Development of Clinically Translatable Technologies for Optical Image-Guided Breast Tumor Removal Surgery

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2014
ABSTRACT

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Abstract

The rate of occurrence and number of deaths associated with cancer continues to climb each year despite the continual efforts to battle the disease. When given a cancer diagnosis, it is particularly demoralizing and devastating news to a patient. Generally, cancer is defined as the uncontrolled rapid growth of abnormal cells with metastatic potential. In the cancer types originating from solid tissue or organ sites, a tumor will grow as a result of this rapid proliferation of cells. Surgical resection is a commonly used as part of the treatment regimen prescribed for these types of cancer.

Specifically in breast cancer, which impacts over 200,000 women a year, surgical intervention is used in almost 92% of treated cases. A specific surgical procedure is known as breast conserving surgery (BCS), where the physician removes only the tumor, while retaining as much normal tissue as possible. BCS is used in 59% of cases and is generally more preferable than the more radically mastectomy procedure where the entire breast is removed.

To minimize the chance of local recurrence, it is vital that the tumor is completely removed and residual cancer cells are not still present in the patient. This diagnosis is made by inspecting the edge of the resected tumor mass, typically known as the surgical margin. If tumor cells are still present at the margin, then a positive diagnosis is given and tumor cells likely remain inside the patient. Unfortunately, since margins are
typically diagnosed using post-operative pathology a patient with a positive margin must undergo a second re-excision operation to remove additional tissue.

For breast cancer patients undergoing BCS, a staggering 20-70% of patients must undergo additional operations due to incomplete tumor removal during the first procedure.

Currently, there are two intra-operative techniques that are used, frozen section analysis and touch prep cytology. Although both have been proven to be effective in reducing re-excision rates, both techniques require

There remains a clinical unmet need for an intra-operative technology capable of quickly diagnosis tumor margins during the initial surgical operation

Optical technologies provide an attractive method of quickly and non-destructively assessing tissue. These techniques rely the interactions of light with tissue, which include absorption, scattering, and fluorescence. Utilizing proper measurement systems, these interactions can be measured and exploited to yield specific sources of contrast in tissue. In this dissertation, I have focused on developing two specific optical techniques for the purpose of surgical margin assessment.

The first is diffuse reflectance spectroscopy (DRS) which is a specific method to extract quantitative biological composition of tissues has been used to discern tissue types in both pre-clinical and clinical cancer studies. Typically, diffuse reflectance spectroscopy systems are designed for single-point measurements. Clinically, an
imaging system would provide valuable spatial information on tissue composition. While it is feasible to build a multiplexed fiber-optic probe based spectral imaging system, these systems suffer from drawbacks with respect to cost and size. To address these I developed a compact and low cost system using a broadband light source with an 8-slot filter wheel for illumination and silicon photodiodes for detection. The spectral imaging system was tested on a set of tissue mimicking liquid phantoms which yielded an optical property extraction accuracy of $6.40 \pm 7.78\%$ for the absorption coefficient ($\mu_a$) and $11.37 \pm 19.62\%$ for the wavelength-averaged reduced scattering coefficient ($\mu'_s$).

While DRS provided one potential approach to margin diagnosis, the technique was inherently limited in terms of lateral resolution. The second optical technique I chose to focus on was fluorescence microscopy, which had the ability to achieve lateral resolution on the order of microns. Cancer is associated with specific cellular morphological changes, such as increased nuclear size and crowding from rapidly proliferating cells. *In situ* tissue imaging using fluorescent stains may be useful for intraoperative detection of residual cancer in surgical tumor margins. I developed a widefield fluorescence structured illumination microscope (SIM) system with a single-shot FOV of $2.1 \times 1.6 \text{ mm}^2 (3.4 \text{ mm}^2)$ and sub-cellular resolution ($4.4 \mu\text{m}$). The objectives of this work were to measure the relationship between illumination pattern frequency and optical sectioning strength and signal-to-noise ratio in turbid (i.e. thick) samples for selection of the optimum frequency, and to determine feasibility for detecting residual
cancer on tumor resection margins, using a genetically engineered primary mouse model of sarcoma. The SIM system was tested in tissue mimicking solid phantoms with various scattering levels to determine impact of both turbidity and illumination frequency on two SIM metrics, optical section thickness and modulation depth. To demonstrate preclinical feasibility, *ex vivo* 50 µm frozen sections and fresh intact thick tissue samples excised from a primary mouse model of sarcoma were stained with acridine orange, which stains cell nuclei, skeletal muscle, and collagenous stroma. The cell nuclei were segmented using a high-pass filter algorithm, which allowed quantification of nuclear density. The results showed that the optimal illumination frequency was 31.7 µm$^{-1}$ used in conjunction with a 4x 0.1 NA objective. This yielded an optical section thickness of 128 µm and an 8.9x contrast enhancement over uniform illumination. I successfully demonstrated the ability to resolve cell nuclei *in situ* achieved via SIM, which allowed segmentation of nuclei from heterogeneous tissues in the presence of considerable background fluorescence. Specifically, I demonstrated that optical sectioning of fresh intact thick tissues performed equivalently in regards to nuclear density quantification, to physical frozen sectioning and standard microscopy.

However the development of the SIM system was only the first step in showing potential application to surgical margin assessment. The nest study presented in this dissertation was to demonstrate clinical viability on a sample size of 23 animals. The biological samples used in this study were a genetically engineered mouse model of
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As an intermediate step prior to diagnosing whole margins, a tissue-type classification model was developed to differentiate localized regions (75x75 µm) of tumor from skeletal muscle and adipose tissue based on the MSER nuclei segmentation output. A logistic regression model was used which yielded a final output in terms of probability (0-100%) the tumor within the localized region. The model performance was tested using an ROC curve analysis that revealed a 77% sensitivity and 81% specificity. For margin classification, the whole margin image was divided into localized regions and this tissue-type classification model was applied. In a subset of 6 margins (3 negative, 3 positive), it was shown that at a tumor probability threshold of 50% only 8% of all regions from a negative margins exceeded this threshold, while over 25% of all regions exceeded the threshold in the positive margins.
Dedication

To my mother and father, Grace and Johnny Fu, whom having given me love and support my entire life. And to Jerry Fu, whom has been a great and supportive older brother and friend.
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Symbols and Abbreviations

\[ \beta_c \] – \( \beta \)-carotene

\[ \mu_a \] – absorption coefficient

\[ \mu_s \] – scattering coefficient

\[ \mu_s' \] – reduced scattering coefficient, \( \mu_s(1-g) \)

\[ \mu_m \] – micrometer or micron

\[ < > \] – average value

\[ \sigma \] – standard deviation

\[ [ ] \] – concentration

AO – acridine orange

AUC – area under the curve

BCS – breast-conserving surgery

CCD – charge-coupled device

CDF – cumulative distribution function

DRS – diffuse reflectance spectroscopy

FOV – field of view

H&E – hemotoxylin and eosin

Hb – hemoglobin or total hemoglobin

HbH – deoxy-hemoglobin

HbO\(_2\) – oxy-hemoglobin
HPF – high-pass filter

MSER – maximally stable extremal regions

NA – numerical aperture

n – sample size

nm – nanometer

RFP – red fluorescent protein

ROC – receiver operating characteristic

ROI – region of interest

SIM – structured illumination microscopy

SLM – spatial light modulator

SNR – signal-to-noise ratio
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thank the members of the Willett lab that have helped with the image processing algorithms, Dr. Zach Harmany and Albert Oh.
1. Background and Significance

Cancer is a complex and devastating disease that is continually among the top causes of death worldwide. Its heterogeneous nature and impact on virtually all cell types and organ sites pose challenges to current treatments. In 2014, it is estimated that 1,665,540 new cases of cancer will be diagnosed, equivalent to about 4,500 new diagnoses per day [1]. For men and women, the most common occurring cancer type is prostate and breast cancer, respectively, with over 230,000 estimated new cases of each. In cancers originating from these two tissue types, the disease manifests itself as a physical tumor due to the unregulated and increased replication of cancer cells. Obviously, this prognosis is not limited to only these two organ sites, but virtually all types of cancer except for leukemias (cancer of the blood). To treat these solid tumors, surgical intervention is commonly used to de-bulk the mass. In prostate cancer, surgery is prescribed as part of the treatment 48% of cases and in breast cancer, surgery is used 92% of cases [2]. Unfortunately, there are specific shortcomings of surgical treatments that will be outlined in this section, with a focus on breast cancer due to its overwhelming prevalence.

1.1 Breast Conserving Surgery (BCS)

In 2009, almost 200,000 women with early stage breast cancer and/or carcinoma in situ (CIS) received breast conserving surgery (BCS) [3]. BCS involves removal of malignant tissue with a surrounding margin of normal breast tissue. BCS is the
preferred method of surgical treatment as opposed to the more radical mastectomy procedure where the entire breast is removed [4]. Post-operative histopathologic assessment of the resected specimen is the current gold standard by which microscopic residual tumor in the margin is detected. Post-operative positive pathologic margin status is an important predictor of local recurrence of an invasive or in-situ cancer after BCS [5, 6]. A re-excision surgery is performed if residual cancer is found within 2 mm of the surface of the excised lumpectomy specimen, in order to reduce the risk of recurrence [7]. The cartoon shown in Figure 1 depicts the difference between a positive and negative margin.

Recently, meta-analyses by the Early Breast Cancer Trialists group showed that one death is averted for every four women in which a local recurrence is avoided. A literature review indicates that as many as 20-70% of BCS patients undergo re-excision surgery because the cancer was incompletely removed during the first BCS [8-13]. This represents an enormous physical burden to the patient (increasing her chances for surgical complications and/or eventual cancer-related mortality) and financial burden to the health care system (effectively doubling the cost of treatment for this group of patients). By 2015, it is expected that the number of patients undergoing BCS will rise from approximately 200,000 to more than 270,000 per year in the U.S., at an annual growth rate of 5.5% [3]. With no industry standard to prevent re-excision, it is expected that there will be a concomitant rise in the number of re-excision surgeries.
Figure 1: A graphic which depicts the difference between a positive and negative surgical margin. A cross-section of each excised sample is shown, where A – cancer cells, B – normal cells, C – margin of lumpectomy. Clearly in the positive margin, the cancer cells are present at the margin which indicates cancer cells remain inside the patient.

1.2 Existing Clinical Solutions

Currently there are two clinically used intraoperative techniques that are employed to assist surgeons in the operating room. The first technique is frozen section, where the specimen is immediately flash-frozen and sectioned [14-17]. These tissue sections are stained with hematoxylin and eosin (H&E) then sent to a pathologist for an immediate diagnosis that is relayed back to the surgeon. The second technique is imprint or touch-prep cytology [18, 19]. Once the specimen has been removed, the margin is touched to a glass slide that is inspected by a trained cytologist. Both of these techniques have demonstrated success in reducing re-excision rates by up to 34% [20].

Intra-operative frozen section analysis and touch-prep cytology are used to assess surgical margins at the time of first surgery at a few select high-volume centers with dedicated resources and personnel. However, these techniques have not been
widely adopted because of (1) the need for pathologists in the operating room, (2) prolonged time during surgery for specimen processing (20-40 minutes), (3) technical challenges associated with processing fatty breast tissues, and (4) suboptimal sensitivity of gross pathologic evaluation to guide where to cut the sample for microscopic margin assessment (since the entire specimen cannot be practically sampled). The most common practice is for the breast surgeon to perform intra-operative gross examination and specimen mammography to determine if an adequate excision has been achieved, with pathologic examination of microscopic disease carried out only after the surgery has been completed. This literature review and analysis of current clinical procedures indicates that there is an unmet need for an intra-operative tool capable of distinguishing positive and negative breast tumor margins.

1.3 Emerging Optical Technologies for Margin Assessment

Optical techniques provide a unique, non-destructive approach to characterizing the biological composition of tissue. These techniques rely on the unique interactions of optical radiation with tissue. These specific interactions include optical absorption, where the optical radiation is simply absorbed by the tissue, and scattering, occurs when the optical radiation is re-directed by the tissue. In some techniques optical fluorescence is used where certain molecules absorb optical radiation but quickly re-emit light of a lower energy. These molecules, known as fluorophores, can either be endogenous, occurring naturally in tissue, or exogenous, an externally added contrast agent to
enhance the fluorescent properties of the tissue. Optical technologies are particularly since optical radiation (400-1300 nm) is non-ionizing and generally safe for biological use.

A number of academic [15, 18, 21-30] and commercial [31-36] groups have worked on or are developing tools for intra-operative assessment of breast tumors. A number of groups have published on the use of optical spectroscopy, in particular diffuse reflectance, fluorescence and/or Raman spectroscopy for the diagnosis of breast cancer - a pair of recent review articles covers these studies in more detail [37, 38]. The majority of groups have studied breast biopsies and collectively shown that the primary sources of intrinsic contrast in breast cancer are alterations in cell density, fat and collagen content as well as tissue vascularity.

1.4 Optical Sources of Contrast in Breast Tissue

The major endogenous absorbers in the visible spectrum in breast tissue are oxygenated and deoxygenated hemoglobin (HbO₂ and HbH) and β-carotene (βc), which are directly related to blood content, oxygenation, and adipocytes. Each of these absorbers exhibits a unique wavelength-dependent spectral characteristics depending on its concentration. In addition to absorption, some biological factors contribute to optical scattering, such as the size and density of cells and the structure of collagen, which provides structure and support for the tissue.
Figure 2: Graphic which details the human breast anatomy. Optical sources of contrast are also specifically indicated. Absorption generally occurs in hemoglobin molecules (located in blood vessels) and β-carotene (stored in fat tissue). Scattering occurs as a result of cells and collagen connective tissue.

Figure 2 is a cartoon of the human breast with arrows pointing to the major sources of optical contrast due to absorption and scattering. Cellular structures and collagen in the breast cause light to scatter. The optical absorbers in breast tissue are HbO₂ and HbH, present in blood vessels and βc, typically stored in adipocytes (fat cells). Additionally, NADH and FAD are fluorescent molecules that re-emit light when illuminated with the proper excitation wavelength.

