± 1.8 (63%)</td>
<td>3.4 ± 2.7 (78%)</td>
<td>0.90, 0.73, 0.62, 0.63</td>
</tr>
<tr>
<td>7</td>
<td>Time delay from peak fluorescence in vessel to node (minutes)</td>
<td>1.0 ± 1.1 (107%)</td>
<td>0 ± 3.6 (NA)</td>
<td>1.3 ± 2.4 (193%)</td>
<td>0.60, 0.54, 0.80, 0.57</td>
</tr>
<tr>
<td>8</td>
<td>Average Normalized Node Fluorescence ($A_N/P_A$)</td>
<td>1.0 ± 0.7 (75%)</td>
<td>1.1 ± 0.4 (37%)</td>
<td>0.3 ± 0.2 (65%)</td>
<td>0.67, 0.02†, 0.08, 0.004*</td>
</tr>
<tr>
<td>9</td>
<td>Average Normalized Afferent Vessel Fluorescence ($A_A/P_A$)</td>
<td>0.13 ± 0.08 (62%)</td>
<td>0.10 ± 0.07 (70%)</td>
<td>0.13 ± 0.05 (38%)</td>
<td>0.48, 0.49, 0.88, 0.77</td>
</tr>
<tr>
<td>10</td>
<td>Peak Normalized Node Fluorescence ($P_N/P_A$)</td>
<td>2.7 ± 1.1 (39%)</td>
<td>2.7 ± 0.6 (23%)</td>
<td>1.0 ± 0.5 (44%)</td>
<td>0.97, 0.006*, 0.01*, 0.0002*</td>
</tr>
<tr>
<td>11</td>
<td>Normalized Retained Node Fluorescence</td>
<td>0.7 ± 0.7 (100%)</td>
<td>0.6 ± 0.4 (62%)</td>
<td>0.2 ± 0.2 (114%)</td>
<td>0.85, 0.12, 0.15, 0.04</td>
</tr>
<tr>
<td>12</td>
<td>Nodal Lymph Flow ($F_{LN}$)</td>
<td>1.5 ± 0.6 (43%)</td>
<td>1.5 ± 0.5 (29%)</td>
<td>0.5 ± 0.3 (48%)</td>
<td>0.90, 0.01*, 0.01*, 0.0002*</td>
</tr>
<tr>
<td>13</td>
<td>Nodal Lymph Volume ($V_{LN}$)</td>
<td>6.2 ± 4.3 (69%)</td>
<td>19.4 ± 15.5 (80%)</td>
<td>2.2 ± 1.1 (48%)</td>
<td>0.19, 0.11, 0.07, 0.03†</td>
</tr>
<tr>
<td>14</td>
<td>Mean Transit Time ($t_{LN}$) [mins]</td>
<td>4.2 ± 2.0 (49%)</td>
<td>11.4 ± 6.6 (58%)</td>
<td>4.6 ± 1.2 (27%)</td>
<td>0.11, 0.13, 0.66, 0.21</td>
</tr>
</tbody>
</table>
measured fluorescence signals. Box plots comparing some of the important measured parameters from all cohorts are shown in Figure 4.10. The large amount of inherent heterogeneity present in fluorophore uptake by healthy nodes was hypothesized to be attributable to heterogeneity in delivery. As seen above, our imaging method provided the opportunity to make measurements on lymphatic vessels thus facilitating their use in accounting for such delivery differences. In an attempt to develop a model that would combine correction of heterogeneity in lymphatic delivery with a model that could provide estimates of a physiologically relevant quantity, as described in the methods section, an afferent vessel input model using nonparametric deconvolution with physiological constraints was used to characterize the relationship between lymph vessel fluorescence signal, \( C_a(t) \), and nodal fluorescence signal, \( L(t) \). Deconvolution of \( L(t) \) and \( C_a(t) \) resulted in the recovery of \( F_{LN} \cdot R(t) \), the impulse residue function, which contains information about lymph flow through node \( F_{LN} \), lymph volume in the node \( V_{LN} \), and mean transit time (\( \bar{t}_{LN} \)) – the mean time between when the lymph enters from the afferent vessel and exits by the efferent vessel. Node fluorescence \( L(t) \), vessel fluorescence curves \( C_a(t) \), and the corresponding \( F_{LN} \cdot R(t) \) functions are shown in Figure 4.9 for all cohorts. These curves were then used to estimate \( F_{LN} \) as described earlier. Figure 4.11 shows \( F_{LN}, V_{LN} \), and \( \bar{t}_{LN} \) plotted for all animals from normal, control and cancer-bearing cohorts. Two-tailed Welch’s t-tests revealed significant differences between \( F_{LN} \) of normal and cancer-bearing groups (\( p = 0.01 \)) and control and cancer-bearing group (\( p = 0.01 \)). Furthermore, while normal and sham control nodes show differences in fluorophore uptake in Rows 1-7 of Table 4.1, due to inherent heterogeneity and possible also due to inflammation from surgery, measurements based
on afferent lymph vessel normalization such as in rows 8, 10, and 12 of Table 4.1, reveal high degree of similarity between these groups. For example, t-test between $F_{LN}$ of normal and control groups revealed a large p-value ($p = 0.9$) indicating no significant differences between these groups, thus allowing us to combine both cancer-free groups. A t-test on this quantity between all cancer-free and cancer-bearing animals showed a p-value of 0.0002, which indicated highly significant differences. $F_{LN}$ is closely related to the previously reported ratio of peak fluorescence in node to vessel $\frac{p_N}{p_A}$; they had a Pearson correlation coefficient of 0.7. $F_{LN}$ was calculated to be $1.5 \pm 0.6$ ml/g/min in normal nodes, $1.5 \pm 0.5$ ml/g/min in sham control nodes, and $0.5 \pm 0.24$ ml/g/min cancer-cell injected nodes. Additionally, $V_{LN}$ was $6.2 \pm 4.3$ ml/g, $19.4 \pm 15.5$ ml/g, and $2.2 \pm 1.1$ ml/g in normal, control, and cancerous nodes, respectively; and $t_{LN}$ for the normal and cancerous nodes was $5.7 \pm 1.7$ min and $5.2 \pm 0.8$ min, respectively. Neither parameter showed significant differences between cancer-free and control nodes ($p = 0.11$ and $p = 0.13$ respectively). Rows 12–14 of Table 2.1 summarize these measurements along with p-values from Welch’s t-tests between various cohorts, and Figure 4.11 shows boxplots compare these modeled parameters for various cohorts. ROC analysis of a binary classification of nodal lymph flow ($F_{LN}$) in cancer-free and cancer-bearing nodes is represented in Figure 4.12a. Area under the curve is reported to be 0.9833. Power of the t-test between $F_{LN}$ of cancer-free and cancer-bearing groups was 0.82, when significance level $\alpha$ was 0.05. Figure 4.12b shows a power curve for various sample sizes, indicating that a much more powerful test can be attained with the addition of data from 4 more cancer-bearing animals.
Figure 4.12 a) ROC Analysis of binary classification of cancer-free and cancer-bearing nodes based on $F_{LN}$. b) Power curve for $F_{LN}$.

4.4 Discussion

This work presents experimental evidence on the ability to use fluorescence measurements of methylene blue (MB) to identify, with statistically significant difference, cancer-bearing from cancer-free lymph nodes in small animals, by using afferent vessel input functions. This is important because MB is commonly used in surgical oncology applications including sentinel lymph node procedures, and so it is possible that its kinetics could be better exploited for further information. The small size of MB (~300 Da), combined with its low affinity for albumin in lymph makes its movement through the lymphatic system very swift. Although we premixed MB in BSA prior to injection, MB can likely dissociate from BSA once it is in the interstitium and then rapidly flow through the lymphatic vessel. We do not see a second methylene blue:Albumin uptake curve in any of our imaging likely due to the fact that the any remaining MB:Albumin would be taken up slowly at the lymph capillaries and maintain a lower concentration than detectable by fluorescence in the lymph. These features allow immediate imaging with little or no wait time post fluorophore administration. Additionally, short imaging-durations of 10 – 20 minutes could produce results similar to those presented (~40
minutes of imaging), as most of the information is contained in the peak of fluorescence signal. While this study focused on using fluorescence imaging, the presented methods have the potential to be extended to dynamic absorption or molecular imaging as well.

Implantation of bioluminescent breast cancer cells directly into the nodes worked well to directly confirm cancer-cell presence in nodes. Animals imaged for MB fluorescence prior to tumor implantation, showed that lymph nodes of the axilla drain the cutaneous and sub-cutaneous regions of the forepaw on their side of the body. A large amount of variability was observed in the uptake of fluorophore into a draining lymph node (~75%), this variability was attributable in a large part to inconsistency in administration, inherent differences in lymphatic vessels, differences in pressure within the lymphatic system, et cetera. Figure 4.8 shows uptake pathways of methylene blue in various healthy and cancer-bearing animals. Within the group of healthy animals that had never been through surgery, significant differences were seen in the lymphatic trafficking routes of the dye. In most animals from our study, lymph was supplied from paw to node via a dorsal lymph vessel. In some cases, animals showed ventral or highly branched routes. On the other hand, a few animals showed multiple routes within the same animal, and uptake by multiple nodes as well. Furthermore, images in Figure 4.9 show high variation in intensities and shapes of uptake curves in both vessels and nodes; a few vessel inputs show double peaks, which could be attributed to lymph propulsion by massaging of the animal paw. It must be noted that in our imaging set up, only those vessels that supplied the bulk of the dye to the draining node and those that were close to the skin were visible. For the purposes of this study and for the sake of simplicity, it was assumed that all input to a visible node arose from this visible vessel, which is a reasonable assumption given the superficial depth of injection of the MB in the paw.
However it is possible that more branches of a lymph vessel could have fed a single node beyond what was imaged, and this could lead to more complex kinetics which may not have been modeled in this study.159

The MDA-MB-231-luc-D3H2LN cell-line53,160 is known to metastasize from a primary tumor to draining lymph nodes in host mice, over a period 10-weeks, but here was examined for establishment of a short term model for lymph node involvement. An intra-nodal cancer-cell injection model has distinct advantages for research imaging studies, because the time to incubate the tumor is dramatically reduced. In this study, the imaging was done at ~5 days post surgery, versus 10 weeks for metastatic invasion from a primary tumor. In this approach, the cancer-cells were easily deposited into lymph nodes with minimal damage and the animal’s lymphatics did not appear to change significantly over a period of 5 days or as a result of surgery. No significant differences were seen between surgery-free and sham-control groups. Also, no significant differences were seen between normal and cancer-bearing nodes when average nodal fluorescence was used as the basis to compare the control and cancer-bearing animals. This was attributable for the most part to significant delivery differences (>50%) through the feeder lymph vessels.

Several parameters based on temporal fluorescence measurements in lymph nodes and lymph vessels were used to differentiate control from cancer-cell bearing nodes. All methods that relied solely on the fluorescence signal from lymph nodes failed owing to the large heterogeneity in lymphatic delivery to these nodes (Table 4.1). On the other hand, methods based on nodal fluorescence normalized by peak fluorescence from respectively vessels performed fairly well, with the ratio of peak fluorescence in node to
peak fluorescence in vessel $\frac{P_N}{P_A}$ producing the most significant difference between normal and cancer-bearing nodes.

In order to estimate a physiologically relevant quantity that correlated with presence of cancer in lymph nodes, an afferent vessel input model of lymphatic flow through nodes, analogous to the arterial input model of kinetic imaging used to measure cerebral blood flow, was used$^{148}$. Based on this nonparametric model, a scaled version of lymph node impulse residue function was calculated by physiologically-constrained deconvolution$^{157}$ of afferent vessel input from node fluorescence to estimate lymph flow through node, $F_{LN}; F_{LN}$ is very closely related to $\frac{P_N}{P_A}$, and is significantly different in cancer-bearing nodes as compared both to control and normal nodes. Furthermore, there was no significant difference between $F_{LN}$ of normal and control nodes that received sham PBS injections, thus indicating that inflammation due to surgery did not significantly contribute to nodal lymph flow. Normal nodes showed nodal flow rates ranging from $\sim 0.5 – 2.5$ ml/g/min; this was comparable to the values of lymph flow rate reported elsewhere$^{51,161}$.

One of the benefits to using a nonparametric model to perform kinetic analysis is that no explicit assumptions are necessary regarding the structure or compartmentalization of the node. Therefore, the shape of the $R(t)$ reflects the true kinetics of the node, rather than an imposed model. Once an understanding of the general dynamics is gained, more specific models can be applied where suitable, for example, to quantify fast and slow components or to estimate binding if the tracer is targeted$^{53}$. These may offer improvements in specificity and sensitivity, when applied to clinical cancer models. While this paper did not attempt to study structural changes in
lymphatic vessels supplying cancer-bearing lymph nodes\textsuperscript{162}, it can be stated with confidence that any changes in the lymphatic architecture will directly affect lymph delivery via lymph vessels to a node downstream of this channel, our method will nevertheless be able to objectively correct for this delivery difference and assess cancer presence in the draining lymph node, but will not be able to detect cancer spread in the lymph vessels themselves.

While the presented methods and results provide evidence to support our claim that $F_{LN}$ can be used to identify cancer-free from cancer-bearing nodes, a technological limitation of this approach that could limit translation to a clinical environment may be the limited depth sensitivity of 700 nm fluorescence signal combined with significant tissue autofluorescence in the 700 nm window. However, this may be overcome by use of 800 nm fluorophores such as ICG\textsuperscript{47} or IRDye 800CW\textsuperscript{163} as the tissue autofluorescence in the 800 nm window is very low. Furthermore, development of an in house hybrid ultrasound guided multispectral fluorescence tomography system has been underway, this system combines the high spatial resolution of high-frequency ultrasound with high specificity and depth sensitivity of the fluorescence tomography system so enable imaging of subcutaneous lymph nodes in humans\textsuperscript{60,164}.

4.5 Conclusions

This study shows large inherent heterogeneity exists in lymphatic delivery to lymph nodes of the axilla in this rat model, for both normal and cancer bearing animals. An intra-nodal cancer-injection model was employed and we demonstrated that incorporation of an afferent vessel input model could successfully account for inherent uptake variability, revealing similarity between normal and control nodes but significant
differences between cancer-bearing and cancer-free (normal and sham control) nodes. Lymph flow in cancer-cell injected nodes is significantly lower than normal nodes, possibly due to cancer-cell mediated disruption of flow through nodes.
CHAPTER 5  Dual Tracer Imaging of Rat Lymph Nodes using two structurally distinct tracers

This chapter describes the methods and results from a group of studies that involved the use of a small, fluorescently cancer-targeted tracer EGFr-Affibody–IRDye800CW, along with methylene blue as an untargeted reference tracer to estimate tumor burden on lymph nodes.

5.1 Introduction

The Lymph Node-Molecular Concentration Imaging (LN-MCI) approach described by Tichauer et al., using a combination of antibody tracers worked well in estimating metastatic burden on axillary lymph nodes in a murine breast cancer model. Both these tracers are not FDA-approved and clinical translation has significant hurdles, especially due to the use of a fluorophore-bound animal derived-IgG antibody tracer. This approach would have mush greater translatability if a combination of previously approved tracers is used. A recent ongoing collaboration between Dartmouth, LI-COR Inc., and Affibody AB was leveraged to produce a GMP production run of tracer ABY-029, which is a fluorescence-labeled Affibody to the EGF receptor, which also recently cleared toxicity tests and is due to receive an institutional review board (IRB) approval at Dartmouth College, Hanover, NH. LN-MCI was identified as a target application of such a tracer. In order to account for non-specific uptake of this tracer, methylene blue was identified as a reference tracer, owing to its approval status and it known uses in
sentinel node mapping. This chapter will discuss in detail the experiments used to test the use and efficacy of the combination of Affibody-IRDye800CW and Methylene Blue in implementing the LN-MCI method to estimate burden of EGFr-expressing cancer on lymph nodes.

5.2 Theory: Dual Tracer compartment modeling to estimate receptor concentration in lymph nodes

The two-compartment model used to estimated receptor concentration in lymph nodes is based on the model described by Tichauer et al.\textsuperscript{53} This has briefly been described here.

![Two-compartment tracer kinetic model as described by Tichauer et al.]({image_url})

Figure 5.1 Two-compartment tracer kinetic model as described by Tichauer et al.\textsuperscript{53}.

Within the lymph node, the targeted tracer is modeled to be freely associated ($C_f$) or bound ($C_b$) to targeted tumor cell receptors, and the measured fluorescence signal $ROI_T$ is proportional to the sum of these,

$$ROI_T(t) = \eta_T [C_f(t) + C_b(t)]$$  \text{Equation 5-1}

Here $\eta_T$ is the detection efficiency of the imaging system for the targeted tracer. The whole system is modeled to be driven by the concentration of tracer in the afferent lymphatic vessels, $C_t$, which enters the lymph node at flow rate, $F_l$. The targeted tracer is modeled to only be able to exit the lymph node into the downstream lymphatics at the
same flow rate, \( F_l \), assuming flow equilibrium conditions. Assuming that the unbound (freely associated) concentration of tracer in the lymph node is always homogeneously distributed and that the concentration of targeted tracer in the lymph node is negligible compared to the concentration of targeted receptor, a system of first-order differential equations can be developed to govern the rate of change of tracer concentration in each compartment:

\[
\frac{dc_f(t)}{dt} = F_l C_l(t) - F_l C_f(t) - k_3 C_f(t) + k_4 C_b(t) \quad \text{Equation 5-2}
\]

\[
\frac{dc_b(t)}{dt} = k_3 C_f(t) - k_4 C_b(t) \quad \text{Equation 5-3}
\]

Here rate-constants \( k_3 \) and \( k_4 \) are used to describe the rate of tracer-receptor association and dissociation, respectively. Similarly, based on the assumption that at equilibrium both tracers have flow rate equal to the inherent lymph flow rate, the untargeted tracer (no bound compartment) concentration \( C_U \) is represented as follows.

\[
ROI_U(t) = \eta U C_U(t) \quad \text{Equation 5-4}
\]

\[
\frac{dc_U(t)}{dt} = F_l C_l(t) - F_l C_U(t) \quad \text{Equation 5-5}
\]

A single-time point estimate of Binding Potential\(^{165} \), \( BP = \frac{k_3}{k_4} \), which is the main parameter of interest, can be obtained by rearranging Equation 5-1 and Equation 5-4, under the assumption that \( C_f(t) \approx C_U(t) \).

\[
\frac{\eta U ROI_T(t) - ROI_U(t)}{ROI_U(t)} = \frac{C_f(t) + C_b(t) - C_U(t)}{C_U(t)} \approx \frac{C_b(t)}{C_U(t)} \approx \frac{k_3}{k_4} \equiv BP \quad \text{Equation 5-6}
\]

### 5.2.1 Accounting for pharmacokinetic differences between tracers

The above equations rely on the assumption that the free and bound concentrations of tracer in the tissue are in instantaneous equilibrium, and that the input function \( C_l(t) \)
is identical for both tracers. The fluorescence signal in the afferent lymphatic vessel can be used as a surrogate for $C_i(t)$, and if these signals vary for the tracers for interest, we can describe the relationship between them as follows\textsuperscript{166},

$$C_{i,T}(t) = C_{i,U}(t) \times y(t) \text{ Equation 5-7}$$

where the subscripts “T” and “U” represent targeted and untargeted tracer respectively, where $y(t)$ is a function that converts $C_{i,U}(t)$ into $C_{i,T}(t)$. $y(t)$ can be estimated using a Tikhonov-regularized deconvolution approach as detailed in Diop \textit{et al.}\textsuperscript{167}, and then applied to correct the untargeted tracer fluorescence signal in the lymph node.

$$R\tilde{O}I_U(t) = ROI_U(t) \times y(t) \text{ Equation 5-8}$$

This $R\tilde{O}I_U(t)$ would now be corrected for the pharmacokinetic differences in delivery of tracer to the lymph node.

\textbf{5.3 Methods and Results}

\textbf{5.3.1 Preparation of Tracers and their Fluorescence Properties}

\textit{5.3.1.1 EGFr Affibody-IRDye800CW}

The targeted tracer was prepared by a method identical to the one described by Sexton \textit{et al.}\textsuperscript{168}. Anti-EGFR Affibody (Affibody AB, Solna, Sweden), was diluted with phosphate buffered saline (PBS) at pH 7.5 to achieve a concentration of 1 mg/ml. As per the manufacturer’s recommendations, the Affibody molecules were reduced by adding dithiothreitol (DTT, mM) and incubated on a magnetic stirrer for two hours at room temperature. Excess DTT was removed by passage through a polyachrylamide 6000 desalting column (Thermo Scientific, Rockford, IL). Recovered protein was concentrated in a centrifuge using a 6-kDa molecular weight cutoff (MWCO) column.
(GE Vivaspin 2, Pittsburgh, PA). At this point the Affibody was ready for binding with a fluorophore. The fluorophore, IRDye 800CW maleimide (LI-COR Biosciences, Lincoln, Nebraska), was suspended in pure water at approximately 2 mg/ml, and was added to the protein solution to achieve a 2.5 molar excess of dye to protein as recommended by LI-COR. The Affibody-IRDye 800CW solution was then incubated on a magnetic stirrer for approximately two hours at room temperature; excess dye was removed by passage through a desalting column, and concentrated in the centrifuge using a 6 kDa MWCO column. A dilution made from the concentrated labeled Affibody solution was examined in a UV-Vis spectrophotometer (Cary 50 BIO UV-Visible spectrophotometer, Varian, Palo Alto, CA) to record the absorption spectrum from 220 – 800 nm. Protein concentrations and dye-to-protein ratios were determined using absorption values at 280 nm and 780 nm as described by LI-COR. All labeled Affibody solutions yielded dye-to-protein ratios between 0.65 and 0.85. Figure 5.2 shows the structure of the Affibody peptide, it is a 3-alpha helix.

![Affibody peptide structure, IRDye800CW Maleimide molecular structure, and IRDye800CW absorption and emission spectra.](image)

Figure 5.2 Affibody peptide structure (left), IRDye800CW Maleimide molecular structure (middle) and IRDye800CW absorption and emission spectra (right).

The fluorophore used, IRDye 800CW is shown in figure 3.1b and its absorption and emission spectra are shown in figure 3.1c. IRDye 800CW maleimide has peak excitation at 774 nm, peak emission at 789 nm, and extinction coefficient 240,000 M⁻¹cm⁻¹ in PBS.
5.3.2 Cancer Cell Model and Animal Preparation

The MBA-MB-231-luc-D3H2LN breast cancer cells line was used in this study. Procedures identical to those described in the previous chapter and were used, these will not be described again here. Rats were grouped into normal and cancer-bearing cohorts. This study did not have sham surgery cohorts. Bioluminescence imaging was used to confirm cancer presence in nodes. Figure 5.3 shows a rat undergoing intra-nodal tumor injection following localization with methylene blue, and Figure 5.4 shows a couple of rats being imaged on the IVIS imager to test for bioluminescence from nodes.

Figure 5.3 Rat undergoing intra-nodal tumor inoculation

Figure 5.4 Bioluminescence imaging
5.3.3 In vivo fluorescence lymphatic imaging of axillary LNs

Prior to imaging, each animal was anaesthetized using 1.5 - 3% isoflurane (Piramal, Bethlehem, PA) in 1.5 L/min oxygen (Airgas, White River Junction, VT). It was then placed on its side with the forelimb on one side of the body stretched out so as to expose lymph nodes of the axilla; shown in Figure 5.6. Next, 25 nmoles of Methylene Blue (Sigma-Aldrich, St. Louis, MO), in 5mM bovine serum albumin (Sigma-Aldrich, St.Louis, MO) mixed with 0.1 nmoles of EGFr Affibody labeled with IRDye800CW (Dye:Protein = 0.82:1) in a 20 𝜇𝑙 volume of PBS, were injected intra-dermally using a 27-gauge syringe-needle into the base of a forepaw of each animal. Care had to be taken to ensure the injection was not too deep; deep injections lead to delayed uptake of dye into the lymph system, as it is the skin that is richly supplied with lymph capillaries. Some of the animals required their paw to be massaged for a few seconds, to improve fluorophore uptake by lymph capillaries. It has previously been shown that lymphatic vessels supplying the axillary and brachial lymph nodes drain the cutaneous regions of the forepaw\textsuperscript{147}. Additionally, the large injection volume produced pressure that caused the lymphatic capillaries to take up dye.

All fluorescence imaging was performed using a Pearl® Impulse system (LI-COR Biosciences, Lincoln, NE) which provides planar surface images in the near infrared band\textsuperscript{53}. The system was set up to acquire grayscale white light, 800 and 700 nm channel images at 2 frames per minute each for 40-60 minutes. The 700 nm channel setting uses a 685 nm laser excitation and a 720 ± 20 nm bandpass emission filter; the 800 nm channel setting uses a 785 nm laser excitation and a 820 ± 20 nm bandpass emission filter. The timing allowed capture of the dynamics of lymph flow through paws, lymph vessels, and lymph nodes. Figure 5.5 shows a rat being set up for imaging. A white light
image taken during dye uptake to nodes is shown in Figure 5.6 along with 800 channel (green) and 700 channel (red) overlays. Regions-of-interest were manually drawn over the lymphatic vessels and lymph nodes to measure fluorescence intensities.