The optical technologies capable of measuring the specific breast composition are listed in Table 1. From previous studies, it was shown that the main sources of optical contrast for differentiating benign from malignant tissues are related to breast vasculature (Hb), fat (indirectly measured via βc content), collagen, and cells.
Table 1: Overview of different optical techniques applied to measuring breast tissue. The specific sources of contrast from each tissue site are also listed

<table>
<thead>
<tr>
<th>Optical Technique</th>
<th>Tissue Interrogated</th>
<th>Source of Contrast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse reflectance &amp; elastic scattering spectroscopy [34, 36, 39-47]</td>
<td>adipocytes cells &amp; collagen blood</td>
<td>βc scattering HbO2 HbH</td>
</tr>
<tr>
<td>Near-infrared spectral imaging [48-52]</td>
<td>water cells &amp; collagen adipocytes blood</td>
<td>water scattering Lipids HbO2 HbH</td>
</tr>
<tr>
<td>Fluorescence spectroscopy [39, 41, 43, 45, 47, 53]</td>
<td>collagen fibers cells</td>
<td>collagen NADH, FAD, retinol, tryptophan</td>
</tr>
<tr>
<td>Raman spectroscopy [21, 54-56]</td>
<td>blood adipocytes variety of cells</td>
<td>heme lipids carotenoids tryptophan cholesterol</td>
</tr>
<tr>
<td>Optical coherence tomography [22, 57]</td>
<td>cells sub-cellular organelles</td>
<td>scattering</td>
</tr>
</tbody>
</table>

1.5 Diffuse Reflectance Spectroscopy for Detecting Biochemical Signatures

Diffuse reflectance spectroscopy (DRS) is one such technique which, when coupled with appropriate light transport models, is capable of extracting quantitative information about tissue absorption and scattering, both of which reflect underlying tissue composition [40, 45, 58-61]. Zhu et al. established the underlying sources of optical contrast between malignant and non-malignant breast tissues that can be exploited for intra-operative margin assessment and her results are very consistent with
other similar studies reported in the literature [45-47, 62]. She found a statistically significant decrease in beta-carotene and total hemoglobin concentration and statistically significant increase in wavelength-averaged reduced scattering coefficient in malignant over non-malignant tissues. Our group has developed a quantitative diffuse reflectance spectral imaging device to exploit these intrinsic sources of optical contrast for the imaging of breast tumor margins [37, 63, 64]. In an initial 48 patients, the technology had a sensitivity and specificity of 80% and 67%, respectively, for detection of residual cancer on the margins of excised specimens [64]. In a later cohort of 92 margins from 72 patients, the technology had a sensitivity and specificity of 89% and 70%, respectively [65].

1.5.1 Diffuse Reflectance Spectroscopy Principles

Briefly, DRS is an optical technique which exploits the absorption and scattering properties of a given sample. Figure 3 shows a graphical depiction of interrogating a tissue specimen with DRS. Traditionally, light is traditionally delivered to the sample using an optical fiber. Once the light has entered the tissue, it will either be absorbed or scattered. Some portion of the light scattered toward the surface of the tissue, which is collected by an adjacent optical fiber. This light remitted from the tissue is known as the diffuse reflectance. The absorption and scattering properties of tissue are wavelength-dependent, so the diffuse reflectance can be measured over a range of wavelengths to construct a full diffuse reflectance spectrum. An example spectrum is shown on the
right side of Figure 3, where the unique absorption properties of β-carotene and hemoglobin (α, β, and Sorét band) are clearly seen. The spectrum is typically analyzed using a predictive photon migration model that is capable of extracting and quantifying the absorption (µs) and scattering (µa) coefficient. The absorption coefficient, µa, is equivalent to the inverse of the mean free path of a photon before undergoing an absorption event. Similarly, µs is the inverse of the mean free path before undergoing a scattering event. Reduced scattering coefficient is often reported, which is simply µs*(1-g), where the anisotropy coefficient, g, is the average cosine of the scattering angle.

**Figure 3**: Basic principle of DRS demonstrating absorption and scattering inside tissue. An example of a measured spectrum is shown on the right

### 1.5.2 Challenges Associated with Diffuse Reflectance Spectroscopy

As previously mentioned, our group recently developed and clinically tested an optical spectral imaging system based on DRS to quantitatively image ex vivo breast
tumor margins for the detection of residual disease [66]. To briefly summarize, the system consists of a Xenon arc lamp, spectrograph, and a 2D CCD camera. Traditionally, a single-channel fiber-optic probe is used for single-point spectroscopy. However, for the margin assessment application, our single-channel probe was replaced with an imaging probe comprised of 8 independent channels, each containing its own set of illumination and collection fibers. Each measured diffuse reflectance spectrum was analyzed using an inverse Monte Carlo model previously developed by our group, to extract beta-carotene concentration, oxy-hemoglobin concentration, de-oxy hemoglobin concentration, and a wavelength-averaged reduced scattering coefficient. While our clinical studies have demonstrated feasibility of optical spectral imaging for the detection of positive tumor margins, the system’s large physical footprint (2 m x 1.5 m x 1 m) and significant cost (~$55,000) potentially limit its widespread clinical utility. In addition, the number of channels in the clinical system is limited by the number of collection fibers that can be imaged by the CCD (while minimizing cross-talk between adjacent channels) and thus multiple placements (and increased time) are required to fully survey breast tumor margins, which can be as large as 10-20 cm².

1.6 Fluorescence Microscopy for Visualizing Tumor Micro-Morphology

The quantitative spectral imaging system provides one optical based strategy for visualizing tumor margins, but there is an inherent lack of spatial resolution due to the
nature of diffuse reflectance spectroscopy. Specifically, spectroscopy is unable to visualize the cellular changes that occur in breast tumors on the microscopic level as optical spectroscopy relies on multiple scattered diffuse photons. In addition, it can be difficult to distinguish scattering signatures from malignant tissues and fibro-glandular tissues, which can result in a reduction in diagnostic accuracy. Increasing nuclear size and crowding of nuclei due to rapidly proliferating cells, are common characteristics in cancer. These changes have long been exploited for diagnosis in ex vivo tissue using Hematoxylin and Eosin (H & E) staining, where pathologists observe microstructural features including nuclear size and shape and nuclear density to determine the pathologic state of the tissue.

There is evidence in the literature of the success of in high resolution optical imaging. For example, Gareau et al. have published a series of papers employing confocal reflectance and fluorescence microscopy in combination with acridine orange (AO) staining of nuclei for tumor margin assessment of skin cancer ex vivo [67-72]. This approach exploited subjective human observation of the images to detect basal cell carcinomas with 97% sensitivity and 89% specificity. A recent study by Nyirenda et al. [73], employed UV-excited DAPI to stain cell DNA ex vivo in rat mammary tumor xenografts and human breast tissue microarrays. The authors demonstrated that they could detect rodent tumors with greater than 95% sensitivity and specificity using automated calculation of inter-nuclear distance. Richards-Kortum et al. have developed
a high resolution fluorescence microendoscope (HRME) and used it in conjunction with acriflavine to image dysplasia and early cancer in the oral cavity and esophagus [74, 75]. Esophageal images collected \textit{ex vivo} were analyzed both subjectively by expert clinicians yielding a sensitivity and specificity of 87% and 61%, and quantitatively through the application of algorithms that focused on spatial frequency content and pixel pair correlation (internuclear distance) yielding a sensitivity and specificity of 87% and 85% [75]. Gmitro’s group has published a number of studies on confocal laser scanning microendoscopy of AO stained tissue [76-79]. In an \textit{ex vivo} ovarian study, they showed that automated classification algorithms leveraging texture could diagnose ovarian cancer with better than 95% sensitivity and 90% specificity [76]. They have developed a mobile confocal endoscopy system, and shown images of microanatomical and nuclear detail from the ovary \textit{in vivo} and \textit{ex vivo} using AO as a contrast agent (under IND approval) [77].

\textbf{1.6.1 Challenges Associated with Fluorescence Microscopy}

Our group has recently demonstrated the viability of visualizing tumor microanatomy using a high resolution micro-endoscope system (HRME) [74, 80]. A coherent imaging fiber bundle is a central component of the HRME system used in the preliminary studies, which requires physical contact with the specimen and provides small field of view (0.63 mm$^2$). These two constraints limit the application of the system in diagnosing the entire tumor surface. It is the goal of this aim to design and construct
a new wide-field optical sectioning microscope that does not require a coherent imaging fiber bundle. The system will also be designed to capture the entire field of view in a single exposure rather than a point by point scanning (i.e. confocal microscopy), due to the speed advantage of wide-field imaging.

Based on the literature of existing and developing technologies, leveraging the changes in tissue microanatomy that occur in cancer development can yield very informative diagnostic information for accurate margin assessment. These cellular changes, particularly related to the cell nuclei, can only be investigated using high resolution optical methods. The current limitation with these optical imaging methods is that there is perceived trade-off between imaging at high resolution and area of coverage. Indeed, a typical breast tumor margin is on the order of 4x4 cm, whereas the field of view on a microscope with a 10x objective may be around 0.1 x 0.1 cm. Another challenge is that in conventional non-contact fluorescence microscopy, the entire field of view is uniformly illuminated with the excitation light. This creates a problem as fluorophores outside the plane of focus are also excited and emit photons, generating a significant amount of unwanted background fluorescence. This in turn significantly degrades contrast of features of interest in the focal plane. This is a common issue in widefield fluorescence microscopy and several specialized techniques exist to reject background fluorescence, such as fluorescence confocal microscopy. In fact, Gareau et al. recently reported a custom fluorescence confocal microscopy system
designed to image and diagnose margins during Mohs procedures for removal of skin cancer [81]. While extremely effective in rejecting background fluorescence and capable of exquisite image quality, confocal microscopy typically relies on sequential pixel scanning, in which a laser beam is focused to a diffraction-limited spot and scanned over the sample in time. Confocal scanning approaches which aim to parallelize the acquisition process, such as spinning disk confocal and line-scan confocal, serve to increase the effective pixel sampling rate, but not without limitations (spinning disk confocal is limited in the adjustment of axial resolution, and line scan confocal with linear CCDs results in possibly asymmetric lateral resolution along the line dimension).

An alternative approach to reject background fluorescence with fully parallel pixel detection is structured illumination microscopy (SIM), in which the entire field of view is illuminated with a defined spatial pattern and scanning of a focal spot is not required [82]. Other than the use of patterned illumination, the illumination and collection geometry is identical to that of conventional widefield fluorescence microscopy, so a standard CCD may be used for detection. The advantages of structured illumination microscopy are that 1) optically-sectioned images are obtained with all pixels in parallel, thereby significantly increasing the pixel sampling rates, 2) CCD detectors with high quantum efficiency can be used making SIM light-efficient, 3) the axial resolution can be tuned by varying pattern frequency, and the lateral resolution is symmetric over the field of view, and 4) it is a relatively low complexity solution with no moving parts.
Structured illumination microscopy has been shown to perform equivalently (and at times better) than confocal microscopy with respect to optical sectioning and SNR, particularly in superficial tissues [83-85]. Disadvantages of structured illumination microscopy include 1) the amplification of shot noise from the out-of-focus background, 2) the reduction of recovered signal when the illumination frequency is near the cut-off frequency of the imaging optics [86], which can reduce the image quality compared to confocal, and 3) its reduced performance in deep imaging as compared to confocal [83]. However the advantages of structured illumination microscopy in terms of imaging throughput and reduced complexity may outweigh the disadvantages in imaging performance when the superficial surface of large areas of tissue are to be scanned (average breast margin size, 20 cm²[66]).

1.7 Objectives

The first objective of this dissertation was to demonstrate the feasibility of designing a low cost, compact optical spectral imaging system for quantitative imaging of tissue optical properties in the visible spectral range that directly addresses the above limitations of our previously developed clinical system. A new prototype system was designed and constructed with significant reductions to cost and physical footprint. The system was thoroughly tested on a set of tissue mimicking phantoms.

The second objective was to design and optimize a SIM imaging system with the requisite parameters to image tumor margins. Specifically, these included the ability to
image fresh tissue immediately after removal from the subject, with sufficient lateral resolution (at least 4 µm) and optical section thickness (50-100 µm) that effectively reduces out-of-focus fluorescence to isolate single nuclei, and a field of view which may be reasonably scaled to cover a large tumor margin. The system was fully characterized on a set of custom-design imaging phantoms with various optical properties.

In the third objective, the SIM imaging system was applied towards the imaging of tumor margins in a transgenic sarcoma model. Additionally, an advanced image processing algorithm known as maximally stable extremal regions (MSER) was optimized and used to segment nuclei in images acquired with the SIM system and a logistic regression model was used for classification. The results of this study demonstrate that the SIM system, in conjunction with proper nuclei segmentation algorithms, is capable of effectively differentiating positive from negative tumor sites, as well as whole positive from negative tumor margins.

In short, the three specific aims of this dissertation are as follows:

**Specific Aim 1** – Develop and test a clinically feasible quantitative spectral imaging system for imaging of tumor margin morphology

**Specific Aim 2** – Develop and characterize a benchtop non-contact high resolution optical sectioning microscope optimized for probing tumor microscopic morphology.

**Specific Aim 3** – To evaluate the diagnostic capability and clinical potential of the system in a pre-clinical study using a transgenic mouse sarcoma model
2. Improvements to the DRS imaging system

In the following chapter, I present the work related to accomplishing specific aim 1 of the dissertation. Significant design changes were proposed and implemented to the clinical DRS imaging system. These modifications were made primarily to decrease the cost and size of the system to increase the clinical viability and accessibility.

2.1 Methods

2.1.1 System Design

Figure 4B and 1C show a schematic of the proof-of-concept compact optical spectral imaging system and the tip of the spectral imaging system that comes in contact with the tissue. Briefly, the design changes implemented in the compact optical spectral imaging system (Figure 4B) involved replacing the spectrograph in the clinical system with a simple 8-slot filter wheel on the illumination end. Instead of a 2D CCD, silicon photodiodes were used for detection by multiplexing the previous single-pixel design reported in the publication by Lo et al. into a 9-pixel 3x3 matrix [87]. The light source for the compact optical spectral imaging system is a broadband 350-Watt Xenon arc lamp (MAX-302, Asahi Spectra). Light from the Xenon arc lamp was immediately passed through one of eight bandpass filters selected using an 8-slot filter wheel. The resulting monochromatic light was launched into a bundle of 9 optical fibers, each 0.6 mm in diameter (FVP600660710, Polymicro), which deliver the light to the tissue through the
centered aperture of each of the nine 5.8 x 5.8 mm silicon photodiodes (S1227-66BR, Hamamatsu) at the distal end of the imaging probe (Figure 4C).

Simultaneous measurements of the diffusely reflected light from all 9 fiber-photodiode pairs can be collected by placing the probe on the surface of the sample. The photocurrent generated by each of the 9 photodiodes was read using a multi-channel transimpedance amplifier (Multiboard, SolGel Technologies GmbH) so that the signal from each photodiode could be read simultaneously. The transimpedance amplifier circuitry was assembled within a small metal housing and powered using a commercial ± 12V power supply. The output voltage (photocurrent converted to voltage via transimpedance amplifier) was read and transmitted to a laptop computer using a USB controlled data acquisition card (NI USB-6210, National Instruments).
Figure 4: Overview of the two spectral imaging systems. A) A system schematic of the current clinical spectral imaging system. This figure contains a block diagram of the system as well as a detailed diagram of the probe tip. B) A system schematic of the compact optical spectral imaging system which details the illumination and collection setup. C) Photograph of the tip of the 3x3 photodiode array. The numbers represent the pixel numbers, which will be referred to throughout the manuscript.

Spectral measurements were accomplished by cycling through each of the eight bandpass filters and recording individual measurements at each wavelength rather than recording a full spectrum as was done with the clinical system. In the end, the collected
data comprised of an 8 wavelength spectral measurement from each of the nine pixels.
The impact of measurements at a reduced number of wavelengths on the extraction of optical properties was previously addressed and investigated by Lo et al [87]. It was demonstrated, through phantom experiments and simulations, that using as few as 5 to 8 discrete wavelengths with a FWHM bandwidth of 20 nm was adequate for extracting absorption and scattering properties of phantoms containing polystyrene spheres and hemoglobin. The same methodology was used to identify eight specific wavelengths between 400 and 600 nm (specifically, 400, 420, 440, 470, 500, 530, 570, and 600 nm) each with a band pass FWHM bandwidth of 10 nm. Filters with these specifications (XBPA, Asahi Spectra) were inserted into the 8-slot filter wheel.

In order to facilitate a seamless interface between all the system components (Xenon light source, filter wheel, multi-channel transimpedance amplifier, and DAQ card), a custom LabView GUI was written and executed on the laptop computer. The program was responsible for synchronizing and controlling all system components and storing all collected spectra, analyzed later for extraction of optical properties.

2.1.2 Theoretical System Characterization:

Monte Carlo simulations were carried out to evaluate the system sensing depth and to compare it to that of the clinical system. The sensing depth is a parameter heavily dependent on the probe geometry and optical properties of the sample. To estimate the sensing depth, a full Monte Carlo simulation was performed for the illumination-
collection geometry of a single pixel of the compact spectral imaging system and a similar approach was used to obtain the sensing depth of a single channel of the current clinical system. Investigating the sensing depth of a single pixel provides a valid estimate for all 9 pixels of the probe since they all have an identical illumination and collection geometry. In the Monte Carlo simulation, the path of each collected photon was individually tracked, and the deepest point reached in media of each photon was recorded. Initial photon positions were determined by a spatially random uniform distribution over a circular region with a diameter equal to the illumination fiber diameter (600 µm). Photons were propagated through the media characterized by a given set of optical properties. Photons were detected if they escaped the surface of the media within a square region defined by the dimensions of the photodiode (5.8 x 5.8 mm) and also within the collecting numerical aperture (NA = 0.96) of the photodiode (excluding the circular region occupied by the illumination fiber). In Monte Carlo simulations, each photon was assigned a weight of 1 from the point of launch into the tissue. The weight was successively decreased after each scattering event, and the final exit weight of the photon was recorded for all successfully collected photons. In addition, the deepest axial depth which the photon traveled to was also recorded. The total collected weight of all collected photons was calculated by summing the weights of all collected. Finally the sensing depth was calculated by finding the exact depth at which 90% of the total collected weight was attained. The sensing depth was heavily
dependent on the optical properties chosen for the Monte Carlo simulations. These optical properties were chosen based on clinically measured optical properties of ex vivo breast lumpectomy specimens. In our clinical measurements, the following three different tissue types were encountered (verified by pathology): malignant, normal adipose, and normal fibro-glandular whose median optical properties are documented in Table 2. The reported sensing depth was simulated for all three tissue types at two wavelengths, 450 nm and 600 nm. These two wavelengths were chosen to estimate shallow and deep sensing depths (450 nm was the shortest wavelength used in the clinical system and 600 nm was the longest). Although 450 nm was not actually used in the compact optical spectral imaging system, the purpose of these simulations was to enable a direct comparison between the two systems, the only difference being the probe geometry. Therefore, a total of 6 sensing depths for each instrument were reported (2 wavelengths x 3 tissue types).

Table 2: The median (over all measured samples) optical properties of three different tissue types encountered in our clinical studies. The number of samples measured of each tissue type are also shown. These optical properties are reported specifically for the shortest (450 nm) and longest (600 nm) wavelengths acquired in the clinical data.

<table>
<thead>
<tr>
<th>Optical Properties</th>
<th>Malignant (n = 10)</th>
<th>Adipose (n = 323)</th>
<th>Fibro-Glandular (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption Coefficient ( \mu_a ) (cm(^{-1} ))</td>
<td>450 nm 20.34</td>
<td>600 nm 1.42</td>
<td>450 nm 11.29</td>
</tr>
<tr>
<td>Reduced Scattering Coefficient ( \mu_s' ) (cm(^{-1} ))</td>
<td>450 nm 9.55</td>
<td>600 nm 8.47</td>
<td>450 nm 7.44</td>
</tr>
</tbody>
</table>
Another important parameter which was assessed using Monte Carlo simulations was the cross-talk between adjacent pixels. For optical property extractions, each pixel was treated as an individual illumination and collection pair. Any diffusely scattered light collected which originated from an adjacent pixel’s illumination fiber was considered cross-talk. Monte Carlo simulations were used to quantitatively assess the amount of cross-talk a pixel would receive from an adjacent pixel’s illumination fiber. To simulate the cross talk, the collection area was defined by the size of the active area for a given pixel (5.8 x 5.8 mm square). For the illumination area, in addition to launching photons from the location of the pixel’s central illumination fiber, they were launched from the location of adjacent pixels’ illumination fiber (assuming 8 mm center-to-center pixel spacing). With this illumination-collection geometry, and given a set of optical properties, we simulated the amount of cross-talk due to photons arriving from adjacent pixels. Cross-talk was simulated for the central pixel (#5) because this represented the worst case scenario as it is surrounded by 8 adjacent pixels. Again, the simulations were performed using the optical properties of the three tissue types encountered in our clinical studies (Table 2) but this time only at 600 nm. This was the only wavelength chosen for this simulation since absorption is at its lowest, which is considered the worst case scenario for cross-talk. Cross-talk was calculated in a similar manner for the clinical system for the purposes of comparison. The illumination area, collection area, and center-to-center pixel spacing were set based on the probe
specifications of the clinical system. However, since the 8 channels of the clinical system are arranged in a 2x4 configuration, the pixel experiencing the worst case scenario for cross-talk only had 5 adjacent pixels. The final value of cross-talk was reported as a percentage by dividing the number of cross-talk photons by the number of signal photons.

2.1.3 Experimental System Characterization

Experimental measurements were required to fully characterize the system. The signal-to-noise ratio (SNR) was an important metric to quantify the precision of the experimental measurements. In order to measure the SNR, a liquid phantom was constructed with known optical properties (wavelength-averaged $\mu_a = 7.00 \text{ cm}^{-1}$ and $\mu_s' = 14.84 \text{ cm}^{-1}$). The imaging probe was placed at the surface of the phantom and 15 repeated measurements were collected. The SNR was calculated by taking the mean and dividing by the standard deviation of these measurements. This SNR measurement from the compact spectral imaging system was compared to the previously measured SNR by Lo et al. [87]. The SNR reported in that study was measured on a benchtop diffuse reflectance spectroscopy system with instrumentation identical to the clinical spectral imaging system. The SNR measurement with that system on a liquid phantom with similar optical properties (wavelength-averaged $\mu_a = 7.50 \text{ cm}^{-1}$ and $\mu_s' = 16.00 \text{ cm}^{-1}$) provided a sufficient benchmark for comparison for the compact spectral imaging system.
The overall drift of the system was also measured in order to determine any change in system measurement over a period of time. The probe was placed on a Spectralon 99% reflectance standard (SRS-99-010, Labsphere Inc) and diffuse reflectance measurements were recorded every 5 minutes over a span of 40 minutes. The entire system (including the probe) was not adjusted or modified during the course of the experiment so changes in measurements strictly represented drift caused by the system. The drift was quantified by dividing the range by the mean of the measurements acquired over this time window. This ratio yielded a percentage that represented the drift of each pixel of the system over the 40-minute period. The drift measurements were taken with both the compact optical spectral imaging system and the clinical system to compare the two systems.

To test the optical property extraction accuracy and robustness of the compact optical spectral imaging system, a tissue mimicking phantom study was designed. The liquid phantoms consisted of hemoglobin (H0267, Sigma Co.) as the absorber and polystyrene spheres (07310-15, Polysciences, Inc.) as the scatterer. Hemoglobin was chosen due to its distinct absorption spectral features and biological significance in tissue measurements.

The exact wavelength-dependent absorption coefficients ($\mu_a$) of hemoglobin were determined using a spectrophotometer (Cary 300, Varian). Polystyrene spheres were used due to their well-defined size and density, meaning the reduced scattering
coefficient (µ′) could be accurately estimated using Mie Theory. Prahl’s Mie scattering program was used to perform this task, using the manufacturer’s specified sphere diameter (1.025 µm), density (2.62%), and refractive index (1.6) [88]. With knowledge of the optical properties of the constituents, the liquid phantoms could be constructed with the desired optical properties by varying the absorber and scatterer concentrations. Table 3 documents the expected optical properties of the 14 phantoms made for this phantom study, constructed using hemoglobin and polystyrene spheres. The optical property range (µa and µ′) was chosen to match those of the previous phantom studies used to characterize the clinical system to enable a direct comparison between the accuracy of the two systems [89].

Table 3: Table detailing the optical properties of 14 liquid phantoms (average value over 400-600 nm) used to test the optical property extraction accuracy of the 3x3 compact optical spectral imaging system. The highlighted phantom was used as the reference phantom for the inversions using the scalable Monte Carlo model.

<table>
<thead>
<tr>
<th>Phantom #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>µa (cm⁻¹)</td>
<td>0.50</td>
<td>1.00</td>
<td>1.50</td>
<td>2.00</td>
<td>2.50</td>
<td>3.00</td>
<td>3.50</td>
</tr>
<tr>
<td>µ′ (cm⁻¹)</td>
<td>20.53</td>
<td>20.09</td>
<td>19.66</td>
<td>19.22</td>
<td>18.78</td>
<td>18.34</td>
<td>17.91</td>
</tr>
<tr>
<td>[Hb] (µM)</td>
<td>2.28</td>
<td>4.57</td>
<td>6.85</td>
<td>9.14</td>
<td>11.42</td>
<td>13.71</td>
<td>15.99</td>
</tr>
<tr>
<td>µa (cm⁻¹)</td>
<td>4.00</td>
<td>4.50</td>
<td>5.00</td>
<td>5.50</td>
<td>6.00</td>
<td>6.50</td>
<td>7.00</td>
</tr>
<tr>
<td>µ′ (cm⁻¹)</td>
<td>17.47</td>
<td>17.03</td>
<td>16.59</td>
<td>16.15</td>
<td>15.72</td>
<td>15.28</td>
<td>14.84</td>
</tr>
<tr>
<td>[Hb] (µM)</td>
<td>18.28</td>
<td>20.56</td>
<td>22.84</td>
<td>25.13</td>
<td>27.41</td>
<td>29.70</td>
<td>31.98</td>
</tr>
</tbody>
</table>

To measure the diffuse reflectance of each of these phantoms, the probe tip was placed flush in contact with the surface of the liquid phantom. The liquid phantom was
created in a container with dimensions of 11 x 7 x 1.6 cm. Its length and width were large enough to accommodate the entire face of the distal end of the probe. The depth of the phantom was sufficiently large enough to simulate a semi-infinite media (roughly 8 times the simulated sensing depth). The liquid phantom was continuously stirred using a magnetic stir bar over the course of the phantom study to ensure homogeneity. Light at each wavelength was successively launched into the illumination fibers and the diffuse reflected light signal was measured with all nine photodiodes. A separate calibration measurement was taken, after all the phantom measurements, on a Spectralon 99% reflectance standard (SRS-99-010, Labsphere Inc.) with the same measurement procedure. The collected spectrum of liquid phantom was divided by that of the reflectance standard to obtain the calibrated diffuse reflectance spectrum, correcting for wavelength-dependent instrument throughput and the spectral shape of the source.

2.1.4 Monte Carlo Model of Diffuse Reflectance

The collected diffuse reflectance spectrum was processed using a fast scalable inverse Monte Carlo model previously developed by our group [26-27]. Our model can quickly generate optical properties (μₐ and μₛ') for a given wavelength-dependent diffuse reflectance and specific probe geometry [50]. Previous studies demonstrating the extraction accuracy and robustness of the model have been reported [51-54].
The forward Monte Carlo model assumes the measured diffuse reflectance spectrum is dependent on the two optical properties, absorption ($\mu_a$) and reduced scattering coefficients ($\mu_s'$). The absorption coefficient is a function of the wavelength-dependent molar extinction coefficient of the absorber and its concentration. The reduced scattering coefficient is described using Mie Theory and is dependent on the size and density of the scatterer. In the inverse Monte Carlo model, an initial guess of the absorber concentration, scatterer size and density is input into the forward model, producing a modeled diffuse reflectance spectrum. These guesses for the optical properties are iteratively updated until the residual sum of squared errors between the experimentally measured and Monte Carlo modeled diffuse reflectance is minimized. The final optical properties that generate the modeled spectrum which most closely matches the measured spectrum are designated as the extracted values.

## 2.2 Results

The compact spectral imaging system was assembled and constructed according to the desired specifications listed in the previous section. With the exception of pixel 8, all pixels appeared to be functional once construction was completed. Unfortunately, the central illumination fiber for pixel 8 was broken during assembly. The individual illumination fiber was already permanently fixed in the center of the detector and bundled with the remaining illumination fibers. While polishing the proximal end of the illumination bundle, the illumination fiber snapped and separated in the middle
resulting in no optical output from pixel 8. Replacing the individual illumination fiber at this stage was not possible, so data was not measured or presented for pixel 8. The results of our simulation and experimental system analysis for the remaining pixels of the compact optical spectral imaging system are presented below.

Figure 5A shows a photograph of the compact optical spectral imaging system alongside the clinical system in Figure 5B. Scale bars are displayed to show the reduction in footprint between the new and original systems. Both the size and cost of the compact optical spectral imaging system were significantly reduced compared to the clinical system. More importantly, the compact optical spectral imaging system can be more easily expanded to a larger and denser imaging array with mature semiconductor technology. The key physical parameters of the compact optical spectral imaging system were compared to those of the clinical spectral imaging system, as shown in Figure 5.

Figure 5: System photographs to compare physical size A) Photograph of the modified 3x3 compact optical spectral imaging system compared to the B) Photograph of the clinical system. The same laptop is pictured in both system photographs in order to compare system scale.
2.2.1 Theoretical Characterization Results

The results of the sensing depth simulations are shown in Table 4. The reported value (in mm) is a range since sensing depth was calculated at the clinical system’s shortest wavelength (450 nm) and the longest wavelength (600 nm). The comparison between the clinical system and compact optical spectral imaging system show that the sensing depths are very similar. In addition, the probe of the compact optical spectral imaging system appears to surpass the minimum criterion of 2 mm for a clear margin.

Table 4: Side-by-side comparison of key physical system parameters between the clinical system and compact optical spectral imaging system. A noteworthy comparison is the large reduction of footprint in the compact system.

<table>
<thead>
<tr>
<th>Spectral Imaging System</th>
<th>Probe Geometry (One Channel)</th>
<th>Number of Channels</th>
<th>Center-to-Center Distance Between Channels (mm)</th>
<th>Footprint of Entire System (L x W x H) (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical System</td>
<td>19, 0.2 mm diameter fibers</td>
<td>4, 0.2 mm diameter fibers</td>
<td>8</td>
<td>2 x 1.5 x 1</td>
</tr>
<tr>
<td>Compact System</td>
<td>0.6 mm diameter fiber</td>
<td>5.8 x 5.8 mm Si PD</td>
<td>9</td>
<td>0.35 x 0.3 x 0.2</td>
</tr>
</tbody>
</table>

The cross-talk simulations were also carried out according to the methods described earlier. The results of these simulations are shown in Table 5 as well and as can be seen, the cross-talk in all three tissue types is significantly smaller in the clinical system than the compact spectral imaging system. A higher cross talk is certainly expected for the compact system, considering the collection area of each pixel in the compact spectral imaging system (5.8 x 5.8 mm = 33.6 mm²) is much larger than each
pixel in the clinical system (4 x 0.2 mm diameter fibers = 0.125 mm²) and more importantly, the number of adjacent pixels were 8 in the compact spectral imaging system as opposed to 5 in the clinical system (due to differences in system configuration). In addition, the center-to-center pixel spacing is closer in the compact spectral imaging system (8 mm) than the clinical system (10 mm) which would also lead to increased cross-talk. However, the results of the tissue mimicking phantom study (presented later in this section) demonstrate that this level of cross-talk between pixels did not significantly impact the ability to quantitatively extract optical properties.