Figure 5.5 Rat placed on the imaging bed of the Pearl Imager with paw taped down.

Figure 5.6 *In vivo* fluorescence imaging

5.3.4 Deconvolution schemes using reference tracer uptake

Figure 5.7 shows the uptakes of targeted and untargeted tracers in lymph nodes and afferent lymph vessels in normal and cancer bearing rats. No observable trend could be determined from the uptake curves by visual inspection. The large amount of heterogeneity present in delivery of both tracers seen in normal rats prevented any reasonable interpretation of results. An approach similar in principle to that described in 5.2.1 was used to observe the relationship between afferent vessel uptake and nodal
uptake in the two cohorts. We reformulated Equation 5-7 to describe the relationship between targeted and untargeted fluorescence in nodes (N) and vessels (V).

\[ ROI_T^{NV}(t) = ROI_U^{NV}(t) * i_{NV}(t) \]  

Equation 5-9

The deconvolution function \( i_{NV}(t) \) corresponds to nodes or vessels respectively. Binding potential was not calculated in this study. Our hypothesis was that, uptake in normal lymph nodes should bear resemblance to the uptake in their afferent vessels, i.e. have \( i_N(t) \) and \( i_V(t) \) approximately similar to each other, while cancer-bearing node should not show such resemblances due to the binding of Affibody with EGFr expressed by cancer-cells in the lymph nodes.

Figure 5.7 Uptake of targeted tracer (green) and untargeted tracer (red) is shown in nodes (large circle markers) and afferent lymph vessels (small markers). Tumor bearing animals are tagged with the word “tumor”. Animals that could not conclusively be determined to bear tumor are marked with “?”. Left side of the body is indicated using “L” and right side using “R”.
Figure 5.8 shows the deconvolution function curves obtained when the uptake of untargeted tracer was deconvolved from the uptake of targeted tracer in both lymph nodes and vessels separately. The solid lines indicate node deconvolution functions \(i_N(t)\) and dotted lines indicate vessel deconvolution functions \(i_V(t)\). We expected that the deconvolution function from lymph vessels could act as a surrogate to the deconvolution function in lymph nodes, which would in effect require the curves to be similar to each other in normal tumor-free lymph nodes. However, this was not the case. While a definitive explanation for this does not exist we suggest that this may be due to the presence of multiple afferent vessels supplying a single lymph node and the presence of multiple pseudo-compartments within each lymph node. More significantly, the rapid clearance of methylene blue from the afferent vessels and lymph nodes posed a major challenge. Most kinetic information about methylene blue uptake is present only within the first 20 minutes of tracer injection, while affibody-IRDye800CW is much slower and would require imaging time longer than 40 minutes to gather enough data. Due to such major incompatibility issues, neither tracer attained steady state during the 40 minutes of imaging – which is essential for application of the LN-MCI method.
5.3.5 Model-free multiparametric analyses using afferent vessel uptake and reference tracer uptake

Failure to account for the pharmacokinetic differences and implement receptor density imaging, called for implementation of simpler, model-free approaches. In an attempt to compare raw fluorescence signal intensities between normal and cancer-bearing groups a number of parameters were considered. These are shown in Figure 5.9 and Figure 5.10. The labels ‘Tar’ and ‘UTar’ are used to indicate IRDye800CW-Affibody and methylene blue respectively. Integrated node fluorescence signal of each tracer from both cancer and non-cancer groups were compared and no significant differences were seen between uptake of either tracer indicating that binding activity between the targeted
agent was masked by uptake variability among all animals. The uptake curves were fit to a single exponential model, \( y = Ae^{-Bt} \) and the fitted parameters were compared as well. Various parameters derived from deconvolution functions from nodes \((i_v(t))\) and vessels \((i_v(t))\) were also compared. These are all shown in Figure 5.10.

![Figure 5.9 Comparison of measured parameters related to uptake of targeted and untargeted tracers in lymph nodes. A and B are described in the text above. Subscripts ‘UTar’ and ‘Tar’ denote untargeted and targeted tracers respectively, and subscript N represents that the signal was measured in the node.](image)

![Figure 5.10 Measured parameters derived from deconvolution functions of node and afferent vessels.](image)

### 5.4 Discussion & Conclusions

The results presented above show that imaging of two tracers very different in size and pharmacokinetics from each other is challenging. Deconvolution techniques seem to be promising, but our experimental data suffers from the lack of methylene blue
retention by lymph nodes at time points >40 minutes post injection. Moreover, in contrast to Erbitux (used by Tichauer et al\textsuperscript{53}), Affibody owing to its small size, is rapidly taken up by the lymphatics into the axillary lymph nodes but shows an almost ~10 times lower binding affinity to EGFr than Erbitux. This implies lower sensitivity to small numbers of cancer cells, and possibly the binding effect is visible much later than 40 minutes. The next step to further the above study would be to use Affibody as a targeted tracer along with the control untargeted Affibody\textsuperscript{166} to eliminate the large variation that we observed between methylene blue and EGFr-targeted Affibody kinetics.
CHAPTER 6 Ultrasound-guided multi-spectral Fluorescence Tomography for Subsurface Fluorescence Quantification


6.1 Introduction

Previous chapters described fluorescence imaging methods to quantify fluorophore distribution in lymph nodes and vessels. This has so far been useful in whole body imaging of small animals, with lymph nodes that are within a millimeter in depth from the skin. In humans, the most superficial lymph nodes can be over 1 cm deep from the skin, and in patients with high body-mass indices, lymph nodes of the axilla can be 4 – 5 cm deep. This demands that an imaging system capable of subsurface fluorescence imaging be designed. Diffuse fluorescence tomography (FT) of tissue can be used for subsurface fluorescence imaging, and has been shown to provide improved quantitative
accuracy as compared to broad-beam surface imaging methods\textsuperscript{169, 170}. Subsurface FT typically employs an array of fiber optic source-detector pairs to measure diffusely remitted light, and the data are used to construct depth images from the different light paths sampled. Ideally, each source-detector pair samples a slightly different light path through the tissue and numerical method solutions for the multiple-scattering paths are used to isolate the local contribution of fluorophore concentration to each measurement. However, mathematical reconstruction of a true, high-resolution fluorescence image from FT measurements is an ill-posed problem because the limited number of measured signals does not provide a unique solution to the fluorescence distribution map for the large number of locations within the domain. Incorporation of structural information as a spatial prior constraint on the fluorescence reconstruction has been shown to provide promising results. In this work, high-resolution spatial prior information from ultrasound was combined with highly sensitive optical information to reduce the ill-posedness of the inversion process and provide region-wise estimates of fluorophore concentrations.

The use of the reflectance geometry in the system setup allows seamless integration with an US transducer and is more relevant to large animal (human) imaging applications. Most current FT methods use transmission geometry, which works well for small animals but requires light transmission through the region of interest, and is thus less applicable to large animal (human) imaging. Fluorescence measurements were made using spectrometer enabling spectroscopic signal decoupling for improved sensitivity and detection accuracy. In the upcoming sections, methods and results pertaining to Aminolevelunic-Acid (ALA) induced protoporphyrin IX (PpIX) production are
presented, as the monitoring of ALA- Photodynamic therapy (PDT) was an early application of this system.

6.2 USFT_v2 System description

A diagram of the USFT version 2 imaging system used is shown in Figure 6.1. The instrument was originally introduced by Gruber et al\(^{71}\) underwent significant hardware and software improvements in version 2, noted in this Chapter; we will also describe instrumentation updates on the 3rd iteration of this system with multispectral fluorescence capabilities in a later section, however all the data processing and analysis methods remain the same. This system is composed of a Sonix Tablet (Ultrasonix, Richmond, British Columbia, Canada) high-frequency ultrasound system with a 40 MHz transducer (L40 8/12, Ultrasonix) coupled to a multi-channel optical tomography instrument. These modalities are integrated at the tissue interface through a custom ‘v-block’ positioning system which facilitates precise positioning for imaging a given plane with both ultrasound transducer and fluorescence tomography fiber probe sequentially, as illustrated in Figure 6.1. Image acquisition begins by using the US system to locate the region of interest, such as a subsurface tumor. Once this imaging plane is located, the positioning arm holding the ‘v-block’ is locked and an US image is captured. Then, the US transducer is slid away and replaced with the FT optical fiber array via the sliding v-block design and all optical projections are acquired. The transducer v-block is designed such that both transducers image the same plane, allowing direct use of segmented US images for FT processing.
Figure 6.1 The ultrasound unit, transducer and custom probe holder of the USFT_v2 system are shown. The image on the right shows a close-up view of the probe holder.

The optical component of the system consists of a linear array of alternating source (4) and detector (5) optical fibers with a center-to-center spacing of 2.5 mm (See inset Figure 6.1). The excitation illumination source is a 633 nm current and temperature regulated laser diode system (Model 7404, Intense Co., North Brunswick, New Jersey). The laser output passes through a filter wheel (FW102C, Thorlabs) containing a 2 OD neutral density filter, a 650-nm short pass filter, and a beam stop, and is multiplexed to the four source channels via a 1×4 fiber switch (Piezosystem Jena, Hopedale, Massachusetts). The output of each channel is coupled to the target tissue by 600 µm low-OH optical fibers (LGOptics, Germany). The five detection fibers channel light from the target tissue through custom moveable filter blocks to five compact spectrometers (USB2000+, Ocean Optics, Dunedin, Florida) with spectral range of 540 to 1210 nm. Sources are illuminated sequentially, while detection fiber spectra are captured in parallel. Dark spectra are captured for each imaging mode using the same
exposure times and light paths with the excitation source blocked by a beam stop in the filter wheel.

Figure 6.2 Optical Components of the USFT_v2 system including a 633nm laser, filter wheels, spectrometers and a white light source.

In addition to the fluorescence excitation source, a shuttered white light source (HL-2000-FHSA, Ocean Optics) has been incorporated into the optical system, through an additional optical fiber, to obtain white light spectroscopy data for explicit recovery of tissue optical properties. This capability is unique in fluorescence tomography systems.

The fiber switch, filter wheel, and spectrometers are connected via a USB hub (DUB-H7, D-link) to the Sonix Tablet Windows-based computer. The detection-side moveable filters, filter position feedback limit switches, and white light shutter are controlled or read via NI DAQs (NI USB 6009, National Instruments). Imaging, including source-detector-pair-dependent auto-exposure for each imaging mode is controlled via LabView virtual instrument software (LabView v8.4.2, National Instruments). Ultrasound image- segmentation, spectral processing, and fitting of
fluorophore distribution were performed using Matlab (R2011b, Mathworks, Natick, Massachusetts) and NIRFAST\textsuperscript{172}.

### 6.3 Data Acquisition: Trimode Spectroscopy

Three modes comprise of every set of optical measurements recorded (See Figure 6.3).

![Trimode Spectroscopy](image)

Figure 6.3 Trimode Spectroscopy involves measurements in the ‘transmission mode’ where unfiltered excitation signal (633 nm and 785 nm on USMFT system) is measured, a ‘fluorescence mode’ where signal emitted from the sample if filtered using long pass filters, and a ‘white-light mode’ where white light signal traversing the tissue is measured to estimate bulk tissue-optical properties. Sample spectra and ultrasound images are shown as insets.

In the first mode, the \textit{excitation-measurement mode}, once the fibers were positioned, excitation intensity is measured for all 20 projections (four sources and five detectors).

During this acquisition, the source intensity was attenuated at the filter wheel using a neutral density filter of OD = 2.0 (NE20B, Thorlabs, Newton, New Jersey) to prevent detection spectrometer saturation. To acquire full projection fluorescence data, during the \textit{fluorescence-measurement mode} the neutral density filter at the filter wheel was replaced with a 650-nm short pass filter (FES0650, Thorlabs), and 655-nm long pass interference
filters (655ELPF, Omega Optics) were automatically lowered into each detection channel via movable filter mounts (MFF001, Thorlabs) attached to custom filter blocks. Finally, during the *white-light spectroscopy mode* a beam stop in the filter wheel blocks the excitation laser, and white light spectra from a single source were measured at each detector position to provide five projections for tissue spectroscopy. LabView frontpanel designed for trimode spectroscopic data acquisition is shown in Figure 6.4.

![LabView frontpanel window of the data acquisition program.](image)

**Figure 6.4** NI Labview frontpanel window of the data acquisition program.

### 6.4 Data Processing

#### 6.4.1 US image segmentation and mesh generation

Ultrasound images were manually segmented into 2 or more regions with at least a fluorescent inclusion region, and background bulk tissue (Figure 6.5) using Matlab to produce a region mask. Because the US transducer field of view is narrower than the FT array, the mask background was extended laterally. This mask was then supplied to a meshing routine described elsewhere\(^{173, 174}\) and was recently built into the NIRFAST...
package along with source and detector fiber locations, node density, and mask pixel dimensions as inputs, to produce a finite element mesh.

Figure 6.5 US image is manually segmented and meshing and an FEM mesh is generated based on known source and detector positions.

6.4.2 Spectral data processing

Following the subtraction of dark signal, individual spectra were normalized with respect to their exposure times. Examples of raw spectra are shown in Figure 6.6.

Figure 6.6 Spectra acquired during the excitation mode (left) and emission mode (right).

A three-point median filter was used to eliminate spikes from each spectrum followed by a 14-point (5nm) moving average smoothing filter. For fluorescence spectra, a linear-least-squares spectral fitting algorithm was used to determine the contributions
from normalized basis spectra for PpIX fluorescence, photoproduct photoprotoporohyrin (pPp), and system and tissue auto-fluorescence (See Figure 6.7). The pPp fluorescence basis spectra is approximated using a Gaussian curve centered at 675nm with 27nm full-width-half-maximum, as reported previously by Kruijt et al.176.

The combined system and target auto-fluorescence spectra were obtained for each detection channel using phantom ingredients without PpIX. When small animals were imaged, average normalized normal skin fluorescence was used as the auto-fluorescence basis spectrum. The spectral decoupling was formulated as follows:

\[ \mathbf{U} \cdot \mathbf{A} = \mathbf{D} \]  

where \( \mathbf{U}, \mathbf{A} \) and \( \mathbf{D} \) are the fit coefficient vector, spectral basis matrix, and data matrix respectively and were defined as follows.
\[
U = \begin{bmatrix}
u_1 \\
u_2 \\
u_3 \\ \vdots \\
u_m
\end{bmatrix}, \quad A = \begin{bmatrix}
a_1^\lambda & a_2^\lambda & \ldots & a_n^\lambda \\
a_1^\lambda & a_2^\lambda & \ldots & a_n^\lambda \\
a_1^\lambda & a_2^\lambda & \ldots & a_n^\lambda \\
\vdots & \vdots & \ddots & \vdots \\
a_1^\lambda & a_2^\lambda & \ldots & a_n^\lambda
\end{bmatrix}, \quad D^T = \begin{bmatrix}
d_1^\lambda \\
d_2^\lambda \\
d_3^\lambda \\
\vdots
\end{bmatrix}
\]

Here \( m \) is the total number of bases, and \( \lambda_i \) for \( i = 1:n \) is the wavelength axis. We can solve for \( U \) using a pseudo-inverse scheme following by a linear least-square inversion, to estimate the fit coefficient for each basis spectrum. A single excitation value was also obtained from the 633nm peak for each projection. The fitted coefficient representing the fluorescence attributable to PpIX within the measured spectrum at each source-detector pair (Figure 6.9) and a corresponding transmitted excitation signal (Figure 6.8) value was then used in the fluorescence reconstruction process.

![Fluorescence signal sinogram](image1)

Figure 6.8 Fluorescence signal sinogram, where red bars S1, S2, S3, S4, S5 denote excitation source that was ON.

![Transmission per channel](image2)

Figure 6.9 Transmitted excitation signal sinogram, where red bars S1, S2, S3, S4, S5 denote excitation source that was ON.


6.5 Fluorescence Reconstruction using a Diffusion-based model

The diffusion-based forward model as described in Chapter 2 was used to simulate the reference surface excitation \( \phi_x^{\text{ref}} \) and expected fluorescence signals \( \phi_m^{\text{calc}} \), where subscripts \( x \) and \( m \) denote quantities at the excitation and emission wavelength, respectively, using target optical properties recovered with white light spectroscopy as described by Flynn et al.\(^{60} \) The measured, excitation-normalized data \( \phi^{\text{BR}} = \frac{\phi_m}{\phi_x} \), (see Figure 6.10 and Figure 6.11) was scaled by the simulated reference data, \( \phi_x^{\text{ref}} \) to provide a calibrated fluorescence measurement for each source-detector pair for reconstruction using the NIRFAST architecture.

\[
\phi_m^{\text{calib}} = \phi^{\text{BR}} \cdot \phi_x^{\text{ref}} \quad \text{……………………..Equation 6-2}
\]

The fluorescence concentration was calculated using an iterative error minimization algorithm of the objective function,

\[
\chi^2 = \| \phi_m^{\text{calib}} - \phi_m^{\text{calc}} \|^2 \quad \text{…………………..Equation 6-3}
\]

This can be reduced to the form

\[
\delta \eta_{af} = (J^T J + \lambda I)^{-1} J^T \delta \Phi \quad \text{…………………..Equation 6-4}
\]

where \( J \) is the Jacobian sensitivity matrix, which conveys the change in the boundary fluence with respect to changes in optical properties, \( \eta_{af} \) is the fluorescence yield, \( \delta \Phi \) is the error between \( \phi_m^{\text{calib}} \) and \( \phi_m^{\text{calc}} \), and \( \lambda \) is the fixed regularization matrix. Spatial segmentation information from the co-registered ultrasound image was incorporated into the reconstruction algorithm, thus drastically reducing the dimensionality of the problem from fitting fluorescence yield at thousands of mesh nodes to fitting fluorescence yield at just a few homogeneous regions. An extensive review of the
benefits of spatial prior in FT is provided by Pogue et al.\textsuperscript{177}. The 2-region mesh obtained from the segmented US image was used to generate a region-mapping matrix $k$ that maps the Jacobian to a lower dimension\textsuperscript{178}:

$$j' \rightarrow jk, \text{ where } k_{ij} = \begin{cases} 1, & \text{node}_i \in \text{region}_j \\ 0, & \text{otherwise} \end{cases}$$

Equation 6-5

And the new update equation was:

$$\delta \eta_{af} = (j'^Tj' + \lambda I)^{-1} j'^T \delta \Phi$$

Equation 6-6

Since FT reconstructions are generally capable only of recovering the effective fluorescence yield, $\eta_{af}$, which is dependent on the quantum yield, molar extinction coefficient, and concentration of the fluorophore), actual fluorophore concentration cannot be calculated without estimates of the other properties. The quantum yield for PpIX in DMSO was determined to be $\eta = 0.0046$ using the comparative method of Williams et al.\textsuperscript{179}, with Kiton Red in DMSO as a standard, but it is well known that this value varies depending on solvent and local environment. The PpIX molar extinction coefficient at 633nm is 4866/Mcm\textsuperscript{180} as found on the Oregon Medical Laser Center website (http://omlc.ogi.edu/). Using this molar extinction coefficient and the known PpIX concentrations, effective quantum yield was calculated for each experimental series.
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Figure 6.10 Sensitivity maps for two measurements, between Source 1-Detector 1 (off the inclusion) and Source 3-Detector 3 (on the inclusion), weighted by the Fluorescence/Excitation value. A shadow of the inclusion location is shown overlaid. Shown below the sensitivity maps are the normalized fluorescence intensities at all nearest-neighbor source-detector pairs, with the two pairs corresponding to the sensitivity maps highlighted. The inclusion to background contrast ratio is 8:1.

Figure 6.11 Excitation-normalized fluorescence is plotted for each detection channel when each of the four sources is ON (black). In addition, the reconstructed fluorescence signal is shown in blue.

6.6 Simulations & Tissue-Simulating Phantom Studies

6.6.1 Simulation Experiments to demonstrate need for optical property estimation

Simulations were performed to study errors in reconstructed fluorescence yield \( \eta \mu_a \) when errors were present in estimates of optical properties assigned to the forward
simulation mesh during reconstruction of a homogenous rectangular phantom with a true fluorescence yield of $4 \times 10^{-6}$ mm$^{-1}$. Absorption coefficient $\mu_a$ was varied from $1 \times 10^{-3}$ mm$^{-1}$ to 0.1 mm$^{-1}$ and reduced scattering coefficient was varied from 1 to 6 mm$^{-1}$. Figure 4.12 shows color maps indicating % error in fluorescence yield. As expected, errors in reconstructed fluorescence yield increase with increasing error in optical properties. Simulations with randomized error incorporation were run 10 times and averaged for each group.

Figure 6.12 Error maps of estimated fluorescence yield when assigned absorption and reduced scattering coefficient values were incorrect.

**6.6.2 Liquid Phantoms with varying optical properties**

In order to examine the sensitivity of the USFT system to changing PpIX concentration in a homogeneous medium, the system was tested using homogeneous liquid phantoms containing Intralipid (Fresenius-Kabi, Bad Homburg, Germany), 5%
Tween20 (P1379, Sigma-Aldrich), porcine whole blood (7204901, Lampire Biological inc.), and [0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8] µg/ml PpIX. Blood and Intralipid concentrations were varied to generate a sampling cross where values of (blood%, Intralipid%) were (1%, 0.5%) (1%, 1%) (1% 1.5%) (0.5%, 1%) (1.5%, 1%). Each phantom was stirred continuously until imaging to ensure consistency and to prevent stacking or settling of RBCs in blood. Three sets of measurements were obtained for each combination of blood, Intralipid, and PpIX concentration, resulting in 15 measurements at each PpIX concentration, with 3 measurements for each of the 5 blood/intralipid concentrations.

Reconstruction was performed in two stages. First, all phantoms were assumed to have equal optical properties based on values found in literature (\(\mu_a,x = 0.2 \text{ mm}^{-1}\), \(\mu_a,m = 0.15 \text{ mm}^{-1}\), \(\mu_s,x = 3.0 \text{ mm}^{-1}\), and \(\mu_s,m = 2.5 \text{ mm}^{-1}\)). Second, reconstructions were performed with phantom-specific optical properties, as measured by white light spectroscopy. The effective fluorescence yields obtained from these approaches were compared. In Figure 6.13 the reconstructed fluorescence yield measured using both the approaches is plotted against known PpIX concentration. The use of the correct optical properties for the reconstruction model lead to significant reduction in reconstruction error, by a factor of 8.9 ± 5.1 (mean ± std). The reconstructed fluorescence values were linear with PpIX concentration, with \(R^2 = 0.96\) for fixed literature based optical properties (data not shown) and \(R^2 = 0.98\) for white light-measured optical properties. Based on reconstructed fluorescence yield, PpIX molar extinction coefficient, and known concentration, the calculated quantum efficiency, \(\eta\), was roughly 0.0025 for homogeneous liquid blood and intralipid phantoms.
6.6.3 Gelatin-based tissue simulating fluorescence inclusion phantoms

To examine the ability of the USFT system to resolve changing PpIX concentration in a small region of interest surrounded by a background with low-level fluorescence, phantoms consisting of two regions, a background and a 10×40×4 mm fluorescent inclusion, were constructed using 10% w/v gelatin (G2500, Sigma-Aldrich), 0.085% w/v TiO$_2$ (14021, Sigma-Aldrich) for optical and ultrasound scattering, 1% porcine whole blood (7204901, Lampire Biological inc.) for absorption, varying concentrations of PpIX (P8293, Sigma-Aldrich), and 5% Tween20 (P1379, Sigma-Aldrich) to prevent PpIX aggregation. Gelatin was dissolved in hot water, stirred vigorously until cooled to 30°C, then mixed with the remaining ingredients and poured into molds. Inclusions were cast using negative molds, and backgrounds were cast with positive molds positioned to leave inclusion-sized holes. The bulk background contained a constant concentration of
0.1µg/ml (177 nM) of PpIX, while the inclusions contained [0.1, 0.2, 0.4, 0.6, 0.8, 1.0] µg/ml PpIX. A 2 mm thick human skin-simulating layer having the same composition as the bulk background was prepared as well, and phantoms were imaged with and without the skin-simulating layer.