Table 5: Comparison of the simulated sensing depth and cross-talk between the clinical system and the compact optical spectral imaging system. The sensing depth was simulated at two wavelengths, 450 nm and 600 nm, while cross-talk was only simulated at 600 nm.

<table>
<thead>
<tr>
<th>System</th>
<th>Tissue type</th>
<th>Sensing depth (mm)</th>
<th>Cross-talk encountered by pixel surrounded by most adjacent pixels (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>450 nm</td>
<td>600 nm</td>
</tr>
<tr>
<td>Clinical System</td>
<td>Malignant</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Adipose</td>
<td>0.7</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Fibro-Glandular</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Compact System</td>
<td>Malignant</td>
<td>0.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Adipose</td>
<td>0.9</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Fibro-Glandular</td>
<td>0.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

2.2.2 Experimental Characterization Results

The SNR was measured on a highly absorbing liquid phantom with optical properties of $\mu_a = 7.00 \text{ cm}^{-1}$ and $\mu'_a = 14.84 \text{ cm}^{-1}$. Measurements were taken and the SNR was calculated at each of the eight wavelengths following the procedure described in the methods section. The SNR is shown in Figure 6A, along with the optical power.
(measured at 400 nm) at the tip of each illumination fiber in Figure 6B. As previously mentioned, data could not be collected from pixel 8 due to a broken illumination fiber. The minimum SNR was about 40 dB (at 400 nm) and the maximum was approximately 65 dB (at 600 nm). The SNR for each pixel at 400 nm illumination was comparable to the 32 dB SNR (measured at 405 nm) of the clinical system [87]. The discrepancy of the optical power among pixels (Fig. 3B) is due to the packaging of the 9 illumination fibers on the proximal end (lamp side). Light from the Xenon lamp could not couple uniformly into all illumination fibers, causing the differences in optical output at the distal end. Although the output power of pixel 9 was noticeable lower, no physical defects in the fiber or photodiode were visible.

Another performance metric which was measured was the long-term drift of the system. The system drift over 40 minutes was approximately ±2% for the compact optical spectral imaging system and ±3% for the clinical system.

![Figure 6](image.png)

**Figure 6:** A) Signal-to-noise ratio (SNR) for all pixels at three different wavelengths (400, 500, 600 nm). The SNR was calculated by taking 15 repeated measurements on a liquid phantom and dividing the mean by the standard deviation.
The optical properties of the phantom used in the SNR measurements were $\mu_a = 7.00$ cm$^{-1}$ and $\mu_s' = 14.84$ cm$^{-1}$. B) The table on the right indicates the optical power output (at 400 nm) from the illumination fiber of each pixel.

The set of 14 liquid phantoms described earlier were constructed and their diffuse reflectance spectra were measured using the compact optical spectral imaging system. The acquisition time for a single wavelength was approximately 0.5 seconds and approximately 1 second to switch between wavelengths. Data was collected simultaneously from all pixels. Therefore, approximately 12 seconds was required to collect the reflectance spectra from a single phantom. Figure 7A shows a comparison between the diffuse reflectance spectra measured with the clinical system and each pixel of the compact optical spectral imaging system. The spectra displayed are from phantom #14 and calibrated to a reflectance standard and reference phantom (#4). Overall there is reasonable agreement of the measured spectra between the clinical and compact optical spectral imaging systems. However, pixel #7 does show considerable deviation from all other measurements, particularly at the shorter wavelengths (400 and 420 nm).
Figure 7: A) Normalized (to reflectance value at 600 nm) diffuse reflectance spectrum collected from phantom 14 using the clinical and compact optical spectral imaging systems, corrected with a reflectance standard and reference phantom. The plots demonstrated reasonable agreement between the two systems. B) Measured and modeled diffuse reflectance spectra from the compact optical spectral imaging system. These spectra are not normalized but are corrected with a reflectance standard.

Examples of the experimentally measured spectrum and final Monte Carlo modeled spectrum for two representative pixels (#5 and #7) are shown in Figure 7B.

While the measured and modeled spectra are in agreement for pixel #5, a small deviation exists between 400-420 nm in pixel #7. The inability to accurately model the spectra stems from the measurement error already described. We believe that this is attributed to a construction defect present in pixel 7. A close-up photograph of pixel 5 and 7 are shown in Figure 8, which reveals the defect in the illumination fiber of pixel 7. This defect is not present in pixel 5, or in any other pixels (not pictured), leading us to strongly believe this was the cause of the variation seen in pixel 7. While our robust Monte Carlo model is capable of accounting for different probe geometries, one requirement is that light exiting the illumination area must maintain a relatively uniform
exit distribution. However, as the fiber is damaged, the illumination becomes non-uniform, as seen in the Fig. 5, and the uniformity assumption is compromised.

![Pixel 5 and Pixel 7](image)

**Figure 8:** Photographs of pixels 5 and 7 demonstrating the physical defect in pixel 7. Note difference in the central illumination fiber in both pixels. As noted in the representative spectra, all pixels match the clinical system aside from pixel #7.

Ultimately, the performance metric which would determine the viability of the system is its ability to quantitatively extract optical properties. Simply measuring the diffuse reflectance would not provide any significant insight into the composition of breast tumor margins. From the final modeled spectra the $\mu_a$ and $\mu_s'$ values were extracted and compared to the known expected optical properties of the liquid phantoms using the previously described inverse Monte Carlo model. One phantom (Phantom #4) was also chosen as a reference phantom to put the measured and modeled diffuse reflectance on the same scale prior to the inversions. The choice of this reference phantom was based on the comprehensive reference phantom characterization reported by Bender et al. [89]. The inverse Monte Carlo model was capable of extracting the optical properties of all pixels in <15 seconds. Figure 9 shows a plot of expected versus extracted values for both wavelength-averaged absorption ($\mu_a$) and reduced scattering.
(\(\mu_s'\)) coefficient for all pixels excluding pixel 7 and 8. These pixels were excluded due to their previously mentioned defects and inability to accurate measure and extract optical properties. Each circle on the plot represented the optical properties of one target phantom (14 total phantoms) averaged over all wavelengths and all functioning pixels (#1-6, 9).

![Graph](image)

**Figure 9:** Plots of the optical property extraction accuracy averaged over pixels #1, 2, 3, 4, 5, 6, and 9 of the compact optical spectral imaging system. The data presented is the wavelength averaged \(\mu_a\) and \(\mu_s'\) for all 14 liquid phantoms. Average extraction errors for each individual pixel are displayed in following figure.

The percent error between the extracted optical properties and expected optical properties are also calculated and summarized in Figure 10 individually for each pixel. A single value for error percentage is reported by averaging over all wavelengths and all target phantoms. Most of the pixels are capable of extracting \(\mu_a\) and \(\mu_s'\) with high accuracy (<8%). However, once again pixel 7 is a clear outlier and shows very poor extraction accuracy. This result was anticipated given the previous discussion on the physical defect of the pixel. Overall, the optical property extractions experimentally
confirmed the appropriateness of the modeling assumptions, i.e. treating each pixel as an independent source-detector pair. In addition, we demonstrated the feasibility of the 3x3 photodiode geometry design for acquiring quantitative spectral images.

![Table of Optical Property Errors](image)

**Figure 10:** Summary of the optical property ($\mu_a$ and $\mu_s'$ averaged over all wavelengths) extraction errors for all pixels. The errors shown here are the average errors over all 14 target phantoms. Phantom #4 ($\mu_a = 2.00$ cm$^{-1}$; $\mu_s' = 19.22$ cm$^{-1}$) was used as the reference phantom for the inversions.

A comparison of all performance metrics between the clinical system and compact optical spectral imaging system are summarized in Table 6. The uncertainty reported in the optical property extraction column is the standard deviation of error over all pixels (excluding pixel #8) and all phantoms. It is clear that the performance of compact optical spectral imaging system is on par with the clinical system, notably the comparison between the $\mu_a$ and $\mu_s'$ extraction errors compared in Table 6.
Table 6: Comparison of performance metrics between the clinical and compact optical spectral imaging systems. The SNR of the clinical system was measured in a previous study (at 405 nm using a phantom with optical properties of $\mu_a = 7.5 \text{ cm}^{-1}$ and $\mu_s' = 16 \text{ cm}^{-1}$)

<table>
<thead>
<tr>
<th>System</th>
<th># wavelengths between 400-600 nm</th>
<th>Drift</th>
<th>SNR @ $\lambda = 400$ nm (dB)</th>
<th>Absorption ($\mu_a$) and Scattering ($\mu_s'$) Extraction</th>
<th>Mean $\mu_a$ Error</th>
<th>Mean $\mu_s'$ Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical System</td>
<td>81</td>
<td>±3%</td>
<td>32</td>
<td>9.03%</td>
<td></td>
<td>7.33%</td>
</tr>
<tr>
<td>Compact System</td>
<td>8</td>
<td>±2%</td>
<td>40</td>
<td>6.04%±7.78%</td>
<td>11.37%±19.62%</td>
<td></td>
</tr>
</tbody>
</table>

2.3 Conclusions

The major advantage of the compact optical spectral imaging system is the significant reductions in both cost and footprint compared to the traditional clinical system. These reductions were accomplished by replacing two specific components in the current clinical system with simpler, cheaper, and more compact components. Rather than using a cooled CCD (20x20x20 cm, ~$20,000), the new system utilized silicon photodiodes and appropriate readout circuitry (20x10x15 cm, ~$1000). The second component targeted was the grating-based spectrograph (50x50x20 cm, ~$12,000) with a filter wheel with 8 slots for band pass filter (20x20x5 cm, ~$900). Replacing these two components resulted in over a 10x size reduction and over a 15x cost reduction.

For the compact optical spectral imaging system design presented here, a Xenon arc lamp was used, the same type used in the current clinical systems. With experimental proof of our 8 wavelength system, most of the broad spectral output of the Xenon lamp is not needed. Alternatively, multiple low-cost discrete wavelength light
sources, such as light-emitting diodes (LEDs), could replace the Xenon arc lamp. LEDs are available in various visible wavelengths, and are considerably cheaper and smaller than an arc lamp. This modification would have the potential of further decreasing the footprint of the system.

In addition to replacing the light source, other light delivery strategies can also be explored. For the probe presented here, optical fibers are still required for delivery of illumination light. However, optical fibers are fragile and cumbersome, important limitations for clinical use. Individually fixing an optical fiber in the center of each photodiode is a cumbersome procedure, particularly when considering a larger pixel array. One possible strategy for light delivery would be to use a robust large aperture fiber bundle which would terminate a short distance behind the photodiode array, rather than breaking into individual fibers for each pixel. Light exiting from the fiber bundle would travel through free space and pass through the holes drilled in the photodiode detectors. If LEDs are used, then the LEDs themselves may be fixed behind the photodiode array, completely eliminating any optical fibers at the tissue interface. These strategies are analogous to shining a flashlight through a mask, although the actual implementation requires further investigation.

One area of interest is to further explore the imaging aspect of our system. While exploring the system’s imaging capabilities, the spatial resolution is a natural parameter of interest. The spatial resolution of our system is equivalent to the pixel spacing, so in
this particular probe design, ~8 mm. The pixel spacing defines the relative spatial location of each measurement with respect to each other. When reconstructing an image, each region of optical properties will be spaced according to this distance. In order to improve the spatial resolution, the probe would be redesigned with smaller pixel spacing. However, this would not be possible using the same 5.8 x 5.8 mm photodiodes used in this chapter. As the photograph of the probe tip in Figure 4C shows, the photodiodes are already packed as close as physically possible. Increasing the number of pixels or pixel density in the imaging probe could be implemented simply by increasing the number of silicon photodiodes or using smaller photodiodes, respectively. Undoubtedly, this would increase the spatial resolution of the system, but may impact other important system parameters. Moving each pixel closer together may result in increased cross talk. One possible solution would be to use an alternated illumination pattern (i.e. take two separate measurements and light every other pixel, so the effective cross-talk distance is twice the pixel distance). Cross-talk may also be used to our advantage, as with greater cross-talk the measurements become an image reconstruction problem. This problem is similar to the one faced in the heavily researched field of Diffuse Optical Tomography (DOT), where a volume of optical properties is reconstructed from reflectance collected from multiple source and detector pairs. Analyzing reflectance data collected with our compact spectral imaging system using a DOT algorithm is certainly feasible and would address cross-talk issues. Reducing pixel
size can also impact sensing depth. Larger sensing depths could be achieved by using a ring shaped detector with an inner radius slightly larger than the illumination radius, resulting in a ring of “dead area” between the illumination and collection area. This would alter the source-detector separation distance and allow deeper penetrating light to be collected.

The compact optical spectral imaging system presented here experimentally demonstrates progress towards developing a clinically viable spectral imaging system for breast tumor margin assessment. Our group has demonstrated that our scalable Monte Carlo model is capable of quantitatively imaging and extracting optical properties, which contain valuable information on tissue composition which is significant in discerning malignant from benign breast tissue. While these measurements can be acquired using our current clinical system, redesigning the system as described in this manuscript could increase the widespread clinical viability of quantitative spectral imaging through increased portability and speed and decreased cost.

The compact optical spectral imaging system presented here experimentally demonstrates progress towards developing a clinically viable spectral imaging system for breast tumor margin assessment based upon using silicon photodiodes for photon collection. While the utility of this system was demonstrated in only liquid phantoms an ongoing project is focused on completing the clinical translation. In collaboration with Dr. Nan Jokherst, custom fabricated silicon photodiodes are being manufactured for a
clinically robust spectral imaging probe. With these custom designed and fabricated systems, the next step is to move into clinics to test on breast tumor margins.
3. Developing a SIM System for Margin Imaging

Chapter 2 presented a heavily modified DRS system with drastically lower cost and footprint. Despite these improvements to increase clinical viability, there are some additional clinical challenges associated with DRS aside from high cost and accessibility. In recent clinical studies by our group, it was found that generally $\beta$-carotene and $\mu'$ were indicative of residual tumor cells on the margin. However, in certain cases, this ratio indicated a region was at risk, however post-pathological analysis revealed the corresponding tissue was benign fibrous glandular tissue. An example of a false positive is shown in Figure 11.