Figure 6.14 shows reconstructed fluorescence yield versus known fluorophore concentration for inclusion phantoms with and without a 2-mm skin-simulating top layer (same fluorophore concentration as background, 0.1 µg/ml), as well as the reconstructed background values (same for all inclusions, 0.1 µg/ml) for each inclusion fluorophore concentration. These reconstructions were performed using spatial priors from segmented US images (See Figure 6.14 (a)) and the actual optical properties were determined with white light spectroscopy. Reconstructed values are linear with concentration for inclusions with and without skin, with R² = 0.90 and 0.98 for series respectively, down to the background concentration and lowest concentration used, 0.1 µg/ml. Figure 6.14 (b) shows the calculated versus known contrast to background ratio (CBR), for inclusions with and without skin-simulating top layers. The linear fit equations are y = 1.21x - 0.95 (R² = 0.78) and y=1.78x - 0.52 (R² = 0.77) for inclusions without and with the skin-simulating top layers, respectively. The mean error in recovered CBR is 23% without a top layer and 74% with a top layer. The CBR slope is near unity for the plain inclusion phantom, but is considerably higher when a skin-simulating layer is added. When all FT fiber pairs sample a uniform layer and spatial information is not available, the solution is non-unique because fluorophore concentration and depth are no longer independent parameters. The addition of US-guidance provides more accurate reconstruction through layers, as Figure 6.14 shows, but does not completely solve this reconstruction issue.
Figure 6.14 Reconstruction of inclusion phantom fluorescence yield (a) Recovered PpIX yield is shown, both reconstructions are linear. Sensitivity, based on intercept, is \( \sim 0.1 \mu g/ml \) PpIX. (b) Inclusion contrast-to-background ratio in both types of phantoms.

Figure 6.15 shows a representative inclusion reconstruction and the benefits of prior spatial information and accurate optical property information. Figure 6.15B shows hot spots in the un-informed (no US guidance, ill-posed) reconstruction and the obviously poor fluorophore mapping when using only FT. Figure 6.15C compares reconstruction fits using literature-based or white light spectroscopy measured optical properties for a single inclusion. Optical properties used from the literature were based on known absorption and scattering of blood, Intralipid, and gelatin. Note the reconstruction algorithm cannot adequately scale the intensity range, based largely on absorption, to reproduce measured data by changing only the fluorophore concentration. In Figure 6.15D note that in two-region reconstruction, there are only two fluorophore values calculated when using hard prior spatial information: homogeneous values in the inclusion region and the background region. While normalized results are represented in Figure 6.14, \( \eta \) calculated as described previously, is 0.0024, which can be expected in a gelatin phantom.
Figure 6.15 Inclusion fluorescence reconstruction improvements with addition of spatial information and white light-determined optical properties are shown (A) US image outline of a phantom inclusion that contains 4:1 PpIX inclusion:background contrast is shown. The phantom includes a 2-mm top layer above the inclusion. (B) Reconstruction without spatial priors. The $\eta \mu_a$ spatial map is overlaid on the ultrasound image. Color axis corresponds to $\eta \mu_a$. Note “hot spots” three orders of magnitude higher than values in (D). (C) Reconstructed source-detector measurement fits with (blue) and without use (red) of white light-measured optical properties. Note that without correct absorption coefficients, varying only fluorophore concentration cannot reproduce the correct range of signal intensities for varying source-detector separations. This is why the fixed optical property reconstruction fails to reproduce the measured data (black). (D) Regional fluorescence reconstruction with use of spatial priors and measurement-specific optical properties.

6.7 *In vivo* Validation studies: Tumor-simulating Matrigel-based injections

After successful testing of the USFT_v2 system with liquid and gelatin phantoms, the next step was *in vivo* validation. PpIX inclusions mixed with matrigel and injected subcutaneously in mouse-skin provided a highly controllable testing ground. Approximately 200 $\mu$L of phenol red-free and growth factor-reduced matrigel medium (#356230, Corning) was mixed at 4°C with 5% Tween 20 (Sigma Aldrich, #P1379) and
PpIX in DMSO to produce solutions having 0 to 0.8 µg/mL PpIX concentration. These were injected subcutaneously into the hind flanks of 6-week old nude mice. Each injection was made using a 27-gauge syringe to resemble a skin tumor and was allowed to solidify over 45 minutes. During this time animals were kept anesthetized using 1-3% isoflurane in oxygen. Prior to matrigel injections fluorescence measurements were captured and averaged to obtain autofluorescence basis spectra. Ultrasound images obtained were segmented manually based on the hypoechoic nature of matrigel injections and processed as previously described. This approach to in vivo imaging was highly controllable. A total of 14 animals each bearing 1 to 2 inclusions each were imaged. Figure 6.16a shows an ultrasound image of a subcutaneous matrigel phantom containing 0.05 µg/ml PpIX on a mouse thigh. The hypoechoic region (inclusion) was segmented, and Figure 6.16b shows the resulting segmented mask. The corresponding finite element mesh is shown in Figure 6.16c. Bulk optical properties were estimated using white light spectroscopy\(^6\) and assigned to the reconstruction mesh. The reconstructed region-wise estimate of fluorescence yield, \(\eta \mu_{af}\) is shown in Figure 6.16c. Measured and reconstructed normalized fluorescence signal is shown in Figure 6.17. The overall result was accurate to a significant degree.

Figure 6.16 a) A sample ultrasound image of a subcutaneous matrigel injection is shown. b) Segmented US image. c) Reconstructed fluorescence yield
6.7.1 Shortcomings of Diffusion-based reconstruction in *in vivo* small animal imaging

Notwithstanding the benefits of using a model-based reconstruction approach, migration to *in vivo* studies revealed several deficiencies in quality of measurements obtained and hence in the quality of reconstructed fluorescence yield estimates. These were in most part due to: 1) poor contact between animal skin and optical fibers especially around bulges, 2) invalidity of the diffusion approximation at several instances, 3) errors in co-registration between US images and FT data due to differences in amounts of compression from each transducer, and 4) poor estimates of optical properties using WLS due to inherent heterogeneity of tissue and inclusion/tumor.

6.7.2 Ultrasound-guided normalized spatial fluorescence measurements

Due to the limitations described above and the need for a simplified approach, we compared reconstructed fluorescence yield with excitation-normalized spatial fluorescence measurements. Figure 6.18 shows spectrally decoupled PpIX fluorescence (from a single SD pair) versus reconstructed fluorescence yield. A linear trend is seen between the two indicating that fluorescence measurements obtained can on their own act as a surrogate for fluorophore concentration provided that fluorescence...
concentration estimation is not critical, which is the case when only contrast-to-background between an inclusion is of interest, such as in a tumor or lymph node.

The ultrasound image obtained from the phantom/animal being imaged was used to estimate depth and thickness of the inclusion or tumor. SD Pairwise Jacobian sensitivity maps were generated previously and regions of maximal overlap between these and the ultrasound image of the inclusion were used to determine the “best” SD pairs that probed the region of interest such as the inclusion and background. Figure 6.19 is used to demonstrate this approach. Inclusion is shown as a gray overlay, and sensitivity maps for 2 sets of source-detector pairs, one separated by 2.5 mm and the other by 7.5 mm, are shown. The excitation-normalized fluorescence intensity from the latter would be used as an estimate of fluorescence yield. In the case of multiple SD pair having maximal overlap with an inclusion, a geometrical average of the measurements was performed. Geometrical average was preferred over arithmetic average to take into consideration the large dynamic range of signals from differently spaced SD pairs.

Figure 6.18 Measured fluorescence versus reconstructed fluorescence

![Graph](image-url)
Results from white-light spectroscopy informed diffusion-based reconstruction were compared with the simplified ultrasound-guided normalized fluorescence measurement approach. The comparison is shown in Figure 6.20. From the former approach, fluorescence quantum yield $\eta$ was calculated to be 0.0025 using a 0.01 $\mu$g/ml matrigel phantom as a reference. It is expected that PpIX effective fluorescence yield \textit{in vivo} would be lower than in DMSO solution. Since the estimated reconstructed fluorescence cannot definitively be mapped to a PpIX concentration value, we consider only the estimated fluorescence yield as a final result with the expectation that a simple scaling factor will be able to convert these results into PpIX concentration. Comparing obtained $R^2$ ($R^2 = 0.57$ and 0.63 for diffusion-based reconstruction and spatial..
fluorescence measurements respectively) values for the two methods shows that the spatial normalized fluorescence approach performs as well as if not slightly better than its diffusion-based counterpart.

Figure 6.20 Comparison of Diffusion-based reconstruction and excitation-normalized spatial fluorescence measurements in matrigel inclusion bearing mice.

All animals imaged have been included in this discussion. Since the inclusions were at some depth below the skin, the source-detector fiber pairs separated by 7.5mm were chosen for analysis, as these maximally probed the inclusion. Therefore, fluorescence signals from individual measurements separated 7.5 mm were averaged to obtain an estimate of PpIX Fluorescence signal from the inclusion. Spectral fitting separated out any contributions from tissue autofluorescence from non-PpIX sources. Figure 6.21 shows a plot of known PpIX concentration versus recovered PpIX fluorescence signal. The results of PpIX fluorescence signal show linear correlation of $r = 0.93$ with the known PpIX concentration and an overall variability of $\pm 25\%$. Inclusions containing 0 ug/ml PpIX formed the control group. The intersection between the control group results and linear trend line estimates noise floor to be at 0.043 µg/ml PpIX.
concentration within the matrigel space. The source of noise is largely PpIX present in the mouse body mainly the skin and varied greatly across animals.

![Graph showing PpIX fluorescence vs known PpIX concentration](image)

Figure 6.21 Recovered PpIX fluorescence versus known PpIX concentration in the inclusion. The shaded region shows measured and spectrally decoupled fluorescence from the control group, PpIX fluorescence here is that from mouse skin.

6.8 *In vivo* Validation studies: Measurement of ALA-induced PpIX production in a preclinical skin cancer model

In this study A431-tumor bearing mice treated with Aminolevulinic Acid (ALA) to study the technical and logistical issues involved in using the developed imager to a preclinical or clinical environment. ALA is a known precursor to PpIX as part of the heme synthesis pathway. This pathway is disrupted in tumors, leading to preferential accumulation of PpIX in them.

6.8.1 Cell culture and skin tumor model

A431 is a squamous cell carcinoma line obtained from human cutaneous squamous carcinoma. Cells were cultured at 37°C in high-glucose Dulbecco’s Modified Eagle
Medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. One million cells (100 µl per spot) were injected subcutaneously into each hind flank of immuno-compromised athymic female nude mice. Tumors were allowed to grow for 10 days before ALA-treatment and imaging. Figure 6.22 shows a photograph of tumor-bearing mouse with its skin surgically removed to reveal the tumor.

![Figure 6.22 Mouse bearing A431 tumor on right thigh.](image)

### 6.8.2 Aminolevulinic Acid - Treatment

All animals were maintained on a fluorescence-free diet for a week before imaging. Prior to imaging, each mouse received an intraperitoneal injection of 200 mg/kg body weight of Aminolevulinic Acid (A3785, Sigma-Aldrich), see figure 4.22. Imaging was performed at various time-points post-ALA injection. Additionally, exposure to light was avoided to prevent photo bleaching of PpIX and light-induced treatment of the skin.

### 6.8.3 In vivo imaging and fluorescence measurement using USFT_v2 system

All animals were imaged after being anesthetized with 1 – 3% Isofluorane in 1 L/min oxygen. Measurements were made using the USFT system (Figure 6.23). Tumor-bearing mice were imaged once pre-ALA injection with the skin intact and at various time-points post-ALA injection with skin folded back to expose the tumor. The skin of
nude mice produced very large amounts of PpIX upon ALA-treatment, and caused washing out of any subsurface PpIX signals. In order to acquire reasonable measurements without the corruption from skin signal, folding back of the skin was an essential step.

Figure 6.23 Imaging A431 tumor bearing mouse using the USFT imager

Ultrasound images of the tumor-bearing site were obtained before and after folding the skin back. Figure 6.24 shows a set of ultrasound images from a single tumor imaged with and without skin intact.

Figure 6.24 Ultrasound images with FEM mesh overlay

The USFT system was used to obtain in vivo fluorescence measurements at the pre-ALA, 4-hrs post-ALA and 24 hours-post ALA time points. Validation required sacrifice of the animal, and hence, the 4 and 24 hour post ALA cohorts are non-overlapping and
distinct animals. Thus, three sets of measurements exist for each tumor-bearing mouse – (i) pre-ALA, (ii) post ALA over non-tumor region through skin and (iii) post-ALA over tumor with skin folded back to reveal the tumor. Each measurement dataset consists of an ultrasound image and trimode spectroscopic data. P-values from unpaired T-tests between PpIX fluorescence from various groups are presented in Figure 6.25. These were seen to be below 0.05 in all cases. The “no ALA” case reported is from pre-injection imaging. Large intragroup variability is seen within in vivo measurements, 117.6%, 72%, 80% respectively in the 4-hrs post-ALA, 24-hrs post-ALA and no ALA cohorts.

Figure 6.25 Boxplots showing PpIX fluorescence signal measured from 3 cohorts.

6.8.4 Ex Vivo validation assays

Tumor, skin, and muscle tissue were removed after euthanizing each animal and tumors were cut into 1mm sections for ex vivo analyses. Imaging was performed on the GE Typhoon fluorescence scanner immediately after tissue resection, to provide a reference of tumor fluorescence intensity for each mouse. The Typhoon scanner
provides flatbed scanning with excitation at 633 nm and a 650 nm long-pass fluorescence emission filter to provide estimates of PpIX fluorescence intensity. It should be noted that this scanner does not have the facility to separate autofluorescence and hence the resulting signal intensities are integrations over all fluorescence emissions from the tissue samples. All tumor slices were fixed in paraffin and stained with hematoxylin and eosin for histological examination. Results from measurement made on the Typhoon flatbed scanner are summarized in Figure 6.26. A sample slide showing tumor slices and additional tissue can be seen in figure Figure 6.26b. The fluorescence signal was calculated by integrating the mean signal intensity across all slices of a single tumor. Sample H&E image of slices labeled 1 and 2 are shown in Figure 6.26c and Figure 6.26d. A large amount of variability in malignancy and vasculature can be seen. The corresponding fluorescence images show large spatial variability in fluorescence signal suggesting that tissue variations may contribute to most of the signal variability.

Figure 6.26 Ex vivo assays performed on tumors show large intra-tumor variability. a) Comparison of ex vivo fluorescence measurements using the Typhoon scanner. b) Sample image from a scan of tumor slices from one animal and H&E images corresponding to
slices ‘1’ and ‘2’ are shown in c. and d. High fluorescence signals appear to correspond to regions with blood vessels, and tumor boundaries. Blue and yellow arrows are used as markers to identify landmarks on typhoon and H&E images.

6.9 Expansion of USFT_v2 to Multi-Spectral Capabilities

The above system was adapted to include an 785 nm laser diode and 800 nm detection filters (FEL800, Thorlabs, NJ) that worked alongside the on board 633 nm laser and 655nm filters. These combinations have been upgraded to operate together but alternately to acquire dual-channel fluorescence tomographic data. We expected to use the LN-MCI method described by Tichauer et al.\textsuperscript{53} to estimate tumor burden on lymph nodes. The USMFT was used to measure uptake of two fluorescent dyes Methylene Blue and IRDye800CW from a mouse forepaw to axillary nodes. Figure 6.27 shows an ultrasound image from a murine axillary lymph node.

![Ultrasound image of an axillary murine lymph node (left). Photograph of the exposed node marked by the arrow (right).](image)

Fluorescence measurements were made using the tomographic fiber-probe, however we found that it was challenging to maintain contact between the fibers and the axillary region, and only 2 pairs could be placed on the skin. The pressure from the ultrasound transducer is different from that of the fibers, and owing to the small size of the lymph nodes (~2 mm longest diameter), they could easily slide under and be missed by the fibers. Ultrasound images could thus not be coregistered to fluorescence.
measurements. Spectral data from one source-detector pair is show in Figure 6.28. The 633 nm and 785 nm lasers were sequentially excited, and respective excitation and emission spectra are shown.

![Fluorescence from Mouse LNs](image)

Figure 6.28 Spectra from 633 nm and 785 nm excitation of a murine lymph node bearing methylene blue and IRDye800CW.

The complexity of the small animal model and the inability to ensure that fibers were probing the lymph node in question, prevented us from further testing this system. However, it does have the ability to image subsurface fluorophores, and can be tested in larger animals where the lymph nodes may not be as prone to movement. Studies described in previous chapters showed more promise and were explored further, while the testing of this system was discontinued.

### 6.10 Discussion

This chapter presents the instrumentation and validation of a combined high frequency US-guided reflectance-mode-FT system, with spectral fitting to decouple multiple signals and novel use of white light spectroscopy to inform optical models for accurate reconstruction of regional, subsurface fluorescence yield in vivo. The data show that the US-guided spatial priors are critical for accurate regional fluorescence...
reconstruction. FT approaches that focus on diffuse recovery exclusively are often limited by an ill-posed problem of estimating both magnitude and spatial location of fluorescence origin within the sampled medium. The benefit of using US images to provide prior spatial information to overcome this limitation is made obvious in Figure 6.16 and Figure 6.17. The non-region-segmented problem is highly under-determined and the reconstruction algorithm cannot yield accurate spatial maps of subsurface fluorescence. Standard reconstruction typically yields sparsely distributed hot spots surrounded by relatively low values. When the reconstruction is constrained to a few homogeneous regions, the problem is no longer ill-posed and yields a more-accurate regional fluorescence yield, with the trade-off that spatial resolution is reduced to a few homogeneous regions.

The data also underscore the benefit of white light spectroscopy and the necessity for knowledge of optical properties for accurate fluorescence tomographic reconstruction. The perturbative influence that variations in background optical properties have on the reconstructions of raw fluorescence spectra is shown in Figure 6.13. While it is possible to estimate a linear metric of fluorescence from reconstructions with assumed background optical property values, the overall reconstruction accuracy of fluorescence estimates are improved significantly when sample-specific optical properties are measured by white light spectroscopy and used to inform reconstruction models. This improvement in accuracy results in reduction in the normalized standard deviation of reconstructed fluorescence yield by a factor of ∼2 across varying phantom optical properties and reduction in reconstruction fitting errors by a factor of ∼9. In many imaging applications, the region of interest may be pathologically altered and thus tissue optical properties may differ from values expected in normal tissue. The data in this
study suggests that incorporation of white light spectroscopy to determine these bulk properties will significantly reduce fluorescence reconstruction errors in vivo.

While measurements in tissue-simulating phantoms showed high accuracy of measurement using hard spatial prior reconstructions based on ultrasound images, obtaining accurate measurements on preclinical animal models was significantly more challenging. The sliding mechanism of the coupled probe-head had a tendency to push the subcutaneous tumor/inclusion as the transducers were switched. This is not so much of a problem if the tumor is flush with the surrounding skin, but is a problem when the tumor has a high surface curvature. Using a matrigel inclusion-based model we found that in such circumstances, FT-reconstructions were not significantly better than spatial excitation-normalized fluorescence measurements using ultrasound data to inform the inclusion depth. This approach was extended to measure ALA-induced PpIX production in a subcutaneous human xenograft-skin cancer murine model. Estimated PpIX concentration was significantly higher at 4-hours post-ALA administration as compared to 24-hours post administration and prior to ALA administration, thus demonstrating that PpIX production was quantifiable, and that PpIX production peaked in the tumor at about 4 hours post ALA-injection. This study was complicated by the high PpIX production in mouse skin upon ALA administration (Figure 6.26b show high skin fluorescence signal), preventing measurements through the skin, i.e. tumors had to be surgically exposed prior to imaging.

After USFT_v2 system was extensively tested, and linear correlations were seen between real and measured fluorophore concentrations in tissue phantoms and in vivo matrigel inclusion phantoms, it was expanded to multispectral capabilities of measuring ~650 nm and ~800 nm fluorescence emissions, with the goal of using tracer
combinations to ratiometrically quantify metastasis in lymph nodes\textsuperscript{53}. However, the complexity of the small animal model and the inability to ensure that fibers were probing the lymph node in question, prevented us from further testing the upgraded USMFT system. It however, does have the ability to image subsurface fluorophores, and can be tested in larger animals where the lymph nodes may not be as prone to movement as in mice and rats. Studies involving imaging approaches described in other chapters of this thesis showed more promise and were explored further, while the testing of this system was discontinued. In order to account for the inherent delivery variability in the lymphatic system, measurements may need to be made in the afferent lymph vessels. The inability to detect lymph vessel on the ultrasound image, and the use of a 2-D tomography instrument, with a single linear-array of sources and detectors, prevents simultaneous measurements on nodes and vessels, but a high-speed 3-D tomography instrument would be more suitable system.

6.11 Conclusions

This study provides a new hybrid imaging/optics approach to region-based molecular imaging of subsurface targets \textit{in vivo}. The presented data demonstrates that ultrasound-guided fluorescence tomography (USFT) can be employed to determine region-based fluorophore concentration at greater depths than standard surface-imaging techniques, for tumor detection and delimitation. The method presented here combines tissue spatial information (US) with an array of diffuse optical samples to reconstruct fluorescence yield in tumor and background regions. Fluorescence spectral fitting is used to isolate the fluorophore of interest from autofluorescence and white light spectral fitting is used to accurately inform the optical models for reconstruction. Expansion to
imaging multiple fluorophore capabilities enabled ratiometric tools for quantification of tumor burden in lymph nodes. However, there was a need to study the underlying mechanisms of nodal metastasis, and the effects of high heterogeneity and variability on imaging outcomes, owing to which further testing of the USMFT system was suspended.
CHAPTER 7  Fluorescence Snapshot Paired agent imaging of lymph nodes

This chapter describes a single fluorescence image-based quantitative metastasis detection method, applied such that tracers were injected peritumorally, much like the current clinical protocol.

7.1 Introduction

Although NIRF-based techniques for nodal staging are growing in use in node mapping, there is still need for surgical resection and pathological testing which is invasive and time consuming, possibly requiring follow-up surgeries. Any preoperative imaging approaches would also need intraoperative imaging and coregistration of preoperative imaging data\textsuperscript{42}. As such, there is need for intraoperative imaging approaches than can simultaneously map and diagnose lymph node involvement, and thus go from metastasis mapping to ‘metastasis sensing’. Antibody-based cancer-targeted imaging has been investigated for several years and Sampath \textit{et al.} provide a detailed review\textsuperscript{52} of antibodies conjugated to NIR fluorophores used in assessment of nodal involvement. Antibodies are beneficial as they are preferentially taken up by the lymphatics and have long lymphatic transit times enabling localization. However most studies using antibodies administer tracers at a distal injection site\textsuperscript{183} or intravenously\textsuperscript{184} rather than peritumorally\textsuperscript{185, 186}. Any imaging is usually performed 16-24 hours after injection, thus limiting the scope of their use in enabling mapping of lymph nodes, and potentially missing nodes resulting in high false-positive rates. Several animal studies demonstrate
utility of targeted antibodies and peptides in identifying node involvement, but very few
attempt to perform peritumoral injections and simultaneously map the tumor-draining
nodes as well as detect metastasis using quantitative imaging. The most commonly used
intravenous administration route requires use of non-tracer doses of imaging agents, and
regulatory approvals further prevent clinical translation. Peritumor injection on the other
hand has been shown to have superior mapping efficiency, and has been shown to be a
suitable route for administration of targeted antibodies for imaging studies, with high
sensitivity in detecting cancer presence in nodes\textsuperscript{185,186}. Furthermore, the high variability in
uptake and non-specific retention confound results. Tichauer et al showed that number
of tumor cells in lymph nodes correlated with epidermal growth factor receptor
concentration imaged using a dual-tracer combination of a targeted and an untargeted
fluorescent tracers; but that a single tracer imaging would not be a reliable metric of
nodal involvement\textsuperscript{53}. Tracers were administered by an interstitial injection in the animal
paw to highlight nodes of the axilla. As described earlier, this method while enabling
efficient dye delivery to potentially involved nodes, can easily miss the tumor draining
sentinel nodes, but due to animal model limitations this effect may not be apparent in
small animals.

In this chapter we apply the approach validated by Tichauer et al.\textsuperscript{53}, to a more
clinically relevant setting involving peritumoral injection, to identify tumor draining
lymphatic vessels, and the nodes with highest likelihood of metastasis using a
combination of targeted and untargeted tracers. We used EGFr-targeting Erbitux-
IRDye800CW Conjugate and a non-targeted isotype control Mouse IgG-IRDye680RD
conjugate. Using two-channel fluorescence imaging and temporal intensity
measurements, we propose a metric for metastasis prediction based on both the node
and vessel signals from both tracers. We found that using such an approach, decisions about node involvement can be made within 2 hours of injection, and potentially data from each time point can be used without prior measurements as long as the afferent lymph vessels are detectable.

7.2 Materials and Methods

In this study lymph node metastasis was produced in mice after implantation of an EGFr-overexpressing breast cancer cell line and a round of resection surgery. Metastasis presence in the nodes was detected and measured by peritumorially injecting a pair of fluorescent imaging agents, one that targets tumor cells and one that behaves as a non-tumor specific control, and tracking their uptake in nodes and lymphatic vessels.

7.2.1 Cancer Metastasis Model

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at Dartmouth College under approved protocols. Bioluminescent human breast cancer cells from MDA-MB-231-luc-D3H2LN (PerkinElmer, Waltham, MA) were cultured at 37°C in high-glucose Dulbecco’s Modified Eagle Medium (HyClone® SH30243.01, Fisher Scientific, Pittsburg, PA) supplemented with 10% fetal bovine serum (HyClone® SH30910.03, Fisher Scientific), and penicillin-streptomycin (#30-002-CI, Cellgro, Mediatech Inc., Manassas, VA). A total of 24, 6-8 weeks old female athymic nude mice (Crl:NU(NCr)-Foxn1nu, Charles River Laboratories, Wilmington, MA) were used in this study. Animals were prepped for surgery, and 1 Million cancer cells in a phenol-red free growth-factor reduced matrigel medium (#356230, Corning Life Sciences, Corning NY) in a total volume of 50 µl were injected into the exposed right inferior mammary fat pad using a 27-gauge syringe needle.
Animals were sutured, and tumors were allowed to grow for ~4 weeks, after which they were surgically resected. The mice were then retained for 6-10 weeks to allow for growth of metastasis in the regional lymph nodes. This cell line has already been shown to metastasize to the axillary nodes in a murine model\cite{160}. Bioluminescence imaging (described in Chapter 4) was performed to track progression of metastasis. In some cases, tumor regrowth was observed at the resection site. Animals from the “normal” cohort (N = 9) were those that did not show detectable presence of metastasis in the imaged lymph node by secondary validation methods described below.

### 7.2.2 Fluorescent Imaging Agent Preparation

The labeling procedures for the targeted and untargeted tracers were identical to those described in\cite{53}. The EGFR-specific antibody, Erbitux (clinical grade from Bristol-Myers Squibb, Princeton, NJ) was labeled with the NHS ester form of IRDye-800CW (LI-COR Biosciences, Lincoln, NE), and mouse IgG (Athens Research and Technology, Athens, GA), an untargeted isotype control of Erbitux, was labeled with the NHS ester form of IRDye-680RD (LI-COR Biosciences, Lincoln, NE). Labeled solutions were concentrated to µM concentrations. The antibodies were initially diluted in phosphate buffer solution (PBS) before being run through a 5-ml polyacrylamide desalting column to remove Sodium Azide. With the antibodies in 500-µl aliquots, 50 µl of 1 M sodium bicarbonate solution was added to increase the pH of the solution for optimal binding to the fluorophore as per manufacturer instructions. The fluorophores were diluted in DMSO and added to the antibody aliquots in a 3:1 dye-to-antibody ratio. These solutions were then left to mix on a magnetic stir plate for 2 hours at room temperature. The dye-antibody conjugates were then separated from unassociated antibody and
fluorophore with a desalting column using PBS to flush. Presence of the conjugate was confirmed in the resulting aliquots with a fluorimeter.

Figure 7.1 Figure shows a summary of injection and imaging performed in this study. a) Bioluminescence imaging following administration of Luciferin, demonstrating the expression of luciferase in tumor-regions, i.e. the mammary fat pad primary tumor and metastasis in axillary node. b) injection of dyes was performed peritumorally after exposing the tumor and region lymph nodes. c-d) show the uptake of the two fluorescent tracers overlaid on white-light grayscale images, and lymphatic dye uptake is seen.

7.2.3 In vivo Bioluminescence Imaging

The chosen cell line has been transfected with the Luciferase gene. The presence of luciferase in only the tumor site allows for the bioluminescence emission upon breakdown of Luciferin. The cancer bioluminescence was imaged to confirm cancer presence in nodes, using Luciferin (#88294 Thermo Scientific) administration, prior to fluorescence imaging using the Xenogen VivoVision IVIS bioluminescence system (Perkin Elmer, Waltham, MA). Primary tumor sites were covered using black shrouds during the imaging process to prevent signal saturation from them. Presence of bright spots in the axilla were noted and reported as potential metastasis candidates.
**7.2.4 In vivo Fluorescence Imaging**

All animals were maintained on a fluorescence-free diet for 3 – 5 days before imaging. Prior to imaging, each animal was anaesthetized using 1.5 – 3% isofluorane (Piramal, Bethlehem, PA) in 1.5 L/min oxygen. It was then placed on the imaging bed in supine position; shown in Figure 7.1. A superficial longitudinal incision was made along the midline of the body from the top of the thorax to the midpoint of the imaginary line joining the lower-most nipples on either side of the body; the skin on the right side of the body (tumor-site) was peeled back to expose the tumor-site, the lymphatics and blood vessels draining it, and the axillary nodes. Next, a combination of 0.05 nanomoles of Erbitux-IRDye800CW conjugate and 0.05 nanomoles Mouse IgG-IRDye680RD conjugate were injected in 10 µl volume of PBS using a 27-gauge needle into the peritumor tissue. When tumor recurrence was absent, injection was performed into the inner membrane, near the primary tumor implantation site. The lymphatic capillaries that drain the tumor take up the antibody tracers and pool them into the lymph vessels that are present on the under side of the skin and lead up to the axillary nodes\(^\text{187}\). All fluorescence imaging was performed using a Pearl® Impulse system (LI-COR Biosciences, Lincoln, NE) which provides planar surface images in the near infrared band. The system was set up to acquire grayscale white light, 700 nm channel images, and 800nm channel images at 1 frame per minute each for 120 minutes. The 700 nm channel setting uses a 685 nm laser excitation and a 720 ± 20 nm bandpass emission filter, and the 800nm channel setting uses a 785nm laser excitation and detects at about 820 ± 20 nm. Nodes that appeared to take up fluorescent tracers were excised, flash frozen in liquid nitrogen, and saved for further testing in a -80°C freezer.
7.2.5 Quantitative PCR for secondary detection validation

The presence of luciferase gene expression in the breast-cancer xenografts was analyzed using real time PCR to estimate tumor presence, as mice do not express the luciferase gene naturally. Total RNA was isolated from flash-frozen lymph nodes using the RNeasy mini kit and Qiashredder (Qiagen, Venlo, Netherlands), and 18-gauge needles were used to homogenize the tissue. To assess gene expression levels, cDNA was prepared from lymph node RNA using the Superscript III First Strand cDNA Synthesis Kit and oligo dT primers (Life Technologies, Grand Island, NY). Reactions (25 µl) were set up using 2× iQ Supermix (Bio-Rad, Hercules, CA), pre-validated Luciferase primer and probe set (Assays on Demand, Applied Biosystems, Life Technologies), and equivalent amounts of template cDNA. Quantitative real-time PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad). Each sample was assayed in duplicate, and 18s rRNA served as the endogenous control for normalization. The expression levels for each gene were calculated relative to a standard curve of known tumor cells added to naïve lymph nodes. For the standard curve, 10, 100, 1,000, 10,000, and 100,000 were added in quadruplicate.

7.2.6 Image Data Analysis and Statistical Tests

The 22-bit images from the Pearl imager were read using Bio-Formats plug-in (LOCI, University of Wisconsin-Madison) and analyzed using a combination of ImageJ and Matlab (Mathworks, Natick, MA) software. Fluorescence measurements were made on temporal fluorescence images by calculating the mean signal intensity from regions-of-interest over lymph nodes and lymphatic vessels. Although uptake data for every minute from the injection time to node excision were recorded, only images from a single time point at ~90 minutes were used so that clinical translation of such an
approach can be validated. The mean signal from an ROI measurement off the node and lymphatic vessel was used to perform background subtraction at \(\sim 90\) minutes. All statistical analysis was performed using Minitab 17 Statistical Software. Measurements on normal and metastasis-bearing nodes were tested for to determine whether or not the data were normally distributed using the Ryan-Joiner test. For testing for statistical significance of results, when the normality assumption was valid, Student’s t-test was performed between the normal and metastasis-bearing node measurements; Mann-Whitney test was performed when the normality assumption was invalid.

Figure 7.2 Tracer uptake seen over time in lymph nodes (solid), and lymphatic vessels (dashed). a–d show data for nodes bearing metastasis, and e–h show data from normal nodes.

7.2.7 Measurements based on Uptake Data

As described previously by Tichauer et al.\textsuperscript{53}, the ratio of targeted tracer uptake to that of the untargeted tracer uptake, can serve as a surrogate to binding potential measurement. We found that the assumption that tracer uptake in the delivery system, i.e. the lymphatic vessels is not identical for the tracer pair injected at the peritumoral site, and hence adopted a deconvolution-based model to account for the differences as
described in detail by Tichauer et al. The deconvolution approach was described in detail in Chapter 5 in Section 5.2.1, and was used here to correct the uptake differences and corrections were applied to to the uptake of untargeted tracer in the vessel \( V_{UTar} \) and lymph node \( N_{UTar} \), and the corrected curves are labeled \( \bar{V}_{UTar} \) and \( \bar{N}_{UTar} \) respectively. This approach does require the use of the full time course of fluorescence uptake, and is not ideal for clinical use. Results with and without the deconvolution correction are presented in the subsequent sections.

7.3 Results

The uptake of targeted tracer (Erbitux–IRDye800CW Conjugate) and untargeted tracer (Mouse IgG–IRDye680RD conjugate) from the injection site into the tissue near the tumor to the axillary nodes through lymphatic vessels was studied using measurements made on temporal image data using ROIs. Figure 7.2 shows examples of agent uptake through nodes and vessels for both tracers. While there is similarity in shapes of the vessel uptake curves, there is obvious subject-to-subject heterogeneity. Measurements made on a tube containing the injected tracer combination, produces fluorescence signal intensities of \( \sim 17 \) and \( \sim 5.5 \) in the 700 and 800nm detection channel respectively, resulting in 800:700 channel ratio of 1:3. This explains the overall intensity scale difference between the uptakes of the tracers shown in Figure 7.2, however subtle differences do still exist in the intensities, as the tracers do not have the same lymphatic kinetics.

In order to detect the presence of cancer metastasis in the axillary node two validation techniques were used, the primary technique was in vivo bioluminescence imaging and the secondary method was qPCR-based estimation of tumor cells in the
lymph node as described previously. A lymph node was categorized as bearing a “tumor” based on both the presence of bioluminescence signal and a positive qPCR finding. Nodes that presented as cancer-bearing in one validation method and not the other were excluded from the study.

The fluorescence intensities of the targeted tracer $N_{\text{Tar}}$ in the tumor nodes at the 90-minutes time point after dye administration was found to be $0.014 \pm 0.01$ units (Mean $\pm$ Standard Deviation), and $0.0114 \pm 0.0188$ in the normal cohort, with no significant differences between the groups ($p = 0.74$, 2-tailed Mann Whitney test). Boxplots corresponding to these data are shown in Figure 7.4a. A similar spread can be seen in Figure 7.4b for $V_{\text{Tar}}$, the targeted tracer fluorescence signal in the afferent lymph vessel feeding the axillary nodes (normal: $0.013 \pm 0.0094$, tumor: $0.012 \pm 0.011$, $p = 1$, two-tailed Mann Whitney test).

The untargeted tracer uptake $N_{\text{Utar}}$ in the nodes of interest were found to have no significant differences between normal and tumor-bearing cohorts. (Figure 7.4d, normal: $0.028 \pm 0.014$, tumor: $0.037 \pm 0.022$, $p = 0.1828$, Mann-Whitney Test). Similar trend was observed in the corresponding vessel signal (Figure 7.4e, normal: $0.03 \pm 0.033$, tumor: $0.05 \pm 0.037$, $p = 0.0563$ Mann Whitney Test, $p = 0.123$ Student’s t-test).

In an approach similar to the LN-MCI approach as described by Tichauer et al.\textsuperscript{53} we used the early time point data at 90 minutes post injection, and looked at the “normalization-free” targeted-to-untargeted tracer ratio in the nodes, $\frac{N_{\text{Tar}}}{N_{\text{Utar}}}$ (Figure 7.4g, normal: $0.239 \pm 0.146$, tumor: $0.343 \pm 0.299$), and could not see any significant effect on this ratio from tumor burden ($p = 0.34$, Student’s t-test). This follows logically from the differences seen between the deliveries of the two tracers through the lymphatics,
causing any specific uptake of the Erbitux–IR-Dye 800CW conjugate to be obscured by non-specific retention.

Under the assumption that the source of the variability in node uptake is in major part from the variability in delivery via lymphatic vessels, we hypothesized that differences between the normal and cancer-bearing nodes should appear in the node signal normalized by the vessel, \( \frac{N_{\text{Tar}}}{V_{\text{Tar}}} \) (Figure 4c, tumor: 1.02 ± 0.237, normal: 1.01 ± 0.32, \( p = 0.97 \), Student’s t-test). We however did not see any significant differences between the two cohorts. Looking at the fluorescence ratio for the untargeted tracer \( \frac{N_{\text{Utar}}}{V_{\text{Utar}}} \) (Figure 7.4f, normal: 1.13 ± 0.034, tumor: 0.842 ± 0.28, \( p = 0.03 \), Student’s t-test) we observed statistical significance in the difference between the groups. We discuss both these observations in detail in the ‘Discussion’ Section.

![Figure 7.3](image)

Figure 7.3 Targeted tracer uptake, and deconvolution corrected untargeted tracer uptake is shown for few animals. Vessel- dotted and open circles, Node- solid line and filled circles.

In order to account for the perceived differences in delivery, we applied deconvolution correction. Figure 7.3 shows examples of the deconvolution corrected untargeted tracer uptake. The “corrected” untargeted tracer uptake in the vessel \( V_{\text{Tar}} \) traces the targeted tracer uptake in the vessel \( V_{\text{Tar}} \). Applying this correction the to untargeted signal in the node, we get \( \tilde{N}_{\text{Utar}} \). The average ratio of targeted to untargeted tracer from 20 to 90 minutes was calculated and compared for the two groups (normal: 0.98 ± 0.2035, tumor...
= 1.1496 ± 0.232, p = 0.09), and was found to not bear statistically significant differences between cohorts. This may be for a large part due to the early time point of imaging and complications from the presence of unbound targeted tracer in the tumor nodes.

Figure 7.4 Boxplots showing comparisons of various uptake parameters in lymph nodes and vessels, between tumor and normal cohorts.

For the above approach to be clinically relevant there is a need for single-time point based identification of normal and tumor-positive nodes. Using the vessel signal as normalization factors, the ratio of targeted to untargeted node signals normalized by the
corresponding vessel signal \( \frac{N_{Tar}/V_{Tar}}{N_{UTar}/V_{UTar}} \) revealed statistically significant difference between normal and tumor-positive cohorts of lymph nodes (tumor: 1.284 ± 0.376, normal: 0.909 ± 0.24, \( p = 0.014 \) by Student’s T-test).

Based on the presented results it is evident that two parameters, (i) vessel normalized untargeted tracer node signal \( \frac{N_{UTar}}{V_{UTar}} \) and (ii) vessel normalized targeted over untargeted node ratio \( \frac{N_{Tar}/V_{Tar}}{N_{UTar}/V_{UTar}} \) can be used to distinguish between normal and tumor bearing lymph nodes. Furthermore, ROC analyses on a simple binary threshold-based classifier, (Figure 7.5 e,f) reveal that both (i) and (ii) have similar areas-under-the-curve of 0.75. For maximum accuracy (79.17% for both), threshold levels of 0.9051 and 0.817 respectively were identified. At these threshold levels, (i) and (ii) showed sensitivities of 86.67% and 100% and specificities of 66.67% and 44.44% respectively. Positive-predictive values were found to be 81.25% and 75%, negative-predictive values of 66.67% and 100%, and showed diagnostic odds ratios of 13 and \( \infty \) respectively. Both of these perform much better than all other classification metrics shown in Figure 7.5 a-d.

7.4 Discussion

Tichauer et al. described an approach to perform molecular concentration imaging in lymph nodes to identify tumor burden and nodal involvement in a murine model using a distal injection site for tracer administration. This approach worked excellently and showed sensitivity down to 200 cancer cells per node\(^53\). However, some of the limitations included the use of a distal injection site, long wait time after injection and the need for normalization of measured signal using an early time point during tracer uptake.
The goal of the work presented in this paper was to expand Tichauer's work to a more clinically appropriate setting and identify its successes, challenges, and hurdles. The key motivations behind peritumoral tracer administration are (i) the need to combine the mapping and cancer detection methods, (ii) similarity to current clinical protocol using blue dyes, and (iii) evidence of restructuring of lymphatics in response to tumor metastasis. The first point is intuitive and easy to appreciate and the second point

Figure 7.5 ROC Analyses of the various comparative parameters for a binary classification of metastasis-bearing from normal nodes.
follows logically. These potential advantages of peritumoral tracer administration can also provide an easy tracer approval pathway as the clinical protocol would need to be modified insignificantly, thus allowing clinicians to identify the direct impact and benefits of peritumoral tracer administration for mapping and metastasis assessment over qualitative visible or fluorescent dye-based node mapping.

Figure 7.6 Fluorescence images from uptake of Mouse IgG before (left) and after (right) injury and inflammation in a mouse paw.

With respect to (iii), Swartz et al. have published extensively on the changes in the lymphatic system during cancer metastasis and inflammation and have detailed the mechanisms behind this in several papers\(^\text{188, 189}\). Kwon et al. have also presented evidence that tumor-draining lymphatics undergo changes in response to tumor proliferation and metastasis that could be visualized using fluorescence imaging\(^\text{190}\). Figure 7.6 shows anecdotal evidence of modification in lymphatic drainage pathway in response to inflammation in the paw cause by repeated injections over the course of 3 weeks. While this is not evidence that such an advanced effect occurs in response to tumor proliferation, it serves to show that the lymphatic architecture undergoes dynamic physiological changes under disease conditions. Several studies from the Sevick-Muraca lab\(^\text{191, 192}\) have demonstrated that broad-beam imaging of subsurface lymphatic
architecture and its propulsion using Indocyanine Green as a contrast agent, thus establishing the feasibility of imaging lymph nodes and vessels in humans.

Through our imaging study of lymph nodes and their afferent lymph vessels, we found that using node fluorescence signals and lymph vessel fluorescence signals independently is unreliable, and we need to treat them as coupled measurements. The data show that neither untargeted tracer signal nor targeted tracer signal in the lymph nodes have sufficient power to predict that status of nodal involvement, owing to the high delivery heterogeneity we discussed in a previous publication\textsuperscript{144} and earlier in this thesis. After incorporating the afferent vessel signal at the 90 minutes post injection time point as a normalizing factor to the node measurement, it was seen that the tumor-bearing nodes showed a reduction dye uptake relative to the normal lymph nodes. This can be seen in the node to vessel ratio $\frac{N_{UTar}}{V_{UTar}}$ (median value $\sim 1$ in normal nodes). The combination of reduced dye transport in the node and dye buildup in the vessel due to obstructions within the node likely contributes to this effect, and significant differences (a significance level of $\alpha = 0.05$ was defined) were observed between tumor-free and tumor-bearing lymph nodes. The vessel-normalized node signal from the targeted tracer, $\frac{N_{Tar}}{V_{Tar}}$ was also investigated, but it failed to show any similar trends. The median values of measurements in both normal and tumor-bearing nodes were close to 1. This is expected as the differences in the tracer transport through the nodes is now confounded by the effect of binding between Erbitux and epidermal growth factor receptor (EGFr), that is known to be overexpressed by the cancer model used in this study. Tichauer et al. have previously demonstrated that Erbitux can be effectively used to study the binding effect on the metastasis-bearing lymph nodes produced using the cancer model studied\textsuperscript{53}. 
We attempted to evaluate nodal involvement independent of vessel signal, using only the measurements from the uptakes of both tracers in the nodes of interest, and found that the normalization-free targeted tracer to untargeted tracer ratio was unsuccessful in revealing any differences in uptake and retention between normal and metastasis-bearing nodes. This is due to the fact that uptakes of the chosen tracers were not equivalent in the animal model we studied, when administration was done peritumorally. The Tichauer paper\textsuperscript{53} relied on the similarity of tracer delivery in the distal injection approach they used, and one of the goals of this chapter was to study the feasibility of the LN-MCI approach in pertiumoral administration. While we do not have conclusive evidence as to why the difference in delivery exists in this study, we hypothesize that the high EGFr concentration (attributable to upregulation of EGFr concentration by the tumor cells) near the injection site may be leading to trapping of injected Erbitux, thus resulting in a differences in its uptake into the lymphatic collector vessels. Differences between shape of Erbitux–IRDye800 and Mouse IgG–IRDye680 were in fact observed in this study, indicating that injection site does influence the lymphatic uptake. A deconvolution-based approach was used to account for the pharmacokinetic differences between tracer deliveries using lymphatic vessels as reference tissues but targeted over untargeted tracer ratio measurements post deconvolution-correction did not reveal significant differences. This may be in part due to the high sensitivity of the deconvolution approach to noise in the signals and the fact that the approach is valid only when tracer transport has attained steady state\textsuperscript{166}.

The metric that we found to have the best distinguishing ability (based on p value, and diagnostic odds ratio) was the vessel-normalized targeted over untargeted tracer ratio (VNTR). Figure 7.6 graphically demonstrates the steps in the calculation of this
parameter. VTNR successfully incorporates the contributions of delivery differences and receptor binding to enable classification of a lymph node as normal or tumor bearing. This metric is advantageous as it can potentially be used to make decisions about nodal involvement based on only single time point measurements, thus enabling snapshot imaging-based metastasis mapping and diagnosis (See Figure 7.7). This approach does however have its limitations. Owing the leaky nature of vasculature supplying and draining tumors\textsuperscript{193}, we observed high background signal from dye presence in the capillary beds. This effect prevented measurements at time points later than 100-minutes post injection administration, and hence we do not have reference data to establish a direct comparison to the results presented by Tichauer et al using a similar tracer combination but a distal injection site\textsuperscript{53}. Plasma excretion curves for Erbitux (Cetuximab) presented by Sexton et al. indicate that the tracer remains in the plasma for >11 hours post administration\textsuperscript{168}; therefore once the tracers are in the plasma, signal contrast between the lymphatics and the background drops, and reliable measurements on the nodes can not be made. We empirically saw that up to 90-100 minutes post injection this effect of leakage into the plasma was not significant, but this may vary with dye administration techniques, distance of the sentinel nodes from the primary tumor, tumor type and stage etc. Furthermore, the complex approval process of diagnostic tracers for human use would restrict the testing and adoption of the proposed approach.

Nevertheless, the changing scenario in fluorescence-guided surgical intervention is paving the way for quicker deployment of novel quantitative imaging techniques that will help accelerate the testing of approaches similar to ours. The results and data from this study suggest that there may be potential for tracers with different lymphatic uptake as long as they remain in the lymphatics at the time of imaging and sufficient contrast-to-
background ratio is available. ICG being the only approved NIR fluorophore may serve as a non-specific reference tracer with a target specific tracer conjugated to a short wavelength far-red tracer. With the growing availability of imaging systems designed for multi-fluorophore imaging, clinical testing of the proposed (or similar) quantitative ratiometric approaches may very well be around the corner.

![Figure 7.7 Summary of the snapshot fluorescence imaging technique for nodal staging.](image)

### 7.5 Conclusions

This paper demonstrates a snapshot based quantitative imaging approach using the ratio of targeted to untargeted tracer uptake in nodes, each normalized by the corresponding afferent lymph vessel uptake signal to identify differences between normal and metastasis-bearing nodes. The injection site affects use of the cancer-specific tracer especially when the site is in near a tumor, and the untargeted tracer on its own was unable to successfully account for variability. On the other hand, the use of an internal normalization method based on afferent lymphatic vessels could account for inherent uptake variability, revealing the differences in lymph flow in normal and metastasis-bearing lymph nodes, and the use of cancer-specific tracer further bolstered the positive discriminatory power of the test in identifying normal from cancer-bearing...
nodes. There is however need to further test this approach and compare it against imaging targeted tracers with long wait times (~24 hours) before we can conclusively discuss its diagnostic potential in comparison with clinically-validated gold-standard methods. Although, the presented results are still far from providing an estimate of the presence of macro- and micro-metastases, they appear to be promising in identifying tumor-free nodes.
CHAPTER 8  Study of Biphasic Uptake within the Lymphatics: Comparing small and large molecular transport

Peristaltic propulsion occurs within the lymph collector vessels due to presence of smooth muscles and valves. The initial lymphatics lack this and passively deposit fluid and dye into the collectors based on the pressure and concentration gradients (described in detail in Chapter 2). Using the Pearl Impulse Imager in “fast” mode we could see the pumping action. However, it is challenging to separate pumping from noise and corruption from nearby structures. Lymphatic flow in the vessels is driven by diffusion, pressure, and lymphatic contractions of the smooth muscles lining the collector lymph vessels. The molecular size, shape, charge, and affinity to proteins in the lymph influence the diffusive uptake of molecules at the injection size into the lymphatics, and hence affect the overall tracer kinetics.