![Figure 11: An example of a DRS image acquired with a clinical system on human lumpectomy specimen. Based on the $\beta$-carotene to $\mu'$ ratio, sites 1 and 2 both appear could potentially be malignant sites. However, as the pathology reveals, only site 1 corresponds to malignancy, while site 2 corresponds to normal fibro-glandular tissue.](image-url)
With this limitation in mind, we set out to investigate another optical technology with a higher spatial resolution capable of resolving changes in tumor microenvironment. Fluorescence-based SIM was selected as an approach due to its ability to achieve resolutions at the micron scale. In combination with the proper stain, specific tissue structures, such as cell nuclei, can be clearly visualized.

While SIM has been applied to a wide range of samples, the majority of previous publications have focused on imaging of cells in culture or optically clear samples [82, 85, 90-92]. A few groups have successfully applied this technique on thick biological tissues. Elson et al. [93] demonstrate the effect of optical sectioning in thin slices of mouse ear. Santos et al. [94] used HiLo microscopy, a specialized form of structured illumination for optical sectioning, through a fiber bundle microendoscope to image rat mucosa ex vivo. More recently, Lim et al. [95] has also utilized HiLo microscopy in the hippocampus region of ex vivo rat brain. There have been limited reports of applying structured illumination to thick, intact, highly scattering biological samples. Mazher et al. [96] have previously demonstrated structured illumination imaging in a thick tissue-mimicking phantom. However, their implementation and analysis focused on the diffusion regime (mm to cm spatial scales), rather than the diffraction regime, which is more relevant to our microscopic imaging application.

The optical section thickness is dependent on frequency selected for illumination. Chasles et al. [83] provided an in-depth theoretical and experimental analysis of the
effect of different grid frequencies on axial resolution and compared structured illumination microscopy directly to other optical sectioning techniques. They determined that the axial resolution was improved by a factor of 1.5 when using structured illumination over conventional widefield microscopy. Karadaglic et al. [97] presented a detailed theoretical analysis showing that the optical sectioning thickness could be appropriately estimated using the Stokseth approximation [98] of the optical transfer function of a defocused imaging system. However, this derivation was calculated assuming the sample was a thin fluorescent sheet scanned axially in the absence of turbidity. The exact effect of a scattering background on the optical section thickness has not been fully explored. In addition, the modulation depth, which is a measurement of the transfer of the illumination pattern contrast to the focal plane, is also directly related to the illumination frequency and scattering of the sample. Understanding this relationship is vital as the modulation depth has an impact on the signal to noise ratio (SNR) of the sectioned image [84] and the optimal illumination frequency has to balance a tradeoff between optical section thickness (which is better at higher frequencies) vs. modulation depth and SNR which degrades as frequency increases.

In this chapter, I present the design for a SIM system specifically designed for imaging surgical margins. The system was intended to meet the specifications listed in Table 7. Once constructed, the system was tested on a set of tissue mimicking phantoms
to measure, resolution, FOV, contrast enhancement, and SNR. Finally, preclinical images of mouse sarcoma were acquired with the system to demonstrate feasibility in a biological system.

### Table 7: Target design specifications for the SIM imaging system.

<table>
<thead>
<tr>
<th>Target Values for SIM system</th>
<th>Resolution</th>
<th>Field of View</th>
<th>Background Rejection</th>
<th>Single Image Acquisition time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Values for SIM system</td>
<td>4 µm</td>
<td>2.25 mm²</td>
<td>12 dB</td>
<td>&lt; 500 ms</td>
</tr>
<tr>
<td>Justification</td>
<td>Based on nuclear size from mouse sarcoma and human breast tissue [99]</td>
<td>Dependent on time needed to cover whole tumor margin</td>
<td>Based upon segmentation ability of algorithm [80]</td>
<td>Dependent on overall required imaging time</td>
</tr>
</tbody>
</table>

### 3.1 Methods

#### 3.1.1 Structured Illumination Theory

The implementation of SI involves illuminating a sample using a sinusoidal pattern defined by the following equation:

\[
s_i(x, y) = 1 + m \cos(\nu x + \phi_i) \quad \text{Eq. 1}
\]

The quantity \(m\) represents the modulation depth (value between 0 and 1), \(\nu\) represents the spatial frequency, and \(\phi_i\) represents the phase shift of the pattern. The measured image intensity of a sample illuminated by this pattern can be described by the following:
\[ I_i(x, y) = d(x, y) + s_i(x, y) f(x, y) \]  \hspace{1cm} \text{Eq. 2}

In this equation, \( d(x,y) \) represents the fluorescence emitted from the sample which is out of focus, and \( f(x,y) \) represents the in-focus fluorescence. As only the in-focus fluorescence from the object is modulated by the sinusoidal component of the illumination pattern, a proper demodulation method can be applied to extract only this information. The most straightforward and commonly used algorithm relies on square law detection to extract and demodulate the in-focus component.

\[ I_{\text{Sectioned}} = \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2} \]  \hspace{1cm} \text{Eq. 3}

As the equation shows, the image, which contains only information from the focal plane, \( I_{\text{Sectioned}} \), is calculated by acquiring three separate images \( (I_1, I_2, I_3) \) which differ only in phase shift \( (\varphi_1 = 0, \varphi_2 = 2\pi/3, \varphi_3 = 4\pi/3) \). The resulting sectioned image thus only contains the modulated portion of the object, which corresponds to the plane of focus.

### 3.1.2 System Design

In order to achieve comparable performance to the current histopathologic methods, the structured illumination microscopy (SIM) system was designed to resolve cell nuclei. The cell nuclei diameter seen in our intended pre-clinical model, a mouse sarcoma tumor, is in the range of 5-15 µm [100]. Furthermore, human breast cancer nuclei sizes are at least 8 µm in diameter [99]. The field of view (FOV) was also an important consideration which was determined by both the size of the sample and
desired imaging time. Because of the relatively large sizes of the preclinical mouse sarcoma margins (15 mm²) and breast tumor margins (20 cm² [101]), the system was designed with the largest per-frame FOV as possible, while maintaining the required lateral resolution which in our case was 2.5 mm² (1.58 x 1.58 mm). AO was chosen as the contrast agent in this study because it has been demonstrated to stain nuclei, skeletal muscle, and collagenous stroma [67, 77]. While our studies involved imaging samples ex vivo, it is worth noting that AO has been approved for use in humans in at least one previous study [77]. As an alternative, other non-propidium iodide nuclear markers, such as proflavine and acriflavine, have similar staining properties and proflavine, in particular, has been deemed safe for human use and is used as a disinfectant for the umbilicus.
A detailed schematic of the system is shown in Figure 12. A broadband super continuum laser (Fianium SC400) was used to provide illumination for fluorophore excitation. This source was chosen due to its low coherence which mitigated the appearance of speckle in the acquired images. The output from the laser traveled through a band-pass filter centered at 480 nm with a bandpass of 20 nm, which corresponded to the excitation peak of AO. This filter can potentially be replaced to match the excitation peak of other intra-vital dyes or even intrinsic fluorophores. The filtered beam was then passed through a 6X beam expander and a polarizing beam splitter that redirected the light toward an LCoS SLM display chip (Holoeye LCR-720).
Following reflection off the SLM chip, the light traveled through a series of 4 lenses, the last of which was the microscope objective (Nikon 4x E Plan Fluor, NA = 0.1). An iris placed one focal length after the first lens was used to spatially filter the diffraction orders created by the sinusoidal pattern on the SLM. The iris was aligned on the optical axis to only pass the 0 and +1 diffraction orders. Allowing these two diffraction orders to pass yielded a sinusoidal pattern at the sample plane, free of undesired higher frequency harmonics, which produce sectioning artifacts if allowed to pass to the sample. The resulting fluorescence generated by the illumination pattern incident on the sample was collected by the objective and imaged onto the CCD (LaVision Imager 3 QE) using a 200 mm focal length tube lens (Nikon MXA20696).

### 3.1.3 System Imaging Parameters Characterization

Once the system was constructed according to the design specifications above, the basic imaging parameters of the microscope were characterized. For a Nikon 4x objective with numerical aperture (NA) of 0.1, the diffraction limited lateral resolution is expected to exceed the value needed for visualization of cell nuclei (3.2 µm, based on the Rayleigh criterion calculation and emission peak of AO). The actual lateral resolution of the system was measured using a standard 1951 USAF Resolution test target. The test target was placed at the focal plane of the objective with a fluorescence calibration test slide underneath (outside of the focal plane). Uniform excitation light was projected onto the sample and fluorescence emitted from a calibration slide through the test target,
which was imaged onto the CCD. The smallest resolvable line pair group was deemed as the measured lateral resolution of the SIM system. In addition, the single frame field of view (FOV) was also measured using a larger line pair group from the same test target.

Because of the finite pixel number and size of the SLM, a limited number of discrete frequencies could be produced for structured illumination. Using the experimentally measured FOV, the value of each discrete spatial frequency (in mm⁻¹) at the sample plane (for each pattern generated by the SLM) was determined by imaging the fluorescence from a uniform calibration test slide and calculating the Fourier Transform of the resulting image.

3.1.4 Tissue Phantom Preparation

A set of phantoms was constructed in order to simulate the type of environment seen in thick tissue samples stained with AO. Each phantom consisted of fluorescence spheres (Polysciences, Fluoresbrite YG Microspheres) and TiO₂ (Sigma, T8141) in a polydimethylsiloxane (PDMS) sample (Dow Corning, Slygard 184). The phantoms were constructed in a petri dish with a cover glass window on the bottom (Mattek, P35G-0-14-C). The first set of phantoms used a layer of 1-µm diameter fluorescent spheres dried on the cover glass to generate an optically thin layer of fluorescence (simulating the superficial layer of AO in tissue). A 1 cm layer of PDMS was added behind the fluorescent layer with variable concentrations of TiO₂ to create three separate phantoms,
each with different scattering levels where the reduced scattering coefficients were $\mu_s' = 0 \text{ cm}^{-1}$, 10 cm$^{-1}$, and 20 cm$^{-1}$. A quantity of 0, 2.25, and 4.50 grams of TiO$_2$ per gram of uncured PDMS was added to the PDMS for each respective scattering level, calculated according to a previously published procedure [102]. The TiO$_2$ was thoroughly mixed with the PDMS prior to curing. Finally the phantom was placed in a vacuum chamber to draw out all residual air bubbles from the mixing process and also to effectively cure the PDMS.

Another solid phantom was constructed using PDMS, 10-µm diameter fluorescent polystyrene spheres and TiO$_2$ spheres. The size of the fluorescent spheres was chosen to simulate the size of targets the system was designed to detect (cell nuclei). The underlying PDMS and TiO$_2$ layer was mixed to generate a single scattering coefficient of 10 cm$^{-1}$, which is a commonly measured reduced scattering coefficient in soft tissues [66, 101].

3.1.5 Structured Illumination Characterization

The phantom consisting of a single layer of 10-µm spheres with a background reduced scattering coefficient of 10 cm$^{-1}$ was used to demonstrate the contrast improvement by computing the signal to background in the structured illumination (i.e. sectioned) and uniform illumination (i.e., non-sectioned) images. The contrast ratio was quantified by taking the mean fluorescence intensity of a sphere and dividing it by the mean intensity of the adjacent background. Additionally, the 10-µm spheres were
representative of typical size cell nuclei both in mouse and human tissue, so this phantom was an appropriate biological model for testing the system.

![Schematic diagram](image)

**Figure 13:** Schematic demonstrating the method used to measure the optical sectioning strength of the imaging system. Also shown is a detailed diagram of the structure of the solid phantom used for measurement. Three separate phantoms were creating with increasing levels, $\mu_s' = 0, 10, 20 \, \text{cm}^{-1}$.

A straightforward procedure was used to experimentally determine the effective optical section thickness of the SIM system, as illustrated in Figure 13. The thin fluorescent phantoms (single layer of 1 µm spheres) were translated axially toward the objective and a sectioned image (using structured illumination) was acquired at each 10 µm step. The mean fluorescence intensity from a region of interest (ROI) within the sectioned image was plotted against axial depth to determine the section thickness of the system. In addition, the section thickness was measured over a range of illumination pattern frequencies, for each of a range of phantoms with different scattering properties.

In order to verify the experimental optical section thickness measurements, the results were compared to predicted theoretical results. The defocus of the structured
illumination pattern has been previously shown to match the Stokseth empirical approximation of the optical transfer function [97, 98].

\[
I(u) = f(\tilde{v}) \frac{J_1 \left[ 2u \tilde{v} \left( 1 - \frac{\tilde{v}}{2} \right) \right]}{2u \tilde{v} \left( 1 - \frac{\tilde{v}}{2} \right)}
\]

Eq. 4

where \( f(\tilde{v}) = 1 - 0.69 \tilde{v} + 0.0076 \tilde{v}^2 + 0.043 \tilde{v}^3 \)

\[
\tilde{v} = \frac{v\lambda}{n \sin(\alpha)}; u = \frac{8\pi}{\lambda} zn \sin^2 \left( \frac{\alpha}{2} \right)
\]

Where \( I(u) \) is the optical sectioning axial response, \( J_1 \) is the Bessel function of the first kind, \( v \) is the grid frequency (as shown in Eq. 1), \( \tilde{v} \) is the normalized grid frequency, \( \lambda \) is the emission wavelength, \( n \sin(\alpha) \) is the NA of the objective, \( z \) is the real axial distance, and \( u \) is the normalized axial distance. The Stokseth approximation provided a valid theoretical optical section thickness value to verify measured results.

Identifying the exact relationship between section thickness and illumination frequency was only the first step in selecting the appropriate frequency for imaging. The modulation depth, \( m \) in the previously shown Eq. 1, is a quantity, which represents the amplitude of the sinusoidal illumination pattern transferred onto the sample. The importance of the modulation depth becomes apparent after combining Eq. 1 and Eq. 2 with Eq. 3 which results in the following [84]:
As expected, we can see that the resulting signal of the sectioned image, $I_{\text{Sectioned}}$, is described primarily by the in-focus fluorescence, $f(x,y)$. However, a coefficient dependent on modulation depth, $m$, appears in this equation and scales $f(x,y)$ accordingly. In addition, modulation depth decreases with frequency because of the natural decay in the shape of the incoherent optical transfer function of the objective.

The following procedure detailed by Hagen et al. [84] was used to measure the modulation depth using the three phase shifted structured illumination images ($I_1$, $I_2$, $I_3$):

$$m(x, y) = \frac{8}{3} \left( \left( \mu_1 - \frac{1}{2} \right)^2 + \left( \mu_2 - \frac{1}{2} \right)^2 + \left( \mu_3 - \frac{1}{2} \right)^2 \right)^{\frac{1}{2}}$$

where $\mu_i = \frac{I_i}{\frac{2}{3}(I_1 + I_2 + I_3)}$

This method can be used to measure the modulation depth, $m(x,y)$ at each pixel within a given image. The median value was chosen to represent the modulation depth over a specific ROI in each image, where fluorescent targets were present. This median modulation depth was measured for a range of frequencies (using the same ROI and sample location) on the same fluorescent phantoms used to characterize the optical
section thickness. The phantom was simply placed in focus at the sample plane and three phase shifted images at multiple illumination frequencies were acquired.

Because of the direct relationship of modulation depth on recovered signal, and the inverse relationship between SNR and modulation depth, an illumination frequency yielding both a sufficient modulation depth and optical section thickness must be selected. The SNR of a sectioned image is a quantitative measurement of image quality, which is directly influenced by modulation depth. Using the derivations of Hagen et al. [84], the following equations were used to calculate the SNR of the uniform and sectioned images respectively:

\[
\text{Uniform SNR} = \frac{\langle I_w \rangle}{\sigma(I_w)} \quad \text{Eq. 7}
\]

\[
\text{Sectioned SNR} = \frac{m}{2} \frac{\sqrt{3} \langle I_{\text{Sectioned}} \rangle}{\sigma(I_w)} \quad \text{Eq. 8}
\]

where \( I_w = \frac{2}{3} (I_1 + I_2 + I_3) \)

Here, \( I_w \) represents the traditional widefield image that is reconstructed from the three phase shifted structured illumination images. The sectioned image, \( I_{\text{Sectioned}} \), is calculated using Eq. 4. The modulation depth (introduced in Eq. 1), \( m \), has a direct impact on the SNR of the sectioned image as shown in Eq. 8. Thus, the measurements of optical section thickness, modulation depth and corresponding SNR characterized at
multiple frequencies, were used to provide insight into selecting a single illumination frequency that provided the best trade-off between these parameters.

3.1.6 Imaging of Tumor Margins in a Primary Mouse Model of Soft Tissue Sarcoma

In order to demonstrate feasibility for imaging tumor margins, the system was tested in a primary mouse model of sarcoma. This model, which was also used for biological verification in previous margin imaging studies [103], provides a controlled environment of spontaneous tumor growth, and serves as an appropriate preclinical test bed for our system. Primary sarcomas were induced by intramuscular injection of mice with conditional mutations in oncogenic K-ras or Braf and p53 with an adenovirus expressing Cre recombinase as previously described [100, 104]. The tumor was grown to approximately 500-700 mm$^3$ and then surgically removed from the animal, then two separate imaging protocols were followed. The first was to establish congruence between the morphology imaged by the SIM system and histopathology by imaging frozen sections. The second was to establish the feasibility of using SIM microscopy to image margins by examining freshly excised sarcoma tissues.

Frozen sections were used to demonstrate that the fluorescent staining approach was capable of highlighting tissue morphology comparable to traditional H&E stained histology slides. Immediately after removal from the mouse, the excised sarcoma tissue was embedded in optimal cutting temperature compound (OTC) and flash frozen in liquid nitrogen. The frozen tissue block was sliced into 50 µm thick sections spaced 50
µm apart and placed on a microscope slide. To prepare the sample for SIM imaging, the tissue sections were allowed to thaw (~5 minutes) and a solution of 0.01% AO (mixed with water) was applied to stain the tissue. The stained tissue sections were then imaged using the SIM system and then immediately fixed in formalin. Next, the fixed tissue sections were stained with H&E and imaged using a standard bright-field microscope.

For the second protocol, the fresh, intact tumor specimen was stained with a 0.02% solution of AO immediately after tumor excision. After ~30 seconds, the tissue was thoroughly rinsed with a phosphate-buffered saline solution to remove any excess contrast agent. A coverglass was placed over the stained tumor tissue to create a flat surface and images were acquired using the SIM system. This procedure was repeated when imaging normal skeletal muscle tissue excised from the normal contralateral hind limb of the mouse. Multiple sites were imaged from a single mouse to acquire a sufficient image dataset for this protocol.

### 3.1.7 Quantitative Image Processing

As previously mentioned, a well-known hallmark of cancer is increased cell nuclear density, due to the increased rate of growth in malignant tissue. To exploit this information, a high pass filter (HPF) algorithm was applied to segment and isolate the cell nuclei from other features within the image. Specifically, a Gaussian filter with a standard deviation of 20 pixels was convolved with each maximum-intensity
normalized image (implemented using MATLAB). The standard deviation was empirically chosen such that a majority of the nuclei were isolated with HPF. Each pixel corresponded to 1.5 µm given the combination of the specific CCD used for detection and 4x magnification objective for imaging. The typical diameter of a cell nucleus is 5-15 µm, which corresponded to approximately 3-10 pixels. Then, a threshold value of 0.1 (10% of the peak intensity) was applied to the output from the HPF, and any pixel that was greater than 0.1 was considered part of a nucleus. Next, a connected components algorithm, in which connected pixels are assumed to belong to the same cell nucleus, was applied to extract the number of cell nuclei from the filtered image.

The quantitative image processing algorithm was first applied to the images acquired in frozen tissue sections. The goal was to determine the expected nuclear density observed in sarcoma tumor tissue. The frozen tissue sections were well-suited for this purpose as they had corresponding and congruent H&E sections, which are widely used by pathologists for diagnosis. Two frozen sections from four different mice (N=4) were acquired and processed using the HPF algorithm to isolate and count the number of cell nuclei per mm². Because of differing tumor sizes, an ROI (350x350 pixels) of solid tumor was manually selected in each image. The average density was calculated over all four mice, which was then used as a benchmark when imaging thick excised sarcoma tumor margins.
However, in contrast to the frozen section samples, background rejection using SIM is vital in thick sarcoma margins to reject background fluorescence and the illumination frequency is an important factor in determining the tradeoff between optical section thickness and modulation depth. Multiple sites (N=5) from a freshly excised intact sarcoma tumor harvested from one mouse was imaged at multiple frequencies. The extracted nuclear density from the optically sectioned thick tissue images using the HPF algorithm was calculated over a manually selected ROI the same size (350x350 pixels) used in the frozen section analysis. The average nuclear density at each illumination frequency was compared to the benchmark nuclear density established from frozen section imaging using a Wilcoxon rank-sum test. For all tests, a p value of less than 0.05 was considered to reject the null hypothesis. Measuring and understanding this information provided further justification for selection of a specific illumination frequency to optimize the extraction of nuclear density in the tumor margin of the genetically engineered mouse model of sarcoma.

3.2 Results

3.2.1 Characterization of Imaging Parameters

The single frame field of view (FOV) and lateral resolution were the first imaging parameters measured using a 1951 USAF resolution target. Using a 4X objective, the smallest element on the target (group 7, element 6) corresponding to a lateral resolution of 4.4 µm, was clearly resolved, with a single frame field of view (FOV) of 2.06 x 1.56
mm (3.21 mm²). This measured resolution met our previously mentioned design criteria both in terms of enabling visualization of individual cell nuclei (in both preclinical mouse models and human tissue) as well as the single-frame FOV to allow imaging of typically sized sarcoma tumor margins.

Table 8 shows a complete list of all illumination frequencies that were achieved at the sample plane, with the corresponding objective lens. The 4X objective was used in the phantom and sarcoma margin imaging studies due to the wider range of higher illumination frequencies at which the samples could be imaged.

<table>
<thead>
<tr>
<th>Objective</th>
<th>Absolute Frequency at Sample Plane (mm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x (NA = 0.1)</td>
<td>101, 67.0, 50.3, 47.7, 40.7, 31.7, 24.1, 19.6</td>
</tr>
<tr>
<td>2x (NA = 0.1)</td>
<td>25.1, 16.7, 12.5, 10.2</td>
</tr>
</tbody>
</table>

### 3.2.2 Characterization of Structured Illumination in Phantoms

Images were first taken on the phantoms consisting of single layer of 10-µm diameter fluorescence spheres. The spheres were embedded in PDMS and TiO₂ giving the surrounding medium a biologically relevant reduced scattering coefficient value (μₛ' = 10 cm⁻¹) to introduce background fluorescence. The resulting images are shown in Figure 14, under both uniform illumination and structured illumination. Each image was normalized by dividing each pixel by the maximum intensity within the image. The
images clearly demonstrate the improvement seen in structured illumination compared to uniform illumination. The specific illumination frequency chosen to acquire these images was 31.7 \( \mu \text{m} \). The contrast ratio was calculated directly in both images by manually selecting ROIs for 5 spheres and dividing by a background ROI (both indicated in Figure 14). The contrast ratio was averaged over these five spheres, which showed a significant quantitative improvement, 889±58\% greater in the sectioned image over the uniform image.

Figure 14: Images of a single layer of 10 \( \mu \text{m} \) fluorescent spheres embedded in PDMS and TiO\(_2\). The reduced scattering coefficient of the phantom is approximately 10 cm\(^{-1}\). Images were taking using a 4x NA = 0.1 objective and an illumination frequency of 31.7 mm\(^{-1}\). The improvement in contrast is clearly seen from the uniform to structured illumination image. The signal-to-background was calculated by taking the intensity of 5 manually selected spheres (indicated by red arrows) and dividing by the background ROI (indicated by the blue square). All scale bars are 100 \( \mu \text{m} \).
The optical sectioning strength for each corresponding illumination frequency (from Table 8) was measured using the phantoms and methods described previously and also depicted in Figure 13. The optical sectioning thickness decreased as the illumination spatial frequency increased, as expected. The results detailing the experimental relationship between optical section thickness and illumination frequency are displayed in Figure 15. The plot on the left shows a single axial scan through one phantom (single layer of 1 µm spheres, \( \mu_s' = 0 \text{ cm}^{-1} \)) using an illumination frequency of 19.6 mm\(^{-1}\). The optical section thickness was defined as the distance at which the mean intensity dropped to 50% of the intensity at the focal plane. The plot on the right shows the measured optical section thickness in all three phantoms (each with a different scattering level) at all available illumination frequencies. Each of the three phantoms is represented with a different symbol. Overall these results clearly show a non-linear relationship between optical section thickness and illumination frequency. Error bars on each measurement were generated by selecting eight different regions of interest within each image stack and measuring the standard deviation among the optical sectioning thickness of all regions. Statistical analysis of the data using a Student’s t-test demonstrated no significant difference between scattering levels. The measured optical section thickness data was compared to a theoretical value acquired using the Stokseth approximation to the optical transfer function (Eq. 4) described in the Methods section. As shown in Figure 15(A) the measured data of the single axial scan matched with...
Stokseth approximation (using the variables \( v = 19.6 \text{ mm}^{-1}, \lambda = 520 \text{ nm}, \) and \( \text{NA} = 0.1 \)).

Also shown in Figure 15(B), the measured optical section thicknesses showed excellent agreement with the theoretical optical section thickness calculated using the Stokseth approximation.

Figure 15: Optical section thickness measurements from solid phantoms. (A) Plot of the mean image intensity as a function of distance from focal plane used to determine optical section thickness. The circles represent data that was acquired on the \( \mu'_s = 0 \text{ cm}^{-1} \) phantom using a 4x NA = 0.1 objective with an illumination frequency of 19.6 mm\(^{-1}\). The solid line represents the Stokseth approximation (Eq. 4) calculated using the same variables. (B) Plot relating optical section thickness to illumination grid frequency for a range of reduced scattering coefficients. The datapoints represent the measured values on phantoms, and the solid line represent the theoretical value calculated using the Stokseth approximation. The dotted arrows show how the optical section thickness was measured on the left and how it was placed on the corresponding plot on the right.

After determining the frequency dependence of optical section thickness, the modulation depth at each illumination frequency was measured using the method described by Hagen et al. [84]. The measured modulation depths in two separate
phantoms (µs' = 0 and 10 cm⁻¹) for each frequency are shown in Figure 16. The modulation depth was calculated, as described in the Methods section, over an identical ROI in each image for all illumination frequencies using the same laser illumination power and CCD integration time. Error bars were generated by computing the standard deviation of modulation depth over 10 images at the same sample location. The plot clearly indicates an inverse relationship between modulation depth and illumination frequency. In addition, a significant decrease in modulation depth at all frequencies is seen in the phantom with µs' = 10 cm⁻¹ compared to the non-scattering phantom.

Figure 16: Modulation depth as a function of grid frequency for non-scattering phantoms and scattering phantoms (two µs' levels). The points labeled A and B are referenced in the Discussion section.

Figure 17 shows a plot of the ratio of uniform SNR to sectioned SNR against modulation depth (i.e., it represents the reduction in SNR of SIM compared to standard widefield microscopy). As the data demonstrates, the ratio decreases with modulation
depth, indicating less degradation in SNR at higher modulation depths compared to lower modulation depths.

![Figure 17: SNR ratio (uniform SNR to sectioned SNR) versus modulation depth for non-scattering and scattering phantoms. The points labeled A and B are referenced in the Discussion section.](image)

3.2.3 Demonstration of System in a Mouse Model of Sarcoma

To demonstrate feasibility of imaging tissue histology with appropriate resolution, the system was tested on 50 µm thick frozen tissue sections cut from a sarcoma tumor harvested from a primary mouse model of sarcoma. Representative images are shown in Figure 18. These images demonstrate excellent congruence between the morphological images acquired by the SIM system and the H&E micrographs. It is clear from these images that the AO stains both the cell nuclei and
skeletal muscle. Importantly, the features of the H&E image are recapitulated in the AO image based on intensity differences: the cell nuclei are more brightly stained than the muscle tissue. The sarcoma tissue can be observed invading into the muscle tissue in both the H&E and AO image. Because these were thin tissue sections, the images did not contain a large amount of background fluorescence. As can be seen, there is not substantial image contrast improvement from the uniform to the sectioned image (acquired at f = 31.7 mm\(^{-1}\)).

![Images of a 50 µm tissue slice demonstrating the correlation between images acquired by the SIM system and H&E histological micrograph. SIM images were taking using the 4x objective and a frequency of 31.7 mm\(^{-1}\). H&E images were taken using a 2.5x objective and the images were cropped to match one another. Site imaged on tissue contains both cross sectional skeletal muscle (M) and sarcoma tumor (T). The arrows on each image point out a site where tumor tissue was invading into the normal skeletal muscle. Scale bars are 100 µm.](image)

To demonstrate the feasibility of imaging of thick tissue, freshly excised sarcoma tumor tissue was imaged using the SIM system. Representative images (uniform and sectioned) of mouse skeletal muscle are shown in Figure 19(A) and sarcoma tumor in Figure 19(B). Because these tissue samples are intact and non-sectioned a large amount of scattered background fluorescence is visible in the uniform illumination images. As a
result, the contrast enhancement provided by structured illumination improves visualization of individual muscle fibers in normal tissue and individual cell nuclei in tumor tissue. Both of these tissue sites were imaged with a frequency of 37.1 mm$^{-1}$, which resulted in median modulation depths of 0.24 and 0.17 in the muscle and tumor, respectively.
Figure 19: Uniform and structured illumination images acquired from mouse tissue. (A) Images of skeletal muscle from mouse. Both longitudinal and cross-sectional muscle can be seen in the region of interest. (B) Image of tumor tissue from mouse sarcoma. Cell nuclei are the only source of contrast apparent in these images. Contrast enhancement is clearly seen in the sectioned images (acquired at f = 31.7 mm−1). Scale bars are 100 µm.