![Diagram of molecules](image)

Figure 8.1 Approximate size comparison of MIgG–680RD and Methylene Blue associated with Albumin (not to scale). MB owing to its low affinity to Albumin exists as both free dye and MB:HSA complex.
In this study we observed the difference in transport of the smaller Methylene Blue (MB) molecule, which may weakly and reversibly associate with protein Albumin in the lymph (Association constant, $K_a = 4.012 \times 10^4$ M$^{-1}$ at 298K$^{151}$, and the larger Mouse Immunoglobulin G (MIgG) conjugated to IRDye 680RD. We identified an active and passive component of uptake through the collector lymphatic vessels, that has been identified in earlier reports$^{73,74}$, and studied how dye size affects them. Figure 8.1 shows the fluorescent molecular tracers used. As described previously in Chapter 4, the small size of MB (~300 Da), combined with its low affinity for albumin in lymph$^{153}$ makes its movement through the lymphatic system very swift. MB likely associates with BSA in the interstitium after administration, but it is the small free dye molecules that rapidly flow through the lymphatic vessel.

Figure 8.2 Uptake of Methylene Blue from the injection site in the paw to an axillary lymph node (white arrow).
8.1 Methods

8.1.1 Tracer Preparation

Two near-infrared fluorescent tracers were used and their lymphatic drainage through murine forepaw and limb were studied and compared. The tracers used were Mouse IgG Isotype control antibody labeled with IRDye680RD and Methylene blue (Sigma Aldrich). The labeling procedures for the former is described in. The antibody Mouse IgG (Athens Research and Technology, Athens, GA), was labeled with the NHS ester form of IRDye-680RD (LI-COR Biosciences, Lincoln, NE). With the antibodies in 500-µl aliquots, 50 µl of 1 M sodium bicarbonate solution was added to increase the pH of the solution for optimal binding to the fluorophore as per manufacturer instructions. The fluorophore was diluted in DMSO and added to the antibody aliquots in a 3:1 dye-to-antibody ratio. The final solution was then left to mix on a magnetic stir plate for 2 hours at room temperature. The dye-antibody conjugate was separated from free antibody and fluorophore with a desalting column using PBS to flush. Presence of the conjugate was confirmed with a fluorimeter.

8.1.2 In vivo fluorescence imaging of collector lymphatics

All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at Dartmouth College under approved protocols. A total of 13, 8 – 10 week old athymic nude female mice (Charles River, Wilmington, MA) were used in this study. Animals were grouped into two cohorts – Methylene Blue-injected, and Mouse IgG–IRDye680RD-injected. All animals were maintained on a fluorescence-free diet for a week before imaging. Prior to imaging, each animal was anaesthetized using 1.5 – 3% isoflurane (Piramal, Bethlehem, PA) in 1.5L/min oxygen. The animal was then
placed on its side with the forelimb on one side of the body stretched out so as to expose lymph nodes of the axilla; shown in Figure 8.2. A pre-injection image was captured to measure autofluorescence background levels. Next, 10 µl volume of one fluorescent tracer in PBS, was injected intra-dermally using a 31-gauge syringe-needle into the base of a forepaw footpad of the mouse. All fluorescence imaging was performed using a Pearl® Impulse system (LI-COR Biosciences, Lincoln, NE) which provides planar surface images in the far-red and near infrared band. The system was set up to acquire grayscale white light, and 700 nm channel images at 1 frame every 2 seconds for the first 10 – 20 minutes and then at 2 frames per minute. The 700 nm channel setting uses a 685 nm laser diode excitation source with an intensity distribution of 2 mW/cm² and a 720 ± 20 nm bandpass emission filter. Imaging was performed for 120 minutes following injection.

Figure 8.3 Cumulative Fluorescence images of lymphatic dye uptake in two mice.

8.1.3 Image Processing and Analysis, and Statistical Analysis

Image analysis was performed using Matlab (R2014b, Mathworks, Natick, MA) and Image J Software (1.47v, NIH). Regions-of-interest were selected over the visible lymph node and its afferent lymphatic vessel. For nodes, circular ROIs of 2020µm diameter were drawn, and for lymphatic vessels, 3 ROIs each ~500 µm in diameter were selected
Care was taken to place the ROIs over regions where there was evidence of pumping function. We expected to see sections with lack of pumping function along each vessel; these regions were avoided for the purpose of this study. To simplify the selection of ROIs, the entire temporal image stack was summed to identify the lymphatic vessel and lymph node, and selected ROIs were confirmed based on these. Figure 8.3 shows samples of summed images for two mice injected with either MB or MIgG–IRDye 680RD tracer. Uptake at various time points after tracer administration was observed in the early time points after injection (transient-phase) and at long-time points, which we treat as representative of the steady-state pump rate.

Statistical Analyses were performed using Microsoft Excel, and equal variances were assumed across all groups. Student’s t-tests were used to identify variables with significant differences between the MB and MIgG cohorts.

Figure 8.4 Active and passive components of lymphatic uptake of methylene blue. Inset shows representative ROI locations (circles were enlarged to be visible, actual ROI size
was ~2 mm for nodes, and 0.5 mm for lymph vessels). ROI circles are color coded to match the uptake curves. Raw data and smoothed curves are shown in the figure.

8.2 Results and Discussion

Figure 8.2 shows the uptake of Methylene Blue from an injection site in a mouse paw to the axillary lymph node. The paw had to be covered to block out the large fluorescence signal from the deposited dye. The transport of tracer appeared to have both an active pumping, and a passive diffusion- and pressure-driven component as expected\(^7\). A sample set of measured Methylene Blue uptake curves in one mouse is shown in Figure 8.4. The overall uptake shape was recovered using temporal smoothing with piece-wise spline fitting. Based on fluorescence intensity signal strength, only one vessel segment was chosen for analysis in each animal. This was generally the vessel segment close to the lymph node, as signal-to-noise was poor at early segments. Periodic vessel contractions were evident in the raw fluorescence measurements, and could be manually counted to identify number of pulses per unit time. Due to the noise in the data, relative intensities of pulses, to each other and to the overall uptake, were not estimated.

Figure 8.5 Comparison of passive uptake components of MB and MIgG-680RD shown in a representative lymph node (solid) and its corresponding afferent lymph vessel
Methylene blue clears rapidly from the node owing to its small size. Inset shows a time-expanded view.

Figure 8.5 shows a comparative set of representative uptake curves for MB and MIgG–IRDye 680RD in the axillary node and its corresponding afferent lymph vessel. The curves were normalized to the peak fluorescence signal in the vessel. IgG accumulation peaks at ~60 minutes in the lymph node, and MB peaks at ~1 minute. The peak fluorescence is attained in the vessel segment much earlier than in the node. The MB vessel peak, is missed in these presented data, owing to the fact that it gets taken up during injection itself, and is instantaneously driven forward. MB is not retained within the lymph node and clears immediately, whereas IgG owing to its large size remains in the lymph node for significantly longer time.

Figure 8.6 a-e Comparison of various measured parameters for MB (N=8) and MIgG–680RD (N = 5) cohorts. f shows comparison bar graphs of transient versus steady-state propulsion rate in all animals. * indicates significant differences with p < 0.009.
The time to reach the peak $T_{\text{peak},N}$ was noted for all animals and found to be $0.85 \pm 0.41$ minutes (Median ± Standard deviation, excluding outliers beyond ± 2σ), in the MB group, and $64.95 \pm 22.69$ minutes in the MIgG-680RD group. Similarly, time to peak in the vessel $T_{\text{peak},V}$ was observed to be $0.57 \pm 0.22$ minutes and $7.92 \pm 5.98$ minutes in the MB and MIgG groups respectively (See Figure 8.6a-b). The differences between these groups was found to be significant with $p \approx 0$ for nodes, and $p = 0.008$ in the vessels.

We have previously shown (in Chapter 4 of this thesis) that raw intensity measurements on the nodes are highly unreliable and prone to high signal heterogeneity, attributable to heterogeneity in delivery by the lymphatic vessel, and so vessel signal-based normalization improves the quantification of signal in the nodes provided that a single vessel delivers tracer, and tracer has not entered the blood stream. We report vessel-peak normalized node fluorescence $\frac{N}{V}$ in Figure 8.6c, and found that this ratio was $1.67 \pm 0.84$ in the MB group and $7.33 \pm 4.36$ in the IgG group, revealing a statistically significant difference between groups with $p = 0.004$. All the above parameters are related to the slower rate of uptake and transport of MIgG as compared to the much smaller MB molecule. The lymph nodes, being clearance organs, retain large proteins and molecules, and hence, the tracer lymphatic kinetics is influenced by this retention. MB appears to be barely retained by the node and is seen in the urine within minutes after injection (data not shown). Other studies suggest that MB has very poor affinity for any plasma proteins due to the lack of sulffonic acid groups, and hence mostly exists as free unassociated dye that can travel swiftly into and out of the node. We do not see a second methylene blue:Albumin uptake curve in any of our imaging likely due to the fact that the any MB:Albumin would be taken up slowly at the lymph capillaries and maintain a much lower concentration in the lymph than detectable by fluorescence imaging. IgG on
the other hand is likely trapped in the lymph node sinuses and clears slowly over the
course of several days after injection. Little is known about the association of IgG with
immune cells in the lymphatic collector vessels, but it appears that the molecules do tend
to linger more than MB. Perhaps, diffusion plays a role in the movement of molecule
within each lymphangion.

Figure 8.6 d–e show the intensity independent “active” pumping components of
uptake. Contraction of smooth muscles lining the walls of the collector lymph vessels
drives the lymph packets from one lymphangion to the next. The number of complete
pulses per minute was manually counted from the raw fluorescence data as shown in
Figure 8.4. The early time points during which tracer signal in the node and lymph vessel
was rapidly changing (0–2 minutes for MB and 0 – 15 minutes for IgG) was labeled
‘Transient pump rate, $p_{trans}$’ and the pump rate over late time points was assumed to be
indicative of the resting pump rate and was labeled, ‘Steady state pump rate, $p_{ss}$’. For the
MB and IgG cohorts, transient pump rates were observed to be $6.07 \pm 1.28$ min$^{-1}$ and
$4.26 \pm 1.16$ min$^{-1}$ ($p = 0.15$), and steady-state pump rates were $3.41 \pm 0.83$ min$^{-1}$ and $3.33$
$\pm 0.48$ min$^{-1}$ ($p = 0.34$). The pump rates were similar for the MB and MIgG cohorts
indicating that molecule size does not affect the lymphatic vessel contractions. Aldrich et
al$^{184}$ observed similar pumping (propulsion) rates in their comparison of indocyanine
green, IRDye800CW and an antibody, though they did not separate transient and steady-
state rates, and instead used the average rate over 20 minutes. Given that there were no
significant differences in pump rates between the MB and IgG cohorts, we pooled the
results from both cohorts and compared the transient ($5.99 \pm 1.39$ min$^{-1}$) and steady-
state rate ($3.41 \pm 0.85$ min$^{-1}$) within each mouse using a paired-Student’s t-test, and
found that the differences were significant with $p \approx 0$, indicating that the collector
lymphatics likely do respond to luminal pressure changes in the initial lymphatic capillaries. Also note that no detectable contraction was observed in the lymph nodes.

8.3 Conclusions

The study of the lymphatic physiology has largely been limited by the inability to image the lymphatic architecture in real-time at a sampling rate greater than the lymphatic smooth muscle contraction rate. Recent attempts using near-infrared and far-red fluorophores and imaging them using microscopy methods such as intravital microscopy\textsuperscript{195} and broad-beam NIRF imaging\textsuperscript{161} have lead the way in quantitative imaging of lymphatic vessels, but do not describe the relationship between tracer size and various components of uptake. Through this work we hope to elucidate the importance of identifying the active and passive uptake components, and we expect that this work will be one piece in the puzzle of developing the right tools to study the lymphatic vasculature and design appropriate physiological models, which will in turn impact the study of lymphatic metastasis.
CHAPTER 9  Miscellaneous Novel Imaging

Methods for improving nodal metastasis detection using fluorescent probes

Various imaging and quantitative measurement approaches other than fluorescence molecular tomography and planar fluorescence imaging were attempted to solve the problem of in vivo non-invasive quantification of metastatic burden on lymph nodes. This chapter is based on the paper “Logarithmic intensity compression in fluorescence guided surgery applications,” by Alisha V. DSouza, Huiyun Lin, Jason R. Gunn, and Brian W. Pogue published in the Journal of Biomedical Optics, and summarizes the Cherenkov-excitation fluorescence measurement methods tested other than conventional fluorescence imaging and tomography, and also describes some visualization tools that were designed to aid lymphatic architecture visualization.

9.1 Adaptive Logarithmic Compression for fluorescence uptake visualization

Fluorescence image guidance is evolving as a paradigm, which could bring different contrast mechanisms into the clinical setting. The implementation of indocyanine green fluorescence for vessel perfusion imaging is now already a widespread commercial/clinical success\(^\text{196}\), and the emergence of other probes, which could offer molecular level information, is showing promise\(^\text{197}\). One of the major problems to solve within this paradigm is that for real-time imaging, the traditional radiologic approach of
window and level adjustment to maximize the display contrast is not feasible quickly, and automated display optimization methods are necessary. This issue is recently compounded by the increasing use of advanced imaging systems that digitize the image information into bit-depths significantly higher than 8-bits, thereby producing images with large dynamic ranges of luminance. Most imaging uses linear display, however ultrasound imaging is an analogous video-rate modality which regularly employs log-compression of the images\textsuperscript{198}. Fluorescence images can be acquired with camera bit-depths of 12 to 16-bits or higher, yet mainstream displays continue use 8-bits per channel. Fluorescence imaging display quality is a mixture of many features, and the detected intensity at the camera can unfortunately easily vary by orders of magnitude. To compensate for undesirable background signals from tissue, non-linear components such as camera filters, and noise, many systems have shifted to high dynamic range cameras, so that simple removal or threshold can be applied to remove the background\textsuperscript{199}. This can work well, and in practice may be the most practical way to proceed, however as dynamic range has expanded, the potential value of image compression is increasing. This value is obviously critical in areas where the real-time video stream is guiding the tissue diagnosis and identification of resection boundaries, such as with Aminolevulinic acid for Protoporphyrin IX fluorescence imaging\textsuperscript{200} or with newer classes of molecular probes\textsuperscript{197}. Logarithmic mapping, on the other hand of high dynamic range images has been explored in detail\textsuperscript{201} as an automatic, fast, high-quality tone-mapping method to improve quality of image display on devices with limited dynamic range. Logarithmic mapping has also found use in audio level compression and is an established step in image pre-processing on ultrasound scanners\textsuperscript{198}, and flow cytometers\textsuperscript{202}. In this chapter,
the concept of optimized logarithmic compression for fluorescence intensity images used in lymphatic imaging and image guidance in during surgery is presented and explored.

9.1.1 Theory

Given a camera with digitization depth of N bits, the intensity values, \( f(i,j) \), at each pixel location \((i,j)\), the image intensity range 0 to \(2^N\) would need to be mapped onto \(2^8\) levels for single-color display\(^{203}\). Assuming a background noise signal offset value, b, the useful signal would then be,

\[ s(i,j) = f(i,j) - b \]  

Equation 9-1

Assuming the system is used with maximum gain and signal is near the maximum dynamic range, then the expression which governs the ideal logarithmic compression base, \(x\), is given by,

\[ 2^8 = \log_x (2^N - b) \]  

Equation 9-2

Where the left hand-side is the output dynamic range is \(2^8 = 256\), and the right is the logarithm base \(x\), for the maximum signal \(s(i,j)\). Solving this expression, the solution for \(x\), is,

\[ x = [2^N - b]^{1/256} \]  

Equation 9-3

This function is plotted in Figure 9.1, which shows that at high bit depths the impact of choice of log base though subtle is significant as bit-depth N varies, whereas for a given camera system (constant bit-depth), the background intensity variation would matter little.

9.1.2 Results and Discussion

Figure 9.2 demonstrates an application of logarithmic compression to visualize lymphatic uptake of IRDye800CW conjugated to an antibody injected at the base of the tail of a
mouse imaged with its skin folded back to reveal shallow lymphatic tracks and nodes. All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee at Dartmouth College under approved protocols. All images were acquired using the Pearl® Impulse system (LI-COR Biosciences, Lincoln, NE) providing planar surface images of the dye. This has image digitization of 22-bits thus providing an exceptionally high intensity dynamic range. The first column shows original fluorescence overlays in green over the white light images for various time points after dye administration (rows).

Figure 9.1 Logarithm base versus background intensity is plotted for various camera bit-depths to demonstrate that at high bit-depths, background signal intensity does not significantly affect the selection of log base x.

Dye administered intradermally at the base of the tail travels via lymph vessels to the inguinal nodes (within 1 minute after injection) and then towards the axillary nodes (5-10 minutes after injection). At the 60 minutes time point dye can be seen in the left and right inguinal nodes and axillary nodes and also in the lymph vessels between these. Image entropy \( E \), was used as a quantitative metric to compare the amount of visual
information supplied by the original and each processed image. Figure 9.2 summarizes the observations.

The original images show very poor contrast as the ranges of intensities occupy only a fraction of the whole intensity range. This can be seen on the histogram on the bottom row of Figure 9.2, for the 60-minute time point, image entropy was calculated to be 1.36. Manual window and level adjustments to the image enable stretching of the histogram to occupy a wider range of intensities, and improve visualization of dye in lymph nodes/vessels. The one-step log-compressed images\(^{1}\), offers a promising alternative or add-on to manual window-level adjustment, and provides a 425% improvement in entropy over the original image. A second semi-automated approach combining Window-level adjustment with contrast-limited adaptive histogram equalization (CLAHE)\(^{205}\) was applied to the original and log-compressed images for comparison. CLAHE applies histogram equalization to small regions of the image so the resulting histogram of each is uniform and the final output image is a bilinear interpolation of the sub-regions. An overall entropy improvement of 456% was achieved over the original image with this approach. The right lymph vessel (white arrows on Figure 9.2) can be seen easily at the last time point in all processed images but is visible only on the log-compressed images at 10 minutes due to the contrast-to-background improvement from 1.22 (original) to 3.58 (log compress + CLAHE + WL). Figure 2 includes a row of fluorescence images of well plates containing various concentrations of IRDye800CW fluorophore (1:2 dilution series from 1 µM to 0.49 nM) with 1% Intralipid and 0.08% India Ink. The original intensity range of 0.002 to 2.31 A.U. is mapped to a range 0.33 to

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\(^{1}\) Compressed image intensity may need shifting/scaling to visible range.
1 A.U. of normalized fluorescence in the compressed image demonstrating dramatic reduction in dynamic range by a factor 381.

Window and level adjustment is the simplest processing tool used to improve the visibility of dim features in images. However, manual adjustments, especially within a clinical and surgical settings dependent on real-time video sequences, are cumbersome. Besides this, windowing-leveling can often be biased by the user, and important low-contrast features can easily be overlooked. On the other hand, optimized logarithmic compression is a tool that offers objective, bias-free contrast-enhancement of images that span several orders of magnitude of intensities.

Figure 9.2 Lymphatic uptake of fluorophore in a mouse is shown as the green overlay on grayscale white-light images. White-light images were windowed and leveled to be dim so
as to facilitate visualization of the fluorescence overlays. Each row corresponds to a time point after dye administration with the first column showing the original image. Each subsequent column shows a processed image \( (x = 1.06) \). Well plates with various concentrations of fluorophore ranging >3 orders of magnitude in concentration and fluorescence intensity are shown. Histograms correspond to 60 minute lymphatic uptake images, these were clipped to not display number of pixels at 0 intensity. Entropy \( E \) is shown inset on the histograms. White arrows indicate a lymph vessel that is hard to detect at early time points without log compression. 5mm scale bars are shown. (See Video 1, Quicktime Movie)

The well-plate images at the bottom of figure 2 demonstrate that the mapping of >3-orders of magnitude onto a visible range of 256 intensities can easily be achieved. Addition of background subtraction or windowing-leveling removes issues of high background, in combination with the automated methods. Log-compression is relatively insensitive to intensity fluctuations, which would enable application of a single window-level adjustment setting at the start of an imaging sequence without the need for further manual adjustment. However, as surgical images especially those from settings involving tumor resection may be complicated by the presence of blood and other absorbers, future studies that apply log-compression to such image sequences may be necessary to test the practical aspects of such an approach.

### 9.1.3 Summary

In summary, fluorescence imaging has a long history in analysis and quantitative spectroscopy, with critical impact in flow cytometry today\(^\text{202}\), attributable to the inherently high biological variance and cell labeling. Fluorescence imaging of molecular contrast should be the same, where variations in signal by orders of magnitude occur spatially from blood vessels to parenchyma on the microscopic level, and can be present on the macroscopic level between regions of high and low perfusion. On top of this biological variation, there are imaging system variations which all contribute to a signal
which ranging over several orders of magnitude. Moreover, as camera systems gain larger dynamic range, the compression of the display to improve information transfer to the user will become critical. Current 12-bit camera systems should likely employ a compression factor of 1.03 in their display, and as more advanced systems are produced higher compression factors may be more appropriate.

9.2 Cherenkov excited fluorescence measurement for dual tracer ratiometric tumor-burden estimation

Several studies in the Optics in Medicine lab at Dartmouth have explored the ability to excite fluorophores using Cherenkov emission produced when MeV radiation travels through tissue, using single fiber-probes\textsuperscript{63} and tomographic arrangements\textsuperscript{62}, connected to spectrometers for detection. The spectrum of Cherenkov emission is inverse-square with wavelength and thus highly weighted in the ultraviolet and blue spectral region when in water\textsuperscript{206}. The Cherenkov emission is dominated by UV/blue wavelength emission, so that it can be used for effective excitation of large-Stokes-shift optical emission probes. The red or near-infrared (NIR) optical emission can then improve transmission up to several centimeters, because of the low absorption of blood\textsuperscript{207}. The ability to shape LINAC delivery combined with highly sensitive optical detection allows the ability to adaptively image/measure Cherenkov stimulated light signals with some direct spatial information in tissue without the need for diffuse tomography algorithms as previously used\textsuperscript{208}. MeV radiation travels deep into tissue with minimal scatter and can be directed to a subsurface such as a lymph node for metastatic burden estimation and directed radiation treatment. Spectrometer-based detection allows for spectral decoupling of measured spectra, as long as an estimate of contributing spectral bases is available.
Spectral decoupling of fluorophore emission from autofluorescence background was described in detail in Chapter 6. Cherenkov emission, which excites the fluorophores, occupies a spectral range that extends into the >650 nm region, and also excites the tissue background and optical fiber probe. These effects demand that the measured emission signal (by the filtered fiber-coupled spectrometer) is spectrally decoupled. Spectral decoupling further allows relative estimation of concentrations of multiple spectrally separated fluorophores.

From previous studies, described earlier in this thesis, dual tracer ratiometric techniques have been shown to be promising in quantifying receptors overexpressed by several cancer types, and also to determine metastatic burden on lymph nodes. We hoped to address the problem of imaging deep nodes (1 – 4cm from skin) using Cherenkov excitation. The initial testing involved measuring fluorescence emissions from liquid phantoms containing dye inclusions as shown in figure Figure 9.3.

The experimental setup included a linear accelerator (Varian Clinic 2100C, Varian Medical Systems, Palo Alto, USA) at the Norris Cotton Cancer Center, a spectrometer (Acton Insight, Princeton Instruments, Acton, USA) with a 500nm longpass filter (FEL500, Thorlabs Inc.) mounted at its entrance, and a fast-gated ICCD camera (PI-MAX3, Princeton Instruments, USA). Since the spectrometer and ICCD were placed outside the radiotherapy room, no shielding from the radiation generated by the LINAC was necessary. A single fiber bundle (CeramOptec, Germany) 15 meters in length, which housed 19, 200-micron diameter silica fibers, conducted emitted light from the treatment room to the spectrometer. This fiber bundle collected the light at the boundary of the phantom at a point location and guided the signal into a vertical line of fibers at the entrance slit of the spectrometer. A LINAC trigger output was used to trigger
acquisitions by the camera at minimum trigger delay equal to the camera insertion delay of 27ns and gate width of 3.25 µs. The ICCD was cooled to ~25°C and the spectrometer grating used in all experiments was 300 lines/mm, with center wavelength set to 700nm (see Figure 9.3).

![Image](image_url)

Figure 9.3 Measurement setup showing the LINAC, and fiber bundle. Adapted from Lin et al.61.