3.2.4 Analysis of Images Using a HPF algorithm for Quantification of Nuclear Density

As shown in Figure 19, application of AO as a fluorescence contrast agent enabled the SIM system to clearly visualize cell nuclei in both frozen sections and intact tissue. While qualitative differences were seen in these images, a quantitative metric such as nuclear density would aid in assessing the effect of illumination frequency on the quality of the sectioned image. As an initial approach, a simple HPF algorithm was used to isolate the cell nuclei described in the methods section. This was first applied to a set of eight images acquired from frozen sections of a sarcoma tumor from four different mice (two images per mouse). The samples were specifically selected so that pure sarcoma tumor tissue (confirmed by H&E staining) was visible and an ROI (350x350 pixels) containing only tumor tissue was manually selected from each image. Quantitative analysis of this image set indicated that the average cell nuclei count was 3561±754 nuclei/mm² over a single ROI of pure sarcoma tumor tissue averaged across the eight images (mean ± standard deviation calculated from different FOVs of sarcoma tissue harvested from one animal).

Next, the HPF quantification algorithm was applied to images of thick in situ sarcoma tumor to determine the impact of different illumination frequencies on the
quantitation of nuclear density. Again, the sample was selected to ensure that tumor
tissue was imaged and the same ROI (350x350 pixels) was imaged using three different
illumination frequencies (24.1, 31.7, 47.7 mm\(^{-1}\)). The original frozen section (50 \(\mu\)m tissue
slice), in situ uniform, and in situ sectioned images (only \(f = 31.7\) mm\(^{-1}\) shown) and
corresponding HPF images are displayed in Figure 20. These images demonstrated that
applying HPF to the in situ uniform image was unable to accurately isolate cell nuclei,
while also applying HPF to the in situ sectioned image isolated more cell nuclei, which
closely resembled the 50 \(\mu\)m tissue slice.

![Figure 20](image)

**Figure 20:** Fluorescent images of mouse sarcoma tissue processed using a
high-pass filter (HPF) algorithm to isolate cell nuclei. The in situ sectioned image
(acquired using \(f = 31.7\) mm\(^{-1}\))

The corresponding sectioned images of sarcoma tissue at each illumination
frequency are shown in Figure 21(A), which qualitatively show the impact of frequency
on isolating cell nuclei. For a quantitative analysis, the mean nuclear density was
calculated on a set of five separate images of in situ sarcoma tumor tissue (different FOVs of sarcoma tissue harvested from one animal). This was done on both the uniform illumination and sectioned images acquired at three illumination frequencies and a comparison to the mean nuclear density from the 50 µm frozen section images are shown in Figure 21. The p-values were calculated using a Wilcoxon rank-sum test, and revealed that the in situ density from the uniform illumination images were significantly lower (p<0.05) than the quantified density from the 50 µm tissue slice images and the sectioned images at all three frequencies. Specifically, the nuclear density from the uniform illumination images was smaller than the density in the tissue slice and sectioned images indicating that the lower contrast between nuclei and the background seen in the uniform illumination (i.e., non-sectioned) images leads to underestimation of nuclear density.
Figure 21: Results demonstrating the impact of illumination frequency on quantification of cell nuclei density. (A) Sectioned images of mouse sarcoma tissue at multiple illumination frequencies. The corresponding HPF processed images are shown which demonstrate the isolation of cell nuclei. (B) Mean density of cell nuclei extracted from images mouse sarcoma tissue. The nuclear density extracted from the 50 µm thick frozen section images (N=9) was compared to density extracted from in situ tissue imaging (N=5), both uniform and sectioned (f = 47.7, 31.7, 24.1 mm⁻¹) images.
3.3 Discussion

Structured illumination is an elegant approach to solve the problem of optical sectioning in microscopy, essentially analogous to frequency modulation techniques used to encode electrical signals. Structured illumination is a low-complexity solution for optical sectioning microscopy of thick tissues that has the potential for clinically feasible high throughput microscopy of tumor margins due to its light efficiency and parallel-pixel detection approach. We have presented a custom SIM microscopy system with a low magnification, low NA objective designed to maximize the single-frame FOV for applications requiring large area tissue surveillance such as is the case for tumor margin imaging. We designed a series of experiments to fully characterize SIM performance for tumor margin imaging, and demonstrated that SIM (optical sectioning) of thick tissue provides equivalent quantitation of nuclear density, a hallmark of carcinogenesis, as compared to frozen section analysis (physical sectioning) using the same fluorescent staining approach.

The choice to use an SLM to generate the structured pattern for illumination allowed us to explore the role of frequency in optical sectioning performance in thick, turbid samples. It is generally understood that using a higher illumination frequency yields a thinner optical section at the focal plane, leading to greater background rejection and enhanced contrast at the focal plane. While this is a desirable result, the caveat of using a higher frequency is a decrease in modulation depth primarily due to the
attenuation of higher pattern frequencies by the optical transfer function of the illumination optics. Furthermore, the effect of sample turbidity on optical sectioning performance with SIM had not been systematically investigated prior to this work. As demonstrated in Figure 15, regardless of the underlying scattering properties of a sample, increasing the illumination frequency yields increasingly thinner optical sections (which follow theoretical calculations of attenuation of signal from a thin fluorescent sheet with defocus). However, the decrease in optical section thickness is not linear with respect to illumination frequency and actually follows an asymptotic relationship. It has been previously shown that the thinnest optical section that can be achieved is using a normalized grid frequency \( \tilde{v} \) (ref. Eq 6) equal to 1 [97]. Given the specific parameters of our system (NA = 0.1, \( \lambda = 520 \) nm), this would correspond to an absolute frequency of 191 mm\(^{-1}\), resulting in an optical section thickness of 36.6 \( \mu m \). Using the Stokseth approximation, 90% of the thinnest optical section (40.6 \( \mu m \)) is achieved at a frequency of 132 mm\(^{-1}\). An additional 10% increase of the optical section thickness to 45.7 \( \mu m \) occurs at an illumination frequency of 106 mm\(^{-1}\). In other words, increasing the illumination frequency from 106 mm\(^{-1}\) to 191 mm\(^{-1}\) (an 80% increase in illumination frequency) only results in a 20% decrease (<10 \( \mu m \)) in optical section thickness. From this data, it is clear that there are diminishing returns when trying to achieve a thinner optical section by increasing the frequency.
In contrast to the optical section thickness, the modulation depth decreases nearly linearly with increasing spatial frequency as shown in Figure 4. This is important because the SNR is directly linked to modulation depth and as Figure 5 demonstrates, this relationship is also non-linear. This indicates that not only is there potentially less benefit in increasing the frequency to reduce optical section thickness as the frequency cutoff of the optical transfer function is reached, but in doing so it would also significantly degrade the SNR of the sectioned image as well. For example, looking specifically at the illumination frequency of 31.7 mm\(^{-1}\), the corresponding optical section thickness at this frequency was 129 µm. If the illumination frequency is increased to 47.7 mm\(^{-1}\), then the expected optical section thickness would be decreased by 29%. However, the SNR would be disproportionally decreased by 217%, assuming that the reduced scattering coefficient of the medium, \(\mu_s'\) is 10 cm\(^{-1}\).

Figure 16 indicates that the achievable modulation depth is also affected by the amount of background signal, which underscores the need to measure modulation depth in the target tissue before choosing the optimum illumination frequency.

Additionally, Figure 17 shows that with knowledge of modulation depth, the SNR reduction in sectioned images can be estimated. A comparison between two specific data points in Figure 6, referred to as point A (illumination frequency = 47.7 mm\(^{-1}\), \(\mu_s' = 0\) cm\(^{-1}\)) and point B (illumination frequency = 31.7 mm\(^{-1}\), \(\mu_s' = 10\) cm\(^{-1}\)), is instructive. While they have noticeably different illumination frequencies and scattering properties, Figure
16 shows that the measured modulation depth of points A and B are actually very similar, 0.248 and 0.252, respectively (~2% difference). And it follows, as no surprise, that in Figure 17, the uniform SNR to sectioned SNR ratio for point A and B are 17.5 and 17.1, respectively, which are also very similar (~2% difference). Regardless of how a particular modulation depth is achieved, either through using a certain illumination frequency, altered by scattering background, or the combination of the two, the SNR reduction is only dependent on the actual measured modulation depth.

Finally, the images from pure sarcoma tumor tissue, which were processed using the HPF algorithm and shown in Figure 20, demonstrated the value of SIM, as standard uniform illumination failed to isolate the majority of cell nuclei and significantly underestimated the nuclear density (shown in Figure 21). Interestingly, the statistical analysis also showed that regardless of the three illumination frequency selected, the quantified nuclear densities from the optically sectioned images were not statistically different from the 50 µm tissue slices. As a compromise between optical section thickness and SNR reduction, the illumination frequency of 31.7 mm$^{-1}$ was selected for this specific system and imaging application. At this frequency, the expected optical section thickness was 129 µm and the median modulation depth over the entire image was 0.137. Although this modulation depth corresponded to a 50-fold reduction in SNR from the widefield case, it was clear that this was outweighed by the improved contrast with respect to not only visualizing, but also properly segmenting tumor cell nuclei. It is
worthwhile to note that this combination of illumination frequency, wavelength, and NA yield a normalized frequency (introduced in Eq.4) of $\tilde{\nu} = 0.165$. Previous studies have shown that a normalized frequency of $\tilde{\nu} = 1$ yields the thinnest possible optical section [83, 97]. While this certainly holds true in the case of our system, we determined that use of the lower spatial frequency provided sufficient benefit from background rejection due to optical sectioning, while still retaining enough signal to identify critical structural information in the sectioned images. Future controlled studies on a larger cohort of animals will determine the accuracy of using this system to differentiate between positive and negative tumor margins.

The conclusion for illumination frequency choice was intended specifically for our system and biological application described in this chapter. In other applications and system configurations, the optimal illumination frequency may be different than the one chosen. We note here that although we used a 4X objective in this implementation to achieve a desired balance between single-frame field of view and resolution, that the resolution could be increased at the expense of single-frame field of view, by going to higher power objectives. Mosaics can be constructed which provide an overall larger field of view, and allow the images to be viewed digitally at a range of magnifications (larger than the single-frame magnification). However, our results demonstrated that the optical section thickness for a given microscope objective could be accurately represented using the Stokseth approximation regardless of the scattering properties of
the tissue. Additionally, the SNR reduction in the sectioned image was only dependent on modulation depth and independent of tissue scattering. While the scattering did affect the modulation depth, it can be measured and quantified directly without knowing the exact scattering properties of the tissue. Therefore, optical section thickness and SNR reduction can be calculated simply by knowing the objective NA and measuring the modulation depth, respectively. This yields a straightforward approach for one to characterize structured illumination and determine the appropriate illumination frequency for a specific application.
4. Preclinical Validation of the SIM system

As the results and conclusions from Chapter 3 demonstrate, the SIM system has the necessary specifications for imaging tumor margins. In the following chapter, I focus on a study where the SIM system has been applied to a larger cohort of animal. In total, 23 margins were imaged from tumors that were surgically excised from mice. The images were quantitatively analyzed using an algorithm called maximally stable extremal regions (MSER) to identify and segment AO-stained regions arising from cell nuclei. Finally, a predictive model was constructed using logistic regression to distinguish between tumor and normal tissue sites and ultimately classify whole margins as positive or negative.

4.1 Methods

4.1.1 Margin Imaging Protocol

To simulate clinical tumor growth and disease progression, a transgenic sarcoma model was selected as a primary testbed for margin classification [100]. Mice with conditional mutations in oncogenic K-ras or Braf and p53 were injected intramuscularly in their hind leg with an adenovirus expressing Cre recombinase to induce primary sarcomas. The spontaneous nature of this model accurately represented a clinical tumor margin compared to a tumor xenograft that would tend to develop a fibrous encapsulation.
After injection of the adenovirus, the sarcoma tumor was allowed to develop and grow to 500-700 mm$^3$ (60-90 days) and then surgically removed from the animal. To simulate clinical treatment, the mouse was anesthetized with isofluorane for the duration of the surgical procedure. During the procedure, the entire leg, including the tumor mass, was amputated to expose the relevant margin. The excised tumor margin was immediately prepared for imaging by topically applying a 0.01% aqueous solution of AO and then rinsed with 0.1M phosphate-buffered solution (PBS). To obtain the highest quality images, a cover glass was placed on top of the tumor margin to create a flat focal plane for imaging.

A brief overview of the imaging procedure is shown in Figure 22. The sample was placed on a 3-axis translation stage under the imaging objective for the purposes of focusing and lateral translation. Due to the large relative size of the tumor relative to the field of view (FOV) of the SIM system, multiple images were acquired to cover entire margin. Beginning at the first image location, the sample was translated to each subsequent image location using a micrometer to ensure the sample was moved an equal distance between image sites. A small amount of overlap between adjacent images was allowed to ensure the entire margin was imaged. The sample was moved 1.7 mm in the x-axis and 2.0 mm in the y-axis, with a typical margin fully imaged using 4-6 sites.
Figure 22: Flowchart demonstrating the imaging protocol immediately after the tumor margin was removed from the transgenic sarcoma model. After the procedure above was completed, the tissue sections were inked to identify the area imaged, stained with H&E and submitted to pathology.

To correlate the imaging results obtained with the SIM system to pathology, a procedure was carefully followed immediately after imaging. Tattoo ink was painted over the region of the tissue that was imaged, which allowed the pathologist to identify the corresponding margin after fixing and slicing the tissue. After applying ink, the tissue was immediately frozen in optimal cutting temperature gel (OCT) using liquid nitrogen. A microtome was used to slice 5 μm thick tissue sections (cross-section to the tissue face painted with ink) spaced 500 μm apart. The tissue sections were then fixed in formalin and then stained with H&E. A pathologist reviewed all slides from a given margin to classify it as a positive or negative margin. The exact number of H&E slides varied based on the physical size of the margin. The criterion used to determine a positive margin was to identify the presence of any tumor cells directly in contact with ink.
4.1.2 Image Processing Algorithm (MSER)

In order to analyze and assess the images obtained of the sarcoma margins, an imaging processing algorithm was optimized and applied to the images in order to segment the AO-stained regions with texture arising from cell nuclei. The goal of the analysis was to leverage the specific increase in nuclear density and changes in nuclear shape and size that differentiate positive from negative margins.

A technique called maximally stable extremal regions (MSER) was used to segment specific AO-stained features from a heterogeneous background of muscle and adipose tissues [105]. MSER has been used previously in the image processing community for automatic reconstruction of 3D scenes, but has also been applied to biomedical microscopy in recent studies [106, 107]. A flow chart with an overview of the MSER algorithm is shown in Figure 23. Briefly, MSER utilizes basic intensity thresholding; however, no global or optimal threshold is sought, rather all thresholds are tested and the stability of the isolated connected components (i.e. nuclei) are evaluated. More specifically, all possible thresholds are applied to an image and all sets of connected components (adjacent pixels with intensity values that exceed the current threshold) that are isolated with each threshold are stored. This yields a data structure in which the area of each connected component is stored as a function of the intensity threshold. Finally, the intensity thresholds that correspond to a local minimum in the rate of change of the area function for each connected component are selected as thresholds producing MSER.
Figure 23: A) Overview of the maximally stable extremal regions (MSER) algorithm. This algorithm, which is used in the automatic reconstruction of 3D scenes was selected as it was a more robust approach than a simple global image threshold. MSER tests all intensities thresholds within the image to ensure local maxima within various background intensities are not lost. B) A detailed description and visual representation of the five variable parameters used in MSER, MinArea, MaxArea, Delta, MaxVariation, and MinDiversity.