### 9.2.1 Liquid Phantoms with two-dye fluorescent inclusions

Liquid phantoms of various concentrations of IRDye680RD and IRDye800CW (LI-COR Biosciences, Lincoln, NE) from 25 µM to 0.1 µM in PBS were prepared in 2ml plastic conical tubes; 1:1 concentration ratio was maintained between dyes. Each tube was then placed in a “background” liquid phantom which contained PBS, or PBS + 1% intralipid (optical scatterer), or PBS + 1% intralipid + 1% whole blood. A blank
phantom tube containing only PBS was also prepared. The phantom setup was placed on a stirring plate, to prevent RBC stacking and inhomogeneity in scatterer concentrations, the tube was positioned inside the ‘background’ phantom such that it was at a distance of 5 mm from the wall at which the optical fiber tip was fixed (See Figure 9.3). The beam shape was set to $1 \times 1 \text{ cm}^2$, which irradiated the inclusion tube from the top, with the LINAC beam isocenter set to be inside the inclusion. This provided sufficient buildup region for the beam passing through the phantom. Five frames of continuous wavelength spectra were acquired were made each with 100–3000 accumulations on the CCD chip (AOC). A background measurement was made with an identical setup and acquisition settings.

9.2.1.1 Spectral data processing

The collected spectra were temporally median-filtered and smoothed (x-axis = wavelength) to produce processed spectra per dye concentration, shown in Figure 9.4.

![Figure 9.4 Processed spectra acquired from phantoms with inclusion containing a range of IRDye680RD and IRDye 800CW concentrations for various AOC settings. Dye concentrations are indicated in the graphs.](image-url)
concentrations are shown in ‘red’ font above each plot. The spectra show fluorescence emission from the two dyes superimposed on the Cherenkov emission background. Green arrows point to the detectable emission peaks.

The acquired normalized background spectrum, which is the sum of Cherenkov emission and the fiber and system autofluorescence, and available fluorescence emission spectra were used as spectral bases to decompose the measured fluorescence spectrum into the contributions from the 700nm and 800nm dyes. The algorithm for spectral decoupling based on a linear least squares solver was described earlier in this thesis.\(^\text{g}\)

![Diagram of spectral bases used for spectral decoupling of fluorescence emission from Cherenkov background + autofluorescence. Absorption bases are also shown (scaled to be <0).](image)

**Figure 9.5** Spectral bases used for spectral decoupling of fluorescence emission from Cherenkov background + autofluorescence. Absorption bases are also shown (scaled to be <0).

### 9.2.2 Subcutaneous Matrigel-based fluorescent inclusions in murine model

Before the above approach could be applied and tested to quantify tracer ratios in animal lymph nodes, a simulated *in vivo* experiment utilizing fluorophores injected subcutaneously in a mouse was performed. In this experiment IRDye 680RD and IRDye 800CW were mixed with clear phenol red-free and growth factor-reduced matrigel

\(^\text{g}\) Section 6.4.2 Spectral data processing
medium (#356230, Corning) such that their concentration was 3 µM each. The matrigel that was stored in 500-µl aliquots at -20°C had to be thawed in an ice tub to raise its temperature to 4°C. Care had to be taken during the handling to prevent overheating the matrigel as this causes it to cross-link and solidify. Dyes in PBS were cooled and mixed with the matrigel. Injection syringes with 27-gauge needles were chilled prior to loading. Animals were anesthetized using 1.5 – 2.5% isofluorane in 1.5L/min oxygen, and placed on a heating pad. Injections of varying volumes (10 µl – 200 µl) were performed subcutaneously such that the matrigel solidified into a spheroid with minimal flattening. Matrigel is a highly viscous substance, care needed to be taken to ensure bubbles were not present in the gel.

![Figure 9.6](image1.png)  
Figure 9.6 Experimental setup for matrigel-inclusion measurements using Cherenkov-excitation.

For the acquisition, the mouse was placed on a black sheet over layers of solid water phantoms (added to 1.5cm thickness), to provide sufficient build-up region. A 6MV radiation beam was directed from below the animal so that the beam size was 1×1 cm², with the isocenter near the fiber tip. The fiber position was fixed and the animal was adjusted to maintain contact between the fiber tip each matrigel blob. A background
spectrum was obtained by measuring a matrigel-free skin region away from the liver and gut. Measured spectra were acquired and processed exactly as described in Section 9.2.1.

9.2.3 Results and Discussion

Figure 9.7 and Figure 9.8 show the processed acquired spectra for various fluorophore concentrations (IRDye680:IRDye800 concentration was 1:1) in an intralipid only and intralipid + blood background medium. The fitted background spectra, and fluorophore emission spectra are shown. The resulting sums of the fitted spectra are also shown. Due to the high cherenkov background signal, only high concentrations of dye were detectable (>3 µM), and at the highest concentrations, i.e. 12.5 µM and 25 µM effects of dye self-absorption and quenching are evident.

Figure 9.7 Phantoms in 1% intralipid background, i.e. absorber-free.

Figure 9.9 shows fit coefficients from all the measured phantoms in both 1% intralipid and 1% intralipid + 1% blood backgrounds. These fits represent the relative contributions of Cherenkov emission and fluorophore emissions to the final acquired
spectra. As dye concentration increased, Cherenkov excitation signal is absorbed, and a linear trend is seen in the detected Cherenkov signal, for dye concentrations >1 µM. This observation is corroborated by the detected fluorescence emissions as well (see on the right side of Figure 9.9). The detected emission of IRDye800CW appears to linear in both phantom types over the range ~1.5 µM to 12.5 µM, whereas estimation of IRDye 680RD emission is linear only from ~1.5 µM to ~3 µM. This may be due to both self-absorption and absorption by IRDye 800CW. Although these ranges are narrow, they do offer a small yet promising window within which ratiometric quantification may be possible.

Figure 9.8 Phantoms in 1% intralipid and 1% whole porcine blood.

Processed and fitted spectra for the murine matrigel inclusions are shown in Figure 9.10. We can see a 800:680 ratio of ~1, and further testing with varying injected 680:800 concentrations to recover the ratio of concentration between the dyes is necessary to confirm the utility of this method. As a next step in this study, we plan to simulate binding between Erbitux and metastasis in lymph nodes by varying the 800:680 ratio to
values of 1.5 and above in various inclusions in phantoms and animals, and then expand this to an *in vivo* metastasis model.

**Figure 9.9** Fit coefficients are representative of relative contributions of the Cherenkov background, and the concentrations.

**Figure 9.10** Spectra acquired from subcutaneous matrigel inclusion in a mouse, shown along with the fit results.
CHAPTER 10  Conclusions & Future Work

10.1 Conclusions

This dissertation presents novel quantitative fluorescence-based tools to non-invasively test for the presence or absence of metastasis in lymph nodes. The current clinical gold standard involves lymphoscintigraphy-guided resection of sentinel nodes to detect metastasis. Although invasive, this method is highly accurate, and any alternative methods would need to match up against it in terms of sensitivity, in order to make clinical impact. The goal of this thesis was to design non-invasive approaches to determine nodal status and identify nodes to resect and nodes to save.

We recognize that the fluorescence-imaging device determines the ultimate usability of an imaging approach, so have we reviewed several FDA-approved and preclinical imagers capable of single- and multi-channel fluorescence imaging, and have identified the key operational goals. We presented a detailed comparison to systems in Chapter 3, and expect that it would only help orient our goals in the right direction, but also serve as a guide to help other research and clinical users in choosing a suitable system for their imaging studies. We also identified the need for on-line compression tools in the context of fluorescence imaging and visualization, which will gain significance as imaging dynamic range grows well beyond the traditional 8-bits (per color channel), and proposed (in Chapter 9) an adaptive logarithmic compression technique that has the potential for successful deployment in clinical settings as it avoids the need for manual window and level image adjustments. All small animals studies were performed using a planar fluorescence imager with dual-channel functionality, under ideal ambient light-free conditions.
Using the small animal fluorescence imager, we confirmed that we could image the active and passive uptake components of lymphatic uptake in vivo and found that tracers with differing pharmacokinetics did not affect the active propulsion in the lymph vessels, although vast differences were observed in their overall nodal uptake pattern and retention. We described the observations in detail in Chapter 9, and expect that this work will aid in furthering the understanding of lymphatic transport, and the development of models of the lymphatic system.

Early tests using non-specific lymphatic tracers revealed that the high heterogeneity in uptake, and the delivery variations prevent quantitative measurements of tracer concentrations in lymph nodes, and that there is a need for methods that account for the delivery differences. We reported in Chapter 4, that incorporation of afferent vessel signals could reduce the inherent biology heterogeneity, and enable use of a non-specific tracer to distinguish between normal and cancer-bearing nodes produced through an intranodal injection cancer xenograft model in rodents. This work bore clinical relevance as it used methylene blue as a fluorescent agent; methylene blue is approved for use in the sentinel node procedure as a visible blue dye, and there appears to be potential for expanding its use to fluorescence-based metastasis sensing. However, our approach involved the use of a slightly artificial model, where the nodes were clogged with tumor, and hence may not necessarily represent a real scenario. The high sensitivity and specificity attained are promising but it do need further evaluation in relevant models, and would also rely on a large-animal (human) imaging system capable of linear performance and relative quantification.

Through previously published research, Tichauer et al.\textsuperscript{53} demonstrated that the LN-MCI approach, where combined use of a cancer-targeted fluorescent tracer and a
reference untargeted tracer with similar pharmacokinetics, could estimate micrometastasis with sensitivity to 200 cells. The work presented in Chapters 5 and 7 detailed approaches that were applied while attempting to overcome some of the hurdles in the clinical translation of the LN-MCI approach, such as, (i) the need for approval of two antibody tracers, and (ii) limitations of tracer administration through distal injection site. In Chapter 5, we attempted to combine methylene blue with cancer-specific affibody as an expansion of the ratiometric node imaging, but found that the large differences in pharmacokinetics was problematic. In Chapter 7, we administered a combination of cancer-specific and non-specific tracers (which were previously shown to have identical pharmacokinetics when administered through a distal injection site in the paw), via a peritumoral injection site, as this is more inline with the current clinical workflow. We found that both the tracers were affected in their uptake by the presence of cancer in the nodes, and while the non-specific tracer signal normalized by the vessel delivery showed reduction in tumor-bearing nodes, the vessel-normalized targeted-tracer showed increased retention. We found that vessel signals provided an internal normalization factor, that could enable snapshot imaging and decision-making. The vessel-normalized node intensities were found to be sensitive but low in specificity. This means that the approach would be able to save nodes that were less likely to metastatic, but not be able to eliminate the need to resect and test suspicious nodes. In terms of clinical translatability, given the current state of lymphatic imaging in humans, we would first need to confirm the ability to image both the nodes of interest and their feeder lymphatic vessels before we can comment on the clinical utility of our proposed approach.
The fluorescence imaging approaches will have limited utility in imaging deep seated lymph nodes, and to address this we modified a previously existing ultrasound-guided fluorescence tomography instrument to enable multi-channel measurements, which was shown to be capable of quantifying nanomolar fluorophore concentrations at ~5 mm depth through skin. We found that owing to the high delivery heterogeneity, we needed to measure signals from vessels and nodes simultaneous, which was not feasible with the 2D tomography instrument, and so research effort was focused more towards imaging techniques, which though limited in depth sensitivity, can have high impact in node assessment in situ during surgery.

10.2 Limitations

Although the final conclusions and accomplishments in this thesis are impactful and motivating, it is important to note some of the limitations of fluorescence lymphatic imaging in the context of this work, these are grouped into biological limitations and technological limitations.

10.2.1 Biological Limitations

The inherent biological limitations pose significant challenges that need to be overcome or circumvented. All of these would apply to human or animal lymphatic imaging.

1) Presence of multiple afferent lymphatic vessels feeding a single node

In this work, we assumed that lymphatic delivery to a node occurred through only one vessel, but in some (excluded) cases, delivery was seen by means of 2 or more vessels, that had differing temporal characteristics. More
complex models are needed to use the combination of vessel signals for eliminating delivery variations in nodes.

2) *Lymphatic uptake is influenced by various physiological factors such as heart rate, respiration etcetera*\(^2\)

As expected, injection pressure (from the injected volume) and style, level of anesthesia, heart-rate, skeletal muscle movements, etcetera can all affect lymphatic uptake and flow. Care was taken to maintain all factors constant, but all elements of human error could not be eliminated. The lymphatic system in particular is highly sensitive to such external factors, and we report the studies in this thesis within the constraints of human error. Effect of some of these errors may be minimized as body mass increase, i.e. in humans, but further testing is needed to study this in detail.

3) *Reduction of tracer flow through lymph nodes that have advanced metastasis*\(^3\)

Through our experiments in Chapters 4 and 7 we found that advanced stages of node metastasis let to lowering of lymph flow through the node, and it is expected that fully tumor-clogged nodes would have very low or no tracer uptake when delivered through the lymphatic system, leading to missing important nodes and hence false negatives. Delivery through the vasculature, i.e. using as intravenous injection route, may circumvent this problem.

4) *Dye presence in skin and vasculature at late time points after administration*\(^4\)

The lymphatic system transports material from the injection site (interstitium) to the blood. As such, any administered tracers will ultimately

\(^{\text{2}}\) See Chapter 7
make their way into the vasculature. Small tracers such as methylene blue enter the blood and are immediately filtered by the kidneys, whereas larger tracers, are retained by the liver and skin. This can lead to high background signals exceeding the signal from the lymph node and also provide a route of dye delivery to the node from outside the lymphatics. This problem is challenging to overcome, and in this thesis we tried to avoid it by imaging at early time-points corresponding to various dyes used (<30 minutes for methylene blue, and <2 hours for antibodies delivered peritumorally, and ~3 hours for antibodies delivered through distal injection site). It is also expected that at wait time on the order of ~24 hours post tracer administration, non-specific uptake of targeted tracers in the vasculature and normal tissues should clear. More testing would be needed to study drug dosages and efficacy in such situations.

5) The lymphatic system is still poorly understood

Owing to the lack of real-time imaging methods until a few decades ago, study of the lymphatic system was limited to excised vessels and nodes. Near-infrared fluorescence and intravital imaging has been shown to have potential in understanding the lymphatics is much greater detail than ever before. However, much is left to be learnt about the lymphatics, and there still is need for development of appropriate models.

10.2.2 Technological Limitations

While the biological limitations seem daunting, there is a lot of scope for improvement on the technology front. The following is a list of limitations that offer opportunities for future research into this area.
1) Limitations on depth sensitivity of planar fluorescence imagers

When micro-doses of tracers are administered, tissue concentration of tracers approaches the nanomolar range, and most commercial planar near-infrared fluorescence imagers have sensitivity limits in this range. In human subjects, sentinel lymph nodes can often be ~1cm or more in depth from the skin, therefore, both the excitation light delivered to nodes, and the resulting fluorescence emissions from tissue are highly diffuse (due to multiple scattering events within the tissue). This limits the utility of planar open-field imagers in imaging deep lymph nodes. Lymphatic vessels can often be shallow, but imaging metrics based solely on lymphatic vessel signals have poor specificity in nodal staging. Tomographic detection methods may be able to push the depth limit deeper, but light delivery to nodes itself is highly diffuse and inefficient at depths greater than ~2cm. Fluorescence imaging can still be useful in mapping and metastasis detection of deeply seated lymph nodes during surgery, and nodes can be probe and diagnosed without the need for resection, thus enabling non-invasive testing.

2) Few FDA-approved fluorophores

There are only 5 fluorescent dyes approved for use in United States by the FDA (2 are used off-label). These are Fluorescein, Proflavin, ALA-induced PpIX, Methylene Blue, and ICG. As described earlier, of these only Methylene Blue and ICG emit in the far-red to NIR wavelength regions where tissue autofluorescence is low, and depth penetration of light exceeds 5 mm. There is a major push towards using disease-target specific fluorescent agents, which most often are protein based ligands covalently
bonded with a fluorescent dye. Such an imaging agent must go through FDA-approvals before it can be used in the clinic; the approval process is long and involves significant time.

3) Lack of devices capable of simultaneous multi-fluorophore imaging

The majority of the fluorescence imagers available today are capable of imaging a single fluorescence channel, most often in the ~800 nm range. Through our imaging studies, we found that multi-fluorophore schemes work well, however clinical implementation will be possible only when approved devices with high sensitivity and capability to image multiple fluorophores simultaneously are available.

4) Inability to simultaneously image lymph nodes and vessels using 2D tomographic techniques

Fiber-contact based fluorescence tomography can probe ultrasound-localized nodes down to ~1 cm, but simultaneous measurements on lymphatic vessels may not always be feasible as their identification on ultrasound images is difficult, owing to their small size and thin walls. 2D tomographic techniques may thus be useful only when structurally identical cancer-specific and non-specific probes are used and measured at steady state, eliminating the need for vessel measurements. Upgrades to high-speed 3D tomographic imaging may overcome this challenge as we can envision a device that locates a node, samples it, and simultaneously samples the surrounding tissue, which should include the afferent and efferent lymphatic vessels.

\[\text{See Chapter 6}\]
10.3 Summary of Achievements

Listed below is an overall summary of achievements through this thesis research.

(1) Commercial FDA-approved and preclinical open-field fluorescence imagers were compared, and evaluated for use in non-invasive lymphatic imaging, and surgical guidance, and the key operational goals for such systems were identified to enable simplification of system selection by end-users, and encourage design improvements by manufacturers. This work is under review for publication in the Journal of Biomedical Optics.

(2) A cancer non-specific tracer based on a clinically approved dye was used to detect tumor-clogged nodes in animals by incorporating lymphatic vessel fluorescence using an appropriate kinetic model.

(3) A novel intranodal cancer-cell injection model was designed using fluorescence image-guidance to implant lymph nodes of interest, and thus produce tumor-bearing nodes with rapid turnaround (<1 week).

(4) Ratiometric quantification with and without the use of lymphatic vessel measurement using combinations cancer-specific and non-specific tracers based on antibodies of similar structures, and smaller protein probes of mismatched sizes when administered through a distal injection site (animal paw) were compared, and it was found that severe size mismatch can prevent any quantification, even when established kinetic models were applied.

(5) A previously designed Ultrasound-guided reflectance-based fluorescence tomography device was tested, and its ability to quantify...
subsurface fluorophores was validated through phantoms and \textit{in vivo} animal models. The system was upgraded to perform multispectral fluorescence measurements through use of additional lasers, and filter sets.

(6) A novel, snapshot, quantitative fluorescence imaging approach using the ratio of targeted to untargeted tracer uptake in nodes, each normalized by the corresponding afferent lymph vessel uptake signal, identified differences between normal and metastasis-bearing nodes. The use of afferent lymphatic vessel could successfully account for inherent uptake variability, revealing the differences in lymph flow in normal and metastasis-bearing lymph nodes. Through this approach, real-time decisions can potentially be made as to whether or not a lymph node is metastasis-free based on only a single time-point set of fluorescence images of targeted and untargeted tracers showing the lymph nodes and afferent lymph vessels.

(7) Tracer injection site was found to influence lymphatic delivery of cancer-specific targeted tracers, and peritumoral injection of such tracers involved several degrees of complexity that needed to be accounted for. Lymphatic vessel normalization proved to be a simple approach to eliminate this effect at early time points after injection, without the need to address underlying causes.

(8) Uptake through lymphatic vessels was studied closely using planar fluorescence imaging to identify, quantify and compare its passive
diffusive and convective components, and active propulsion when various tracers of differing pharmacokinetics were administered.

(9) Novel, real-time fluorescence image processing tools using adaptive logarithmic compression was demonstrated to have overall improvements over conventional window and level adjustments, and will likely have impact on streamlining fluorescence imaging workflows in clinical use\textsuperscript{137}.

(10) Cherenkov-excitation could be used to simultaneously excite fluorophores in the far-red and near-infrared wavelength regions in tissue simulating phantoms, and relative fluorescence signal contributions could be determined using spectrometer-based detection.

Each of the chapters 3-9 of this thesis has been published in a peer-reviewed journal, is under review for publication, or is in preparation for submission. We expect that a total of seven publications (four have already been published) based on this thesis work will have been published at the end of 2016. A complete list of papers was provided in Chapter 1.

10.4 Future Directions

As a continuation of the work presented in this thesis, dual-fluorophore animal experiments using Cherenkov-excitation signals is proposed, and work towards realizing this is already underway (see Chapter 9). Cherenkov light is produced when ionizing radiation travels through tissue, all along its path. As such, Cherenkov light sources are all within the tissue of interest, and the fluorophores can effectively be excited at any depth that the radiation beam can penetrate. This eliminates one half of the limitations
of fluorescence tomography and imaging, as the excitation signals reaching the deep lymph nodes are no longer multiply scattered. However, the detected emission signals will still be diffuse, and limit the depth sensitivity. The depth sensitivity will nevertheless be improved significantly.
References


160. Jenkins, D.E., Hornig, Y.S., Oei, Y., Dusich, J. & Purchio, T. Bioluminescent human breast cancer cell lines that permit rapid and sensitive in vivo detection of


203. Reinhard, E. et al. High dynamic range imaging: acquisition, display, and image-based lighting (Morgan Kaufmann, 2010).


Appendix I: Matlab Codes

Codes pertaining to Chapters 4, 5, 7 & 8

Deconvolution Functions

```matlab
%% This function performs numerical deconvolution of targeted and
% un-targeted tracer concentration curves
function [DeconvolvedData2, t2] = deconvolve2(tar, utar, t, plotting)
%% Make sure the data are in the following structure
% If [x y z] = size(curves); where all the data are in curves
% then x = 41: number of time points 0:1:40 min
% y = 2: number of tracers, [targeted untargeted]
% z = 4: number of animals
% Idea for deconvolution:
% targeted = conv(untargeted curve,function) in BP=0 region

%% Interpolate to minute resolution (0:40)
step = round(t(end)/(numel(t)));   % Make sure data points are
% t22 = round(t(1)):step:round(t(end));
t22 = 0:step:40; %extrap to 40 mins
%% Interpolate to minute resolution (0:40)
t2 = interp1(t,tar,t2,'spline','extrap');
utar2 = interp1(t,utar,t2,'spline','extrap');

%% Deconvolve the data
A = zeros(numel(t2));
for i = 1:numel(t2)
    if i == 1
        A(:,i) = utar2;
    else
        A(:,i) = [zeros(i-1,1);utar2(1:numel(t2)-i+1)];
    end
end
L = second_dif_operator(A);
[U,sm,X,V] = cgsvd(A,L);
regparam = FindRegParam(U,sm,tar2,'dsvd',L,V);
regparam=0.1; %modified by alisha
DeconvolvedData2 = tikhonov(U,sm,X,tar2,regparam);

%% Testing
if plotting == 'y'
    figure;
end
```
fitted = conv(DeconvolvedData2,utar2);
convCurve = fitted(1:numel(t2));
plot(t2, tar2, 'b*', t2, fitted(1:numel(t2)), 'r', t2,
utar2, 'g');
end

function [x_lambda,rho,eta] = tikhonov(U,s,V,b,lambda,x_0)
%TIKHONOV Tikhonov regularization.
% [x_lambda,rho,eta] = tikhonov(U,s,V,b,lambda,x_0)
% [x_lambda,rho,eta] = tikhonov(U,sm,X,b,lambda,x_0) , sm =
[sigma,mu]
% Computes the Tikhonov regularized solution x_lambda. If the
SVD
% is used, i.e. if U, s, and V are specified, then standard-form
% regularization is applied:
% min { || A x - b ||^2 + lambda^2 || x - x_0 ||^2 } .
% If, on the other hand, the GSVD is used, i.e. if U, sm, and X
are
% specified, then general-form regularization is applied:
% min { || A x - b ||^2 + lambda^2 || L (x - x_0) ||^2 } .
% If x_0 is not specified, then x_0 = 0 is used
% Note that x_0 cannot be used if A is underdetermined and L =~
I.
% If lambda is a vector, then x_lambda is a matrix such that
% x_lambda = [ x_lambda(1), x_lambda(2), ... ] .
% The solution norm (standard-form case) or seminorm (general-
% form case) and the residual norm are returned in eta and rho.
% Per Christian Hansen, IMM, April 14, 2003.
% Reference: A. N. Tikhonov & V. Y. Arsenin, "Solutions of

% Initialization.
if (min(lambda)<0)
    error('Illegal regularization parameter lambda')
end
m = size(U,1);
n = size(V,1);
[p,ps] = size(s);
beta = U(:,1:p)'*b;
zeta = s(:,1).*beta;
l1 = length(lambda); x_lambda = zeros(n,l1);
rho = zeros(l1,1); eta = zeros(l1,1);

% Treat each lambda separately.
if (ps==1)
    % The standard-form case.
    if (nargin==6), omega = V'*x_0; end
for i=1:ll
    if (nargin==5)
        x_lambda(:,i) = V(:,1:p)*(zeta./(s.^2 + lambda(i)^2));
        rho(i) = lambda(i)^2*norm(beta./(s.^2 + lambda(i)^2));
    else
        x_lambda(:,i) = V(:,1:p)*...
            (zeta + lambda(i)^2*omega)./(s.^2 + lambda(i)^2));
        rho(i) = lambda(i)^2*norm((beta - s.*omega)./(s.^2 + lambda(i)^2));
    end
    eta(i) = norm(x_lambda(:,i));
    end
    if (nargout > 1 & size(U,1) > p)
        rho = sqrt(rho.^2 + norm(b - U(:,1:n)*[beta;U(:,p+1:n)'*b])^2);
    end
elseif (m>=n)
    % The overdetermined or square general-form case.
    gamma2 = (s(:,1)./s(:,2)).^2;
    if (nargin==6), omega = V\x_0; omega = omega(1:p); end
    if (p==n)
        x0 = zeros(n,1);
    else
        x0 = V(:,p+1:n)*U(:,p+1:n)'*b;
    end
    for i=1:ll
        if (nargin==5)
            xi = zeta./(s(:,1).^2 + lambda(i)^2*s(:,2).^2);
            x_lambda(:,i) = V(:,1:p)*xi + x0;
            rho(i) = lambda(i)^2*norm(beta./(gamma2 + lambda(i)^2));
        else
            xi = (zeta + lambda(i)^2*(s(:,2).^2).*omega)./
                (s(:,1).^2 + lambda(i)^2*s(:,2).^2);
            x_lambda(:,i) = V(:,1:p)*xi + x0;
            rho(i) = lambda(i)^2*norm((beta - s(:,1).*omega)./
                (gamma2 + lambda(i)^2));
        end
        eta(i) = norm(s(:,2).*xi);
    end
    if (nargout > 1 & size(U,1) > p)
        rho= sqrt(rho.^2+ norm(b - U(:,1:n)*[beta;U(:,p+1:n)'*b])^2);
    end
else
    % The underdetermined general-form case.
    gamma2 = (s(:,1)./s(:,2)).^2;
    if (nargin==6), error('x_0 not allowed'), end
    if (p==m)
        x0 = zeros(n,1);
    else
        x0 = V(:,p+1:m)*U(:,p+1:m)'*b;
    end
    for i=1:ll
        xi = zeta./(s(:,1).^2 + lambda(i)^2*s(:,2).^2);
x_lambda(:,i) = V(:,1:p)*xi + x0;
 rho(i) = lambda(i)^2*norm(beta./(gamma2 + lambda(i)^2));
 eta(i) = norm(s(:,2).*xi);
 end


% CGSVD Compact generalized SVD of a matrix pair in regularization
% problems.
% 
% [U,sm,X,V] = cgsvd(A,L)
% [U,sm,X,V,W] = cgsvd(A,L) ,  sm = [sigma,mu] 
% % Computes the generalized SVD of the matrix pair (A,L). The dimensions of
% % A and L must be such that [A;L] does not have fewer rows than columns.
% % If m >= n >= p then the GSVD has the form:
% % [ A ] = [ U  0 ]*[ diag(sigma) 0 ]*inv(X)
% % [ L ] [ 0 V ] [ 0 eye(n-p) ]
% % where
% % U is m-by-n ,  sigma is p-by-1
% % V is p-by-p ,  mu is p-by-1
% % X is n-by-n .
% % Otherwise the GSVD has a more complicated form (see manual for
details).
% % A possible fifth output argument returns W = inv(X).
% Reference: C. F. Van Loan, "Computing the CS and the
generalized
% Per Christian Hansen, IMM, March 17, 2008.

% Initialization.
[m,n] = size(A); [p,n1] = size(L);
if (n1 ~= n)
 error('No. columns in A and L must be the same')
end
if (m+p < n)
 error('Dimensions must satisfy m+p >= n')
end

% Call Matlab's GSVD routine.
[U,V,W,C,S] = gsvd(full(A),full(L),0);

if (m >= n)
 % The overdetermined or square case.
 sm = [diag(C(:,1:p)),diag(S(:,1:p))];
 if (nargout < 2)
 U = sm;
 else
% Full decomposition.
X = inv(W');
end
else
% The underdetermined case.
sm = [diag(C(1:m+p-n,n-m+1:p)),diag(S(n-m+1:p,n-m+1:p))];
if (nargout < 2)
    U = sm;
else
    % Full decomposition.
    X = inv(W');
    X = X(:,n-m+1:n);
end
end

if (nargout==5), W = W'; end

function RegParam = FindRegParam(U,sm,b,method,L,V)

npoints = 200;
smin_ratio = 16*eps;

% [m,n] = size(U); [p,ps] = size(sm);
if (nargout > 0), locate = 1; else locate = 0; end
beta = U'*b; beta2 = norm(b)^2 - norm(beta)^2;
if (ps==1)
    s = sm; beta = beta(1:p);
else
    s = sm(p:-1:1,1)./sm(p:-1:1,2); beta = beta(p:-1:1);
end
xi = beta(1:p)./s;

if (strncmp(method,'Tikh',4) | strncmp(method,'tikh',4))
    eta = zeros(npoints,1); rho = eta; reg_param = eta; s2 = s.^2;
    reg_param(npoints) = max([s(1),s(1)*smin_ratio]);
    ratio = (s(1)/reg_param(npoints))^(1/(npoints-1));
    for i=npoints-1:-1:1, reg_param(i) = ratio*reg_param(i+1); end
    for i=1:npoints
        f = s2./(s2 + reg_param(i)^2);
        eta(i) = norm(f.*xi);
        rho(i) = norm((1-f).*beta(1:p));
    end
    if (m > n & beta2 > 0), rho = sqrt(rho.^2 + beta2); end
    marker = '-' ; txt = 'Tikh.';
elseif (strncmp(method,'tsvd',4) |strncmp(method,'tgsv',4))
    eta = zeros(p,1); rho = eta;
    eta(1) = abs(xi(1))^2;
    for k=2:p, eta(k) = eta(k-1) + abs(xi(k))^2; end
    eta = sqrt(eta);
    if (m > n)
        if (beta2 > 0), rho(p) = beta2; else rho(p) = eps^2; end
    else
        rho(p) = eps^2;
    end
end
for k=p-1:-1:1, rho(k) = rho(k+1) + abs(beta(k+1))^2; end
rho = sqrt(rho);
reg_param = (1:p)'; marker = 'o';
if (ps==1)
    U = U(:,1:p); txt = 'TSVD';
else
    U = U(:,1:p); txt = 'TGSVD';
end

elseif (strcmp(method,'dsvd',4) | strcmp(method,'dgsv',4))

    eta = zeros(npoints,1); rho = eta; reg_param = eta;
    reg_param(npoints) = max([s(p),s(1)*smin_ratio]);
    ratio = (s(1)/reg_param(npoints))^((1/(npoints-1)));
    for i=npoints-1:-1:1, reg_param(i) = ratio*reg_param(i+1); end
    for i=1:npoints
        f = s./(s + reg_param(i));
        eta(i) = norm(f.*xi);
        rho(i) = norm((1-f).*beta(1:p));
    end
    if (m > n & beta2 > 0), rho = sqrt(rho.^2 + beta2); end
    marker = ':';
    if (ps==1), txt = 'DSVD'; else txt = 'DGSVD'; end
elseif (strcmp(method,'mtsv',4))

    if (nargin~=6)
        error('The matrices L and V must also be specified')
    end
    [p,n] = size(L); rho = zeros(p,1); eta = rho;
    [Q,R] = qr(L*V(:,n-1:n-p),0);
    for i=1:p
        k = n-p+i;
        Lxk = L*V(:,1:k)*xi(1:k);
        zk = R(1:n-k,1:n-k)\Q(:,1:n-k)'*Lxk); zk = zk(n-k:-1:1);
        eta(i) = norm(Q(:,n-k+1:p)'*Lxk);
        if (i < p)
            rho(i) = norm(beta(k+1:n) + s(k+1:n).*zk);
        else
            rho(i) = eps;
        end
    end
    if (m > n & beta2 > 0), rho = sqrt(rho.^2 + beta2); end
    reg_param = (n-p+1:n)'; txt = 'MTSVD';
    U = U(:,reg_param); sm = sm(reg_param);
    marker = 'x'; ps = 2;
else
    error('Illegal method')
end

if (locate)
    RegParam = regcFunction(rho,eta,reg_param,U,sm,b,method);
end

end
Codes pertaining to Chapter 6 & Section 9.2

Data Processing and Spectral Fitting

function
[tr,f1,auto,mse]=spectral_data_processing(path,dataname,autofl_basis,w_l,w_u,plot_arg)
%Written by Alisha Dsouza
%last modified Oct 8 2012

%autofl_basis must be a cell array of 20 cells, make sure this is formateded
%correctly
%the path need not have a slash at the end of the string
%w_l is the lower limit of window of fit and w_u is the upper limit
%plot arg must be 0 or 1. 1 implies plots will be displayed as 4x5 plots in one figure window for each datapoint. 0 implies no plots

%load the basis spectra
load
'/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/Basis_spectra/ppix_basis.mat';%from omlc website
load
'/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/Basis_spectra/pp_p_gauss.mat';

%window
w_l_fit=w_l;
w_u_fit=w_u;

%create figure window
if plot_arg
f=figure('Position',[0,0,1680,1050], 'paperpositionmode', 'auto');
end

%load data into individual arrays
data_matrix=load([path,dataname, '_ProcData_Lambda_T_F_W.txt']);

wavelengths=data_matrix(:,1:5);
%dim 1= each wavelength value, dim2=detectors, dim3= sources
Transmission=reshape(data_matrix(:,6:25),2048,5,4);
Fluorescence=reshape(data_matrix(:,26:45),2048,5,4);

meas=0;
for s=1:4%source index
    for d=1:5%detector index
        meas=meas+1;
        ppix=ppix_basis;
        %load the data
%% transmission data
trans=Transmission(:,d,s);
trans=medfilt1(trans,3); % 3 point avg to remove spikes
wv=wavelengths(:,d); % wavelength

% window for transmission data

w_l= max(find(wv < 610));
w_u= max(find(wv < 740));
tr(meas)=max(trans(w_l:w_u)); % take only the max value within the window

%% fluorescence data
% preprocess
fluor_raw=Fluorescence(:,d,s);

diff_fl=medfilt1(fluor_raw,3); % spike removal
smooth_fluo=(smooth(diff_fl,14)); % smoothing filter
data_fl{meas}=smooth_fluo; % hold the data in this cell array
data_wv{d}=wv;
data_tr{meas}=trans;

% % fitting
% choose the right autofl basis spectrum for each detector.
%
if d==4
  autofl=autofl_basis{d}{4};
elseif d>1
  autofl=autofl_basis{d}{d-1};
else
  autofl=autofl_basis{d}{d};
end

autofl(:,2)=autofl(:,2)./max(autofl(:,2)); % normalize the autofl spectrum to the max value

bases{1}=autofl;
bases{i6}=ppp;
[fitted{meas},U]=linear_fit(wv,data_fl{meas},w_l_fit,w_u_fit,ppix,bases);

wv=fitted{meas}.wavelength;

if plot_arg
  nor=tr(meas);
  subplot(4,5,meas);
  plot(data_wv{d},diff_fl/nor,'color','k','linewidth',2); % raw data
  hold on
plot(data_wv{d},data_fl{meas}/nor,'color',[0.3,0.3,0.3],'linewidth',2);

mse(meas)=mean(abs(interp1(data_wv{d},data_fl{meas},wv)-
fitted{meas}.data)./abs(interp1(data_wv{d},data_fl{meas},wv)));

mse=mse';

legend([num2str(100*mse(meas)),'']);

plot(wv,fitted{meas}.ppix/nor,'b','linewidth',2);

plot(wv,fitted{meas}.autofl/nor,'g','linewidth',2);

plot(wv,fitted{meas}.ppp/nor,'c','linewidth',2);

plot(wv,fitted{meas}.data/nor,'r','linewidth',2);

plot(w_l_fit*(ones(100,1)),linspace(0,max(data_fl{meas})/nor,100),'--k','linewidth',1);

plot(w_u_fit*(ones(100,1)),linspace(0,max(data_fl{meas})/nor,100),'--k','linewidth',1);

hold off;
drawnow;
xlim([658,770]);

grid on;

xlabel('Wavelength [nm]', 'fontsize', 20);
ylabel('Intensity [A.U.]', 'fontsize', 20);
title([Source num2str(s) Detector ',num2str(d)],'fontsize',15);

set(gca,'fontsize',10);

end

fl(meas)=max(fitted{meas}.ppix);
auto(meas)=fitted{meas}.autofl;

clear trans_v;
clear fluor_raw;
end
end

Ultrasound Image Segmentation

clc
clear all;
close all;

conc=[0,0.025,0.05,0.1,0.2,0.4,0.8];

Location={RightHindLeg,LeftHindLeg,Abdomen};

dir='/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/Febr uary/Matrigel_PpIX_mice_02-14/Spatial Priors/';

for i=3
    for j=1
imname=[dir,num2str(conc(i)),'_',Location{j},'.png'];
img=imread(imname);
imname=[num2str(conc(i)),'_',Location{j},'.png'];
img=rgb2gray(img);
img=double(img);
img=img/max(img(:));

img2=img(67:542,61:661,:);
figure;
h_im=imshow(img2);
h=roispline(img2);
title('Select upper boundary');
h2=roispline(img2);
title('Select inclusion');

mask=im2uint8(zeros(size(img2)));
mask(h)=100;
mask(h2)=200;
figure
imshow(mask);
[m,n]=size(mask);
mask=im2double(mask);
pixel_dim=0.021;
% add 10 mm on either side and at the bottom

num_pixels=ceil(10/pixel_dim);
long_mask=zeros(m+num_pixels,n);
long_mask(1:m,:)=mask;
long_mask(m+1:end,:)=repmat(mask(end,:),[num_pixels,1]);
mask=long_mask;

ext_mask=zeros(m+num_pixels,2*num_pixels+n);

% ext_mask=zeros(m,2*num_pixels+n);

left_rep=repmat(mask(:,1),[1,num_pixels]);
right_rep=repmat(mask(:,end),[1,num_pixels]);

ext_mask(:,:,)=left_rep,mask,right_rep;
[m,n]=size(ext_mask);
subplot(3,4,3:4)
imshow(ext_mask);

% add 1 mm boundary
num_pixels=ceil(5/pixel_dim);

final_mask=zeros(2*num_pixels+m,2*num_pixels+n);

final_mask(num_pixels+1:num_pixels+m,num_pixels+1:num_pixels+n)=ext_mask;
final_mask=im2uint8(final_mask);
subplot(3,2,3:4); imshow(final_mask);
imwrite(final_mask,[dir,'Segmented_',imname]);
FEM Mesh Generator

clc;
clear all
close all;

path=['/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/February/Matrigel_PpIX_mice_02-14/'];

measmt={['S1-D1'], ['S1-D2'], ['S1-D3'], ['S1-D4'], ['S1-D5'], ...
        ['S2-D1'], ['S2-D2'], ['S2-D3'], ['S2-D4'], ['S2-D5'], ...
        ['S3-D1'], ['S3-D2'], ['S3-D3'], ['S3-D4'], ['S3-D5'], ...
        ['S4-D1'], ['S4-D2'], ['S4-D3'], ['S4-D4'], ['S4-D5']};

ppix=[0.2];
location={['RightHindLeg'], ['LeftHindLeg_regions'], ['Abdomen'], ['RightHindLeg2'],};
LOC={['R'], ['L'], ['A'], ['R2']};

for i=1:mouses
    mouse=i;%mouse number
    for j=1:length(LOC)
        mask=imread([path,'Spatial Priors/Segmented_',num2str(ppix(i)),'_',location(j),'.png']);
        imwrite(mask,'tempim.bmp');
        mask2mesh_2D('tempim.bmp',0.0206,1,100,'test','fluor');
        mesh=load_mesh('test');
        mesh=fix_fibers_usgft(mesh);
        mesh_plot(mesh);
        save_mesh(mesh,[path,'/Meshes/Coarse_Mesh_',num2str(ppix(i)), '_',LOC {j}]);
        delete('tempim.bmp');
        delete('test.*');
    end
end

White Light Spectroscopy

% Calibrate white light ultrasound-spatially resolved diffuse reflectance
%(US-SRDR) probe
clc
clear all
close all

%%
%Specify paths for analysis code
% addpath
/Users/stephen_c_kanick/Documents/SpectralData/Dartmouth/USDosimeter
/USDosimeter_AnalysisCode/Export/
addpath
/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/WLS_from_Cha
d/USDosimeter/USDosimeter_AnalysisCode/Export
%Specify paths for basis spectra
addpath
/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/WLS_from_Cha
d/USDosimeter/USDosimeter_AnalysisCode/BasisSpectra/

%Specify paths for data
addpath
/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/February/WLS
addpath
/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/February/Matrigel_PpIX_mice_02-14/Data

analysisdir=''/Users/alisha_dsouza/Desktop/Thayer/Research/USGFT/2013/February/Matrigel_PpIX_mice_02-14/Data'/;

% General Analysis inputs
% rho    = [22.5,17.5,12.5,7.5,2.5];  % Vector listing source
detector separations [mm]
% SD     = [5];                      % Select source-detector
distances included in fit (specify values corresponding to index in
rho)
% %Note: SD allows you to select which fibers to include in fit, specified by the index in rho)

% Tomography mode
rho_mat=[-2.5:5:17.5;...
  -7.5:5:12.5;...
  -12.5:5:7.5;...
  -17.5:5:2.5];
% rho{s} where s is the source number
% SD can be made to change depending on the source

% Plotting options
PLOTCAL=0; % binary switch to show calibrated raw data plots (yes
=1, no =0) [default =0]
PAUSEON=0; % binary switch to pause for each raw data plot (yes
=1, no =0) [default =0]
GRAPH = 0; % generate plot of fitting algorithm DURING fit 1 =
ON, 0 = OFF (NOTE: very slow when active)
figureprefix='USWLS_Fit_Mouse'; % Text prefix for saved plot of
reflectance fit, saved in analysisdir (eps file)

% Load calibration factor
load ILCalData_tomo.mat % Load 'C' calibration factor to convert
measurements in counts/sec to photons/mm^2
% Conversion factor generated using SRDRCal.m
% [Note: this step requires measurement in Intralipid phantom
with known
% dilution and comparison of those data with diffusion theory prediction
% Load spectra
% This requires the specification of measurement file text
% 'prefix'
% This could potentially be replaced by a matlab command that searches for
% all filenames within a specified directory!
% datalist={'a2012_10_09_14_50_Calibration',
%     'a2012_10_09_14_57_Calibration',
%     'a2012_10_09_15_09_Calibration',
%     'Mouse1_Preinjection_ProcData_Lambda_T_F_W',
%     'Mouse1_Postinjection_1_ProcData_Lambda_T_F_W',
%     'Mouse1_Postinjection_2_ProcData_Lambda_T_F_W',
%     'Mouse1_Postinjection_3_ProcData_Lambda_T_F_W',
%     'Mouse4_Preinjection_ProcData_Lambda_T_F_W',
%     'Mouse4_Postinjection_1_ProcData_Lambda_T_F_W',
%     'Mouse4_Postinjection_2_ProcData_Lambda_T_F_W',
%     'Mouse4_Postinjection_3_ProcData_Lambda_T_F_W',
%     'Mouse4_Postinjection_4_ProcData_Lambda_T_F_W',
%     'Mouse4_Postinjection_5_ProcData_Lambda_T_F_W',
% to be changed each time

dataname={'Calibration_1pctIL',
    'Calibration2_1pctIL'};

k=3;
ppix=[0.8,0.4,0.2,0.1,0.05,0.025,0];
location={[ 'RightHindLeg'],
    [ 'LeftHindLeg'],
    ['Abdomen'],[ 'RightHindLeg2']};
for i=1:7
    for j=[1,2,4]
        dat=([ 'Mouse',num2str(i),'_',location{j},'_',num2str(ppix(i))]);
        if ~exist([analysisdir,datalist{M}]);
            dataname{k}=dat;
            k=k+1;
        end
    end
end
for i=1:length(dataname)
    datalist{i}=[dataname{i},'_ProcData_Lambda_T_F_W.txt'];
end

% Parse raw spectra into data handles in structure 'W'
for M=1:length(datalist) % loop through each measurement file
    Data{M}=load([analysisdir,datalist{M}]);%load the file
    lambdaset=Data{M}(::,1:5); % grab the spectrophotometer-specific
wavelength basis spectrum

\[
\text{lambdafull} = \text{lambdafull}(:,1);
\]
% specify the wavelength-basis to be used as standard

\[
\text{Wrawset} = \text{reshape}([\text{Data}(M)(:,46:65)], 2048, 5, 4);
\]
% old code \( \text{Wrawset} = \text{eval}(\text{sprintf('"%s(:,[46:50])"', datalist{M}))} \);
% grab the raw white light spectrum

\[
\text{eval}(\text{sprintf('W.set\%g=Wrawset;',M))};
\]
% subtract dark noise from the raw spectrum

\[
\text{eval}(\text{sprintf('W.setI\%g=W.set\%g(:,1,1:4);',M,M))};
\]
% rename white light spectrum (dark noise corrected)

%% Interpolate data onto standard basis spectrum

\[
\text{for } s=1:4 \text{ % all sources}
\]
\[
\text{for } i=2:5 \text{ % we already have this for } i=1
\]

\[
\text{eval}(\text{sprintf('W.setI\%g(:,i,s)=interp1(lambdaset(:,i),W.set\%g(:,i,s),
\text{lambdafull)};',M,M))});
\]
end
end

%% Plot dark-noise corrected white light reflectance spectra

\[
\text{if (PLOTCAL)}
\]

\[
\text{figure}
\]
\[
\text{for } s=1:4
\]
\[
\text{subplot(2,2,s)};
\]
\[
\text{plot(lambdafull,eval(\text{sprintf('W.setI\%g(:,,:;s);',M))}',x'))}
\]
\[
\text{set(gca, 'yscale', 'log')}
\]
\[
\text{xlim([560 1000])}
\]
\[
\text{legend('rho1', 'rho2', 'rho3', 'rho4', 'rho5')}
\]
\[
\text{xlabel('Wavelength (nm)')}
\]
\[
\text{ylabel('Reflectance (counts/sec)')}
\]
\[
\text{title(['Raw White light spectra, log scale filename=
\text{', datalist{M})])};
\]
end
end

%% Calibrate data to absolute units (photons / mm2)

% Wavelength dependent calibration constant (for each fiber)
% Note this version of the code assumes that the first 3 measurements in
% datalist are measurements on the calibration reference (this must be
% manually adjusted if there are different numbers of ref data for this
% version.

\[
\text{CalData} = (W.setI1 + W.setI2)/2; \quad \text{\% Averages reference spectra}
\]
\[
\text{C = CalData/CalData; \quad \% Ratio represents conversion factor of expectation [model (photons/m2)] divided by measurement [reference (counts/msec)]}
\]

% Convert measurements to absolute units using calibration constant derived
% from measurements in Intralipid
for k=1:length(datalist)
    eval(sprintf('W.abs%g=C.*W.setI%g;',k,k)); %multiply dark
corrected time normalized data by calibration constant to convert
units from (counts / msec) to (photons / mm2)

%Plot calibrated white light reflectance spectra
if(PLOTCAL)
    figure
    for s=1:4
        subplot(2,2,s)
        hold on
        plot(lambdafull,eval(sprintf('W.abs%g(:,1:5,s);',k)),'x')
        set(gca,'yscale','log')
        axis([560 1000 10^-15 1])
        xlabel('Wavelength (nm)')
        ylabel('Reflectance (photons/mm^2)')
        legend('rho1','rho2','rho3','rho4','rho5')
        title(['Calibrated White light spectra, log scale filename='
               ',datalist{M}]);
        hold off
    end
end

% Smooth reflectance spectra using 'boxcar' moving average filter
bin=7; %specify width of boxcar (in terms of measured pixels)

[lambda,W]=smoothUSspectra_tomo(lambdafull,W,length(datalist),bin);
%return reduced wavelength and smoothed intensity spectra
%Note: lambda and adjusted outputs of W are a reduced data set

close all

% Fit estimates of musp and mua for non-calibrated Intralipid

phantoms

%Select wavelength range to perform fitting
wavestart=570; %wavelength to begin fit [nm]
waveend=910; %wavelength to end fit [nm]

%(Optional) specify range of wavelengths to weight in fit
%(Not utilized in current version of code)
weightwavestart=700; %wavelength to begin weighted region [nm]
weightwaveend=760; %wavelength to end weighted region [nm]
weightfactor=1; %weighting factor [default= 1, reduced to
unweighted fit]

%Find indecies for data included in fit
wvbd =
gt_pixel_index(wavestart,lamba):get_pixel_index(waveend,lamba);
   v =
gt_pixel_index(weightwavestart,lamba):get_pixel_index(weightwaveen
d,lamba); % for use as nm(u(v)) in emphasizing fit to the 500-600
nm range, to specify oxy/deoxy hemoglobin

%Input fit parameters / initial guesses
\[ S = 0.655; \]  \% Tissue oxygen saturation (mixture of arterial and venous blood) [fraction]

\[ a = 0.5; \]  \% Scale the skin scattering (Rayleigh + Mie)

\[ \text{const} = 0.99; \]  \% Estimate verticle offset (and influence of index of refraction mismatch between sample and reference)

\[ \text{Col} = 0.1; \]  \% Estimate collagen [fraction]

\[ B = 0.01. \ast \text{ones}(1, \text{length}(SD)); \]  \% mean blood volume fraction in skin [fraction]

\[ B = 0.01; \]

\text{start} = [S a \text{const} Col B]; \%build initial guess vector

\%Alternatively, estimate single BVF for all sampled depths

\[ \% = 0.01; \]  \% mean blood volume fraction in skin [fraction]

\%Assumed paramters (not estimated in current version of code

\[ \text{Wat} = 0.65; \]  \% Assumed water content [fraction]

\[ \text{Fat} = 0.2; \]  \% Assumed water content [fraction]

\[ n = 1; \]  \% Specify relative index of refraction [default estimate for tissue = 1.37]

\%Parameters not fully utilized in current code (for mouse skin)

\[ \text{Mel} = 0.0005; \]  \% average volume fraction of melanosomes in 60-um-thick epidermis [fraction] (0.0005 for homogenous melanin)

\[ \text{MELFILTER} = 0; \]  \% Model melanin only in epidermis and not distributed throughout tissue (on=1, off=0) [Default on; initial parameters / model may need to be adjusted to perform fit without this assumption]

\[ \text{nmOff} = 0; \]  \% Estimate wavelength-dependent shift (Not enabled in this version)

\%Specify parameter of fitting algorithm (lsqnonlin)

\[ \text{OPTS} = \text{optimset}(\text{'Algorithm'}, 'levenberg-marquardt', \text{'Display'}, 'on', \text{'TolFun'}, 0.00000001, \text{'TolX'}, 0.00000001, \text{'MaxFunEvals'}, 99999, \text{'MaxIter'}, 99999, \text{'ScaleProblem'}, 'Jacobian'); \]

\%Load Basis spectra for fitting

[Basis]=get_Basis(lambda(wvbd))

\%Fitting algorithm

\begin{verbatim}
for j=3:length(datalist) \% loop through each measurement file
[Note loop starts at index AFTER calibration file(s)]
    for s=1:4
        for d=1:5
            rho=abs(rho_mat(s,d));
            eval(sprintf( 'Rdata=W.SFRsmooth\%g(:,d,s);',j,j)); \% Grab intensity spectra included in fit
            eval(sprintf( 'Rstd=W.SFRsmoothstd\%g(:,d,s);',j,j)); \% Grab standard deviation of intensity spectra included in fit
            \%Perform fit using lsqnonlin- perform fit iteratively, using initial result as basis for re-initialization of algorithm with perturbation made to vascular physiological parameters
            \[\text{result}(j)(:,:,:),RSENS,RRESIDUAL,EXITFLAG,OUTPUT,LAMBDA,J\]
            =lsqnonlin(@fitmouseskinUSspectrum,start,[],[],OPTS, lambda(wvbd),

\end{verbatim}
Rdata(wvbd), Rstd(wvbd), rho, Basis, n, v, Wat, Fat, GRAPH, MELFILTER, nmOff);

% result{j}(:,d,s)=abs(result{j}(:,d,s)) ;
% CI = nlparci(result{j}(:,d,s),RESIDUAL,'jacobian',J);
%calculate 95% confidence intervals for parameter estimates
% result{j}(:,d,s)=abs(result{j}(:,d,s))
%parameters allowed to enter negative space- make non-negative
% result_CIhw{j}(:,d,s)=(CI(:,2)-CI(:,1))./2;
%calculate confidence interval half-widths
end
end
end

%%
%Plotting algorithm
k=1;
for j=3:length(datalist)%loop through each measurement file
[Note loop starts at index AFTER calibration file(s)]
for s=1:4
for d=1:5
rho=abs(rho_mat(s,d));
eval(sprintf('Rdata=W.SFRsmooth%g(:,d,s);',j,j)); % Grab
intensity spectra included in fit
eval(sprintf('Rstd=W.SFRsmoothstd%g(:,d,s);',j,j)); % Grab
standard deviation of intensity spectra included in fit

%Generate output plots for each measurement
%Including: (1) reflectance (For each source-detector
distance);
% (2) absorption coefficient spectrum;
% (3) reduced scattering coefficient spectrum
% Also calculate mean residual error between model fit and
data (expressed as % of data)
figure;
[mse{k}(:,d,s),muatot_est{k}(:,d,s),musptot_est{k}(:,d,s)]=plotoutput
ts_USmousespectra_tomo(result{j}(:,d,s),lambda(wvbd), Rdata(wvbd),
Rstd(wvbd), rho, Basis, n, Wat, Fat, MELFILTER, nmOff,
datalist{j},[analysisdir,'WLS/'],figureprefix);

if (PAUSEON)
pause
end
end
result_final{k}=abs(result{j});
close all
k=k+1;
end

%%
% Estimate optical properties at selected wavelengths
% Specifically: mua(lambdax, lambdam) and musp(lambdax, lambdam)
lambdax = 633;
lambdam = 700;
indX = get_pixel_index(lambdax, lambda(wvbd));
indM = get_pixel_index(lambdam, lambda(wvbd));

for j = 1:length(datalist) - 2
    for s = 1:4
        for d = 1:5
            mua_output_X(s, d, j) = abs([muatot_est{j}((indX), d, s)]);
            mua_output_M(s, d, j) = abs([muatot_est{j}((indM), d, s)]);
            musp_output_X(s, d, j) = abs([musptot_est{j}((indX), d, s)]);
            musp_output_M(s, d, j) = abs([musptot_est{j}((indM), d, s)]);
        end
    end
end

dlmwrite(fullfile(analysisdir, 'WLS/', dataname{j+2}, '_WLS_SD_muaX.txt'), mua_output_X(:, :, j), 'delimiter', '\t', 'precision', 5);
dlmwrite(fullfile(analysisdir, 'WLS/', dataname{j+2}, '_WLS_SD_muaM.txt'), mua_output_M(:, :, j), 'delimiter', '\t', 'precision', 5);
dlmwrite(fullfile(analysisdir, 'WLS/', dataname{j+2}, '_WLS_SD_muspX.txt'), musp_output_X(:, :, j), 'delimiter', '\t', 'precision', 5);
dlmwrite(fullfile(analysisdir, 'WLS/', dataname{j+2}, '_WLS_SD_muspM.txt'), musp_output_M(:, :, j), 'delimiter', '\t', 'precision', 5);

mua_output_Xavg(j) = abs(mean2(mua_output_X(:, :, j)));
mua_output_Mavg(j) = abs(mean2(mua_output_M(:, :, j)));
musp_output_Xavg(j) = abs(mean2(musp_output_X(:, :, j)));
musp_output_Mavg(j) = abs(mean2(musp_output_M(:, :, j)));

dlmwrite(fullfile(analysisdir, 'WLS/', dataname{j+2}, '_WLS_avg.txt'), [mua_output_Xavg(j), mua_output_Mavg(j), musp_output_Xavg(j), musp_output_Mavg(j)], 'delimiter', '\t', 'precision', 6);
dlmwrite(fullfile(analysisdir, 'WLS/', dataname{j+2}, '_WLS_result.txt'), result_final(j), 'delimiter', '\t', 'precision', 6);
end

% Write away fitted parameters
% output_params_export=result;
% for s = 1:4
%     for j = 1:length(datalist) - 2
%         output_param_export(
eval(sprintf('dlmwrite('%s/MouseParams.txt',output_params_export,'delimiter','\t','precision',6),analysisdir));

output_optprop=[mua_output_Xavg,mua_output_Mavg,musp_output_X,musp_output_M];

eval(sprintf('dlmwrite('%s/Mouse_OptProp.txt',output_optprop,'delimiter','\t','precision',6),analysisdir));

% % Estimate transport resistance 'X' factors
% [T_X]=Xfactor_est(mua_output_Xavg,musp_output_X,rho)
% [T_M]=Xfactor_est(mua_output_Mavg,musp_output_M,rho)
% %
% T_Avg=(T_X + T_M)./2;
% T_Diff=100.*(T_M - T_X)./T_X;

Custom Nirfast-based Reconstruction routine for USFT

function [fwd_mesh,pj_error] = reconstruct_fl_region_usgft(fwd_mesh,...
  frequency,...
  data_fn,...
  iteration,...
  lambda,...
  output_fn,...
  filter_n,...
  region)

% function [fwd_mesh,pj_error] = reconstruct_fl_region(fwd_mesh,...
%   frequency,...
%   data_fn,...
%   iteration,...
%   lambda,...
%   output_fn,...
%   filter_n,...
%   region)
%
% region-based reconstruction program for fluorescence meshes
% fwd_mesh is the input mesh (variable or filename)
% frequency is the modulation frequency (MHz)
% data_fn is the boundary data (variable or filename)
% iteration is the max number of iterations
% lambda is the initial regularization value
% e.g. [20 20 15; 20 20 15] for a 3D mesh with 2 regions
% output_fn is the root output filename
% filter_n is the number of mean filters
% region is an array of the regions (e.g. [0 1 2])
%
% always CW for fluor
frequency = 0;

% Read data
data = load_data(data_fn);
if ~isfield(data,'amplitudefl')
    errordlg('Data not found or not properly formatted','NIRFAST Error');
    error('Data not found or not properly formatted');
end
% remove zeroed data
ind = data.link(:,3)==0;
data.amplitudefl(ind,:) = [];
clear ind
anom = log(data.amplitudefl);
% Only reconstructs fluorescence yield!

% load fine mesh for fwd solve: can input mesh structured variable
% or load from file
if ischar(fwd_mesh)==1
    fwd_mesh = load_mesh(fwd_mesh);
end
if strcmp(fwd_mesh.type,'fluor')
    errordlg('Mesh type is incorrect','NIRFAST Error');
    error('Mesh type is incorrect');
end
fwd_mesh.link = data.link;
clear data
etamuaf_sol=[output_fn '_etamuaf.sol'];

% Initiate log file
fid_log = fopen([output_fn '.log'],'w');
fprintf(fid_log,'Forward Mesh   = %s
',fwd_mesh.name);
fprintf(fid_log,'Frequency      = %f MHz
',frequency);
if ischar(data_fn) ~= 0
    fprintf(fid_log,'Data File      = %s
',data_fn);
end
if isstruct(lambda)
    fprintf(fid_log,'Initial Regularization  = %d
',lambda.value);
else
    fprintf(fid_log,'Initial Regularization  = %d
',lambda);
end
fprintf(fid_log,'Filtering        = %d
',filter_n);
fprintf(fid_log,'Output Files   = %s',etamuaf_sol);
fprintf(fid_log,'Initial Guess muaf = %d
',fwd_mesh.muaf(1));
%fprintf(fid_log,'Output Files   = %s',tau_sol);
fprintf(fid_log,'
');

% get direct excitation field
% Flag mesh to not calculate the intrinsic emission and fluorescence
% emission fields
fwd_mesh.fl = 0; fwd_mesh.mm = 0;
if isfield(fwd_mesh,'phix')~=0
    fwd_mesh = rmfield(fwd_mesh,'phix');
end
% calculate excitation field
data_fwd = femdata(fwd_mesh,frequency);
data_fwd.phi = data_fwd.phix;

%**********************************************************
% initialize projection error
pj_error=[];
%**********************************************************
% modulation frequency
omega = 2*pi*frequency*1e6;
% set fluorescence variables
fwd_mesh.gamma = (fwd_mesh.eta.*fwd_mesh.muaf)./(1+(omega.*fwd_mesh.tau).^2);

%**********************************************************
% Calculate region mapper
disp('calculating regions');
if ~exist('region','var')
    region = unique(fwd_mesh.region);
end
K = region_mapper(fwd_mesh,region);

%**********************************************************
% Calculate part of Jacobian which does not change at each iteration
% (call it "pre-Jacobian")
[Jpre,datafl,MASS_m] = prejacobian_fl(fwd_mesh,frequency,data_fwd);

%**********************************************************
% Iterate
for it = 1 : iteration

% Update Jacobian with fluorescence field (changes at each iteration)
if it == 1
    [Jwholem,junk] = update_jacobian_fl(Jpre,fwd_mesh,frequency,data_fwd,MASS_m);
    clear junk
else
    [Jwholem,datafl] = update_jacobian_fl(Jpre,fwd_mesh,frequency,data_fwd,MASS_m);
end
Jm = Jwholem.completem; clear Jwholem

% Read reference data
clear ref;
ind = datafl.link(:,3)==0;
datafl.amplitudem(ind,:) = []; clear ind
ref(:,1) = log(datafl.amplitudem);
gauss_weight=zeros(20,1);
gauss_x=[-10:9];
gauss_weight=normpdf(gauss_x,0,2);
gauss_weight=gauss_weight/max(gauss_weight(:));
%     gauss_weight(2)=1;
%     gauss_weight(end-1)=1;
data_diff = (anom-ref).*gauss_weight;
pj_error = [pj_error sum(abs(data_diff.^2))];

% Screen and Log Info

disp('---------------------------------');
disp(['Iteration_f1 Number = ' num2str(it)]);
disp(['Projection_f1 error = ' num2str(pj_error(end))]);

fprintf(fid_log,'---------------------------------
');
fprintf(fid_log,['Iteration_f1 Number = %d
',it]);
fprintf(fid_log,['Projection_f1 error = %f
',pj_error(end)]);

if it ~= 1
    p = (pj_error(end-1)-pj_error(end))*100/pj_error(end-1);
disp(['Projection error change = ' num2str(p) ' %']);
    fprintf(fid_log,['Projection error change = %f %%
',p]);
    if (p) <= 2
        disp('---------------------------------');
        disp('STOPPING CRITERIA FOR FLUORESCENCE COMPONENT REACHED');
    fprintf(fid_log,'---------------------------------
');
    fprintf(fid_log,'STOPPING CRITERIA FOR FLUORESCENCE COMPONENT REACHED
');
    % set output
    data_recon.elements = fwd_mesh.elements;
    data_recon.etamuaf = fwd_mesh.etamuaf;
    break
end
end

%*************************
clear data_recon

Jm = Jm*diag([fwd_mesh.gamma]);

% reduce J into regions!
Jm = Jm*K;

% build Hessian
[nrow,ncol]=size(Jm);
Hess = zeros(nrow);
Hess = Jm*Jm';

% add regularization
reg = lambda.*(max(diag(Hess)));
disp(['Regularization Fluor = ' num2str(reg)]);
fprintf(fid_log,'Regularization Fluor            =
%f
',reg);
Hess = Hess+(eye(nrow).*reg);

% Calculate update
u = Jm'*(Hess\data_diff);

% use region mapper to unregionize!
u = K*u;
u = u.*{fwd_mesh.gamma};

% value update:
fwd_mesh.gamma = fwd_mesh.gamma+u;
fwd_mesh.etamuaf = fwd_mesh.gamma.*(1+(omega.*fwd_mesh.tau).^2);
% assuming we know eta
fwd_mesh.muaf = fwd_mesh.etamuaf./fwd_mesh.eta;

%%% ind=find(fwd_mesh.muaf<0); %added by alisha
fwd_mesh.muaf(ind)=1e-20; %added by alisha
%%% clear u Hess Hess_norm tmp data_diff G

% filter
if filter_n ~= 0
    disp('Filtering');
    fwd_mesh = mean_filter(fwd_mesh,filter_n);
end

%**********************************************************
% Write solution to file

if it == 1
    fid = fopen(etamuaf_sol,'w');
else
    fid = fopen(etamuaf_sol,'a');
end
fprintf(fid,'solution %d ',it);
fprintf(fid,'-size=%d ',length(fwd_mesh.nodes));
fprintf(fid,'-components=1 ');
fprintf(fid,'-type=nodal\n');
fprintf(fid,'%g ',fwd_mesh.etamuaf);
fprintf(fid,\n');
fclose(fid);

end
fin_it = it-1;
fclose(fid_log);
Jacobian Sensitivity Map

```matlab
function sensitivity_plot(loaded_mesh)
% alisha dsouza
% mesh must be loaded for this to work
% mesh plot with nodes and elements

mesh=loaded_mesh;
J=jacobian(mesh,0);
J=J.completem;
J=sum(J);
figure;
c=[0.5 0.5 0.5];
trisurf(mesh.elements,mesh.nodes(:,1),mesh.nodes(:,2),double(J),'
facealpha',0.5)
colormap(hot); view(2); shading flat;
axis equal;
end
```

Codes pertaining to Chapter 9

Log-Compression function

```matlab
function im_log=logcompression(image, logbase)

temp=image; temp=log10(temp)/log10(logbase); temp(isinf(temp))=0;
temp(isinf(-temp))=0; temp(isnan(temp))=0; temp=temp-min(temp(:));
im_log=temp/max(temp(:));
```

Adaptive Log-Compression of static images

```matlab
clc;
clear all;
close all;
k=0;

Project_dir=['/Users/alisha_dsouza/Desktop/Thayer/Research/Post Thesis Proposal/...'
'Log Compression Image Displays/Test Cases/phantom imagings to Alisha/phantom imagings to Alisha/phantom results on Pearl/ABY_029'];

StartFilenumber=1;
Images=1;
%% read the data
Folder800=['/800 Images/'];
cd([Project_dir,Folder800]);
files800=dir(fullfile('*TIF'));
```
FolderWhite=['/White Images/'];
cd([Project_dir,FolderWhite]);
filesWhite=dir(fullfile('*.*TIF'));

for i=Images
    reader = bfGetReader([Project_dir,Folder800,files800(i).name]);
    image800(:,:,i) = bfGetPlane(reader, 1);
    reader = bfGetReader([Project_dir,FolderWhite,filesWhite(i).name]);
    imageWhite(:,:,i) = bfGetPlane(reader, 1);
end

f1=figure;
set(f1, 'Position', [0 0 1680 1050 ], 'PaperPositionmode','manual');
f2=figure;
set(f2, 'Position', [0 0 1680/2 1050 ], 'PaperPositionmode','manual');
load '/Users/alisha_dsouza/Desktop/Thayer/Research/Post Thesis Proposal/Log Compression Image Displays/bio_parula.txt'
% colormap(bio_parula/255);
c=zeros(256,3);
c=[zeros(256,1),[0:255]',zeros(256,1)];
c=c/255;

k=0;
for j=Images
    k=k+1;
    im=image800(:,:,j);
    imWL=imageWhite(:,:,1);

    %convert all images to double
    im(im<0)=0;
    im(isinf(im(:)))=0;
    im(isinf(-im(:)))=0;
    im=im-min(im(:));
    im=medfilt2(im,[5,5]);
    im=double(im);
    im=im/max(im(:));
    imWL(isinf(imWL(:)))=0;
    imWL=imWL-min(imWL(:));
    imWL=double(imWL);
    imWL=imWL/max(imWL(:));

    if k==1
        figure(f2);
        imshow(adapthisteq(imWL,'nbins',65000));
        [~,h]=imcrop;
    end

    im=imcrop(im,h);
imWL=imcrop(imWL,h);

imWL=imadjust(imWL,[0;0.02],[0;1]);
imWL(imWL(:)>0.5)=0.1;
imWL=medfilt2(imWL,[9,9]);

%generate rgb images
im_rgb=zeros(size(im,1),size(im,2),3);
im_rgb(:,:,2)=im;
imWL_rgb=zeros(size(im,1),size(im,2),3);

figure(f1);
set(gcf,'Position', [0 0 1680 1050 ],
'PaperPositionmode','manual');
subplot(261);imshow(imWL_rgb+im_rgb);
subplot(267);imhist(im);colormap(c);
set(gca,'fontsize',15);

%WL
im_adj=imadjust(im,[0;0.01],[0;1]);
im_rgb(:,:,2)=im_adj;
subplot(262);imshow(imWL_rgb+im_rgb);
subplot(268);imhist(im_adj);colormap(c);
set(gca,'fontsize',15);
hgram=imhist(im_adj,256);

%adaptive histogram equalization
im_ha=adapthisteq(im,'nbins',256);
im_ha=imadjust(im_ha,[0;0.3],[0;1]);
im_rgb(:,:,2)=im_ha;
subplot(263);imshow(imWL_rgb+im_rgb);
subplot(2,6,9);imhist(im_ha);colormap(c);
set(gca,'fontsize',15);

%log compression
base=1.06;
temp=im;
temp=log10(temp)/log10(base);
temp(isinf(temp))=0;
temp(isinf(-temp))=0;
temp(isnan(temp))=0;
temp=temp-min(temp(:));
temp=temp/max(temp(:));
%log
im_log=temp;
im_rgb(:,:,2)=im_log;
subplot(264);imshow(imWL_rgb+im_rgb);
subplot(2,6,10);imhist(im_log);colormap(c);
set(gca,'fontsize',15);
hgram=imhist(im_log);
im_log_adj=imadjust(im_log,[0.01;0.7],[0,1]);
% im_log_adj=imadjust(im_log,[0.2;1],[0,1]);
im_rgb(:,:,2)=im_log_adj;
subplot(265);imshow(imWL_rgb+im_rgb);
subplot(2,6,11);imhist(im_log_adj);colormap(c);
set(gca,'fontsize',15);
%log adaphist
im_log_h=adapthisteq(im_log,'nbins',256);
im_log_h_adj=imadjust(im_log_h,[0.35;1],[0;1]);
im_rgb(:,:,2)=im_log_h_adj;
subplot(266);imshow(imWL_rgb+im_rgb);
subplot(2,6,12);imhist(im_log_h_adj);colormap(c);
set(gca,'fontsize',15);
end

Additional Miscellaneous functions

Rename and Restructure Image Data from Pearl Imager
clc;
clear all;
close all;
for j=[323]Folder=['/Volumes/UNTITLED/MDA_MB_Mammary Tumors DEc 2015/Mouse',num2str(j),'/'];% FolderNames=dir([Folder]);
%700 images
mkdir([Folder,'700 Images']); mkdir([Folder,'800 Images']); mkdir([Folder,'White Images']); mkdir([Folder,'Acq']);
for i=5:length(FolderNames)
  if length(FolderNames(i).name)<4
    continue;
  end
  files700{i}=dir([Folder,FolderNames(i).name,'/*700.TIF']);
  files800{i}=dir([Folder,FolderNames(i).name,'/*800.TIF']);
  filesWL{i}=dir([Folder,FolderNames(i).name,'/*White.TIF']);
  if ~isempty(files700{i})
    movefile([Folder,FolderNames(i).name,'/',FolderNames(i).name,'_700.TIF'],[Folder,'700 Images/']);
  end
  if ~isempty(files800{i})
    movefile([Folder,FolderNames(i).name,'/',FolderNames(i).name,'_800.TIF'],[Folder,'800 Images/']);
  end
  if ~isempty(filesWL{i})
    movefile([Folder,FolderNames(i).name,'/',FolderNames(i).name,'_White .TIF'],[Folder,'White Images/']);
  end
  movefile([Folder,FolderNames(i).name,'/',FolderNames(i).name,'_acq.txt'],[Folder,'Acq/']);
  end
end

Extract Timestamps from Image Data from Pearl Imager
function [TimeAxis,PreInj]=extractTime(a,injection_time)%extracts
time axis relative to injection time from Acq files from Pearl imager. Function also returns PreInj which is the index of the preinjection timepoint.

```matlab
[Time_vec]=gettime(a);
Time_axis=etime(Time_vec,repmat(injection_time,[length(Time_vec), 1]));
TimeAxis=Time_axis/60; PreInj=find(TimeAxis<0, 1, 'last'); clear Time_axis;
end
function [times] = gettime(Folder)
    Time_files=dir(fullfile([Folder,'/Acq'], '*.txt'));
times=zeros(length(Time_files),6);
    for i=1:length(Time_files)
        a=importdata([Folder,'/Acq/',Time_files(i).name]); fileID = fopen([Folder,'/Acq/',Time_files(i).name]); C = textscan(fileID, '%s %f32 %f32 %f32 %f32 %f32 %f32', 'CommentStyle', '#', 'Delimiter', '	'); fclose(fileID);
        dateTime=str2num(C{1,1}{2,1}(11:end));
times(i,:)=[0 0 0, dateTime(1,4:6)];
    end
end
```

Boxplots with datapoints

```matlab
function boxy(A,group,Label)
    bh=boxplot(A,group,'plotstyle','traditional','width',0.4,'whisker',2); hold on; groupspread=group+(-0.1+0.2*rand(1,length(A)));
    plot(groupspread,A,'o', 'color',[0,0.5,0], 'markersize',8, 'markerfacecolor',[0,0.5,0]);
    set(gca,'xtick',unique(group)); set(gca,'XTickLabel',Label);
    set(gca,'fontsize',20); set(bh(:,:),'linewidth',2);
end
```
Appendix II: LabView Front Panel and Block

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