4.1.3 MSER Optimization Procedure

In order to apply MSER specifically to our images, five tuning parameters associated with MSER, which are detailed in Figure 23B, needed to be selected. The descriptions of all variables are provided in Table 9. The two most straightforward parameters are MinArea and MaxArea, which are related to the expected size of the
connected components (i.e. nuclei). These parameters were selected based on the biologically expected range of nuclear diameters because the goal of applying MSER was to segment AO-stained features primarily generated from the cell nuclei.

Specifically, MaxArea was set to 15 pixels, which corresponds to greater than 20 µm in diameter, which is the largest nuclear size for the sarcoma mouse model. MinArea was set to 3 pixels, which correspond to less than 5 µm in diameter, which is the smaller nuclear size for the sarcoma model. The next set of parameters was related to the intensity thresholds and included MaxVariation, MinDiversity, and Delta. These intensity parameters were systematically tuned through the range of values seen in Table 9 on 30 representative images containing distinct tissue types, 10 tumor, 10 muscle, and 10 adipose.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Range tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>MinArea</td>
<td>Minimum allowed size of connected component region. Selected based on minimum expect nuclei size.</td>
<td>[3] (pixels)</td>
</tr>
<tr>
<td>MaxArea</td>
<td>Maximum allowed size of connected component region. Selected based on maximum expect nuclei size.</td>
<td>[15] (pixels)</td>
</tr>
<tr>
<td>MaxVariation</td>
<td>Maximum intensity variation allowed within a region</td>
<td>[0 50] (bit-depth)</td>
</tr>
<tr>
<td>MinDiversity</td>
<td>When the relative variation of two nested regions is &lt; MinDiversity, then only the most stable region is selected</td>
<td>[0 1] (relative units)</td>
</tr>
<tr>
<td>Delta</td>
<td>Related to the intensity stability of a region of connected components. The stability of a region is the relative variation of the region area when the intensity is changed of ±0.5*Delta.</td>
<td>[2 50] (bit depth)</td>
</tr>
</tbody>
</table>

Table 9: The MSER parameters that are related to intensity. A short description of the function of each parameter and range of values used during the optimization procedure is given.
These specific tissue types were chosen due to their common occurrence in the original 23 mice margin dataset. Each image was a region of interest cropped from a margin to ensure that only the specified tissue type was present. The images were processed multiple times using the MSER algorithm while slightly varying the parameters in each iteration. The area fraction, segmented area divided by total image area, was the metric calculated to compare each subsequent image output. The method for calculating area fraction is shown in Figure 24A.

![Figure 24A: Methodology for calculating area fraction of a MSER segmented image. Area fraction was the metric used to quantify the performance of the MSER algorithm.]

4.1.4 Tissue Type Classification Model

To approach this, we developed and optimized a predictive model to distinguish between images of distinct tissue types. A total of 30 images (10 of each tissue type) were manually selected as a training dataset for constructing this site level-predictive model. Each image was a 350x300 pixel region of interest (ROI) from the original dataset of 23 margins. These smaller ROIs were selected to ensure that each This ROI size was
chosen because it was roughly equivalent to the FOV of a 10x objective, a typical
magnification used by pathologists when studying a suspicious region.

One additional step was taken to further divide each image into smaller elements
and each image was broken into 42 evenly spaced 50x50 pixel bins. This procedure is
shown visually in Figure 25. The rationale for this step was if classification was carried
out on the 350x300 pixel ROIs, it is likely that small focal areas of tumor would not be
detected. A bin size of 50x50 pixels was chosen because this corresponded to the
diameter of a single skeletal muscle fiber or a single adipose cell. After this step, the true
number of measurements in the training dataset was N=1260 bins (30 images with 42
bins per image) with N = 420 tumor bins and N = 420 muscle bins, and N = 420 adipose).

![Flow chart which demonstrates the quantitative analysis carried out on the site specific ROIs. The purpose of this analysis was to develop a predictive model to differentiate tumor tissue from muscle and adipose. A total of 30 images were used as a training dataset to develop the model. Each image was further divided into smaller bins to ensure very localized disease could be detected. Finally, three quantities were calculated for each bin, area fraction, average diameter, and average shape, and used as input variables for a logistic regression model. Using the segmentation output from the MSER algorithm, three different variables were calculated for each bin, area fraction, average diameter, and average](image)

Using the segmentation output from the MSER algorithm, three different
variables were calculated for each bin, area fraction, average diameter, and average
shape (perimeter/area). Area fraction was selected as a variable due to the expected increased density of AO-stained features (such as cell nuclei) in tumor tissue over normal tissue. Nuclear diameter was chosen to represent the expected increase in nuclear size of tumor cells and shape was selected to identify the pleomorphic nature of tumor cells.

4.1.5 Logistic Regression

Once area fraction, average diameter, and average shape were quantified for each bin, the goal was to construct a predictive classification model to differentiate tumor bins from normal bins. While the training dataset contained three distinct tissue types, a binary logistic regression model was chosen for the classification algorithm. To reduce the three group training dataset to a binary dataset, the adipose and muscle tissue were classified together as normal tissue sites.

Briefly, logistic regression is statistical classification model used to predict a binary response given specific variables from a data point. In this case, the data points corresponded to each 50x50 pixel bin (N = 1260) and the binary response corresponded to known classification (1 = tumor, 0 = normal). The logistic regression model was implemented using the statistical software package R. The general form of a 3 variable logistic regression model is as follows:

\[ g(x) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \]

Where \( g(x) \) is the model predictor value which is a fitted logistic function, \( \beta_n \) represents the model coefficients, \( X_n \) and are the variable values. In our case, \( X_n \) are the quantified variables from each bin which where:

\( X_1 \) – Area Fraction

\( X_2 \) – Average Diameter – average value of all segmented regions contained within the grid element
\[ X_2 \text{ – Average Shape – average value of all segmented regions contained within the grid element} \]

For each bin, these variables, along with the diagnosis (1 for tumor, 0 for muscle or adipose), were input into the R logistic regression code which estimated the coefficients \( \beta_0, \beta_1, \beta_2, \text{ and } \beta_3 \). The model predictor value, \( g(x) \), is then calculated for each bin, where a higher value corresponds to a higher likelihood of presence of tumor. Furthermore, the probability (between 0 and 100%) a given bin is tumor can be calculated from \( g(x) \) using the following equation:

\[
\text{Probability (\%) of tumor} = \frac{1}{1 + e^{-g(x)}}
\]

### 4.2 Results

#### 4.2.1 Margin Imaging Results

In total, 23 mice were imaged following the procedure in the Methods section. In addition to imaging with the SIM system, each margin was also diagnosed by a highly trained pathologist who specializes in connective tissue pathology. The specific breakdown of mice is shown in Table 10. From our dataset of 23 total margins, 15 margins were positive and 8 margins were negative. It should be noted that Table 10 also specifies a portion of mice where the tattoo ink was lost during the tissue processing and the pathologist was unable to locate any ink in the H&E slides. However, these margins were still diagnosed based on alternate criteria. A margin was diagnosed as negative if a tumor was not circumferentially present. However, if tumor cells were visible at the edge of the tissue sections, the margin was diagnosed as positive.
Table 10: Enrollment table for the number of mice imaged in this study. The respective number of negative and positive margins as diagnosed by a trained pathologist using post-operative H&E sections is also shown. In some cases, the ink used to demarcate the relevant margin was lost during the fixing and staining process. Within each classification, it is further specified whether the pathologist was able to find the ink on corresponding H&E.

<table>
<thead>
<tr>
<th></th>
<th>Diagnosed as NEGATIVE</th>
<th>Diagnosed as POSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ink visible on H&amp;E</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Ink lost on H&amp;E</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

4.2.2 MSER Optimization Results

The first step in the image analysis was to optimize the MSER specifically for the images acquired with the SIM system to segment and quantify AO-stained features. However, as mentioned in the methods section, three specific parameters (MaxVariation, MinDiversity and Delta) were tuned to ensure the algorithm was accurately segmenting AO-stained features primarily arising from nuclei. A dataset consisting of 10 tumor, 10 skeletal muscle, and 10 adipose images were used to optimize the parameters. One representative image of each tissue type is shown in Figure 26.
Figure 26: Representative SIM images of three different tissue types commonly found in margin images, tumor, muscle, and adipose tissues. For the smaller ROIs, both the sectioned and uniform images are shown to demonstrate the enhanced contrast that SIM provides. Examples of H&E micrographs are also shown, but it should be noted the H&E is not taken from the exact site of the corresponding SIM images.
The results from the optimization procedure are shown in Figure 27 where the corresponding area fraction (averaged over all 10 images of each respective tissue type) is plotted for the case where one parameter is varied while the other two are held constant.

![Figure 27: Plots that demonstrate the effect of three different MSER parameters on segmented area fraction for tumor, muscle, and adipose images (Shown in previous figure). The parameter Delta shows the most impact on area fraction, while MaxVariation and MinDiversity have a minimal effect.](image)

The MSER output appears to not be sensitive to MaxVariation and MinDiversity, in that varying these parameters has minimal effect on area fraction. For the MaxVariation parameter, it appears that the area fraction begins to reach a plateau after values higher than 10. It was calculated that the area fraction only increased by about 10% after exceeding a MaxVariation of 10. In MinDiversity, almost the entire range is a plateau and the area fraction shows very little variability regardless of the setting (15%). In contrast, varying Delta had the most significant impact on area fraction with the only visible plateau occurring when all area fractions fell to 0. Ultimately, a MaxVariation of 10 and MinDiversity of 0.5 were selected as these were within their respective plateau ranges. However, selecting Delta was much more challenging as there was no
reasonable plateau range to choose within. To determine the best Delta value, the segmented images for three representative images for Delta values between 2 to 14, shown in Figure 28, were visually inspected. Images with Delta > 14 were not included in visual inspection because the area fraction value of the tumor began to converge with the adipose, which does not match the physiological expectation.

Figure 28: Series of images which demonstrate the impact of changing Delta on MSER AO-stained feature segmentation. The calculated area fraction for each image is displayed on the bottom right. As shown in the previous plot, the
segmented area fraction varies greatly as Delta changes. Ultimately, a Delta value of 10 was chosen as the segmented nuclei appeared to be the most physiologically accurate.

In the set of images shown in Figure 28, the original SIM images have been overlaid with false-coloring to represent the segmented areas identified using MSER. Specifically, green regions were smaller areas with a diameter of less than 10 µm and red regions were larger areas with diameters larger than 10 µm. The diameters were calculated by measuring the longest axis of a segmented region. Additionally, the distributions of measured segmented region diameters are also shown on the bottom row of Figure 28.

Delta = 10 yielded the most appropriate and physiologically accurate in segmentation results across the representative images. At Delta values of 2 and 6, it was clear that the images were over-segmented. This was particularly obvious when inspecting the representative muscle images where extensive areas inside the fiber bundles were segmented. While the whole muscle fiber is indeed stained with AO, the features closely related to nuclei should be located on the periphery of the fiber bundles, not in the center. For Delta = 14, the images were under-segmented, meaning some AO-stained features were not identified. Specifically, the adipose image under this Delta value clearly displays nuclei that are not highlighted. The three representative images of tumor, muscle, and adipose are shown in Figure 29 with the finalized MSER parameter
set. Both the sectioned and uniform images are included to demonstrate the importance of contrast enhancement through SIM.

![Figure 29: Application of the finalized set of MSER parameters to both the uniform and sectioned representative image set. It is evident that the sectioned images provide a clear benefit with contrast enhancement which assists the MSER algorithm in accurately identifying nuclei. The finalized set of MSER parameters used for these segmentation results were MinArea=3, MaxArea=15, MinDiversity=0.5, MaxVariation=2.5, and Delta=10.](image)

4.2.3 Tissue Type Classification Model Results

As expected, the margin-level images clearly contained a mix of various tissue types including tumor, muscle, fat, blood vessels, nerves, and more. The goal was not necessarily to separately identify every tissue type within a margin, but simply to identify any presence of tumor tissue. This would lead to the conclusion that the presence of tumor is positive and conversely, the absence of tumor would be negative.
Following the procedure in the methods section, the training dataset of 30 tissue specific images was subdivided into \( n = 1260 \) 50x50 pixel bins. The area fraction, average diameter, and average shape were calculated for all bins based on the MSER segmentation output. These calculated values of all bins from the training dataset are displayed as boxplots and shown in Figure 30.

![Boxplots of calculated values from the training dataset.](image)

**Figure 30:** Distribution functions of the three variables, area fraction, diameter, and shape. These include all 50x50 pixel bins from the training dataset, \( N = 420 \) tumor bins and \( N = 840 \) normal bins (420 muscle and 420 adipose). A Wilcoxon rank-sum test was used to compare the distributions and calculate the corresponding \( p \)-value. Based on the results, the tumor and normal distributions were statistically different in all three variables, so they were included as input to the logistic regression model.

This data was input into the logistic regression to estimate coefficients for \( \beta_0, \beta_1, \beta_2, \beta_3 \). First, the classification model was constructed using all three of input variables. Three additional classification models were constructed using two input variables, where one variable was left out of each iteration and the estimated coefficients of each model is shown in Table 11.
Table 11: The estimated coefficients from the logistic regression model for classifying tissue types.

<table>
<thead>
<tr>
<th>Variables Used in Logistic Regression</th>
<th>Area Fraction</th>
<th>Average Diameter</th>
<th>Area Fraction</th>
<th>Average Shape</th>
<th>Average Diameter</th>
<th>Average Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area Fraction</td>
<td>2.659</td>
<td>1.374</td>
<td>-4.460</td>
<td>1.3587</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Diameter</td>
<td>44.074</td>
<td>43.818</td>
<td>30.12</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Shape</td>
<td>-0.962</td>
<td>-0.870</td>
<td>N/A</td>
<td>-0.2114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area Fraction</td>
<td>-0.8652</td>
<td>N/A</td>
<td>12.09</td>
<td>-5.1622</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Using these estimated, the predictor value $g(x)$ was calculated for each bin. Then using this predictor value, $g(x)$, as the binary classification parameter, a receiver operating characteristic (ROC) curve was generated by varying the classification threshold and is shown on the left side of Figure 31. For comparison, three other ROC curves are also shown that were generated from a logistic regression models built using only two of three variables.

Figure 31: The receiver operating characteristic (ROC) curve on the left was generated when logistic regression model was applied to the original training dataset of 30 site specific ROIs (10 tumor sites and 20 normal sites). The specific combinations of variables used as input to the logistic regression model are shown in
the legend. The ROC curve on the right was the result of applying the same logistic regression model on separate validation dataset containing 25 new site specific ROIs (10 tumor sites and 15 normal sites).

As shown by the ROC curve, the model performed exceptionally well (AUC=0.9), although this was expected since the model was simply applied to its training dataset. To test the true efficiency of the site level model, a separate validation dataset was selected. This validation dataset contained 25 additional images (10 tumor, 10 muscle, 5 adipose) that were separate from the training dataset. Only 5 adipose images were selected for this validation dataset due to the smaller number of adipose sites found in the original dataset of 23 margins. As with the training dataset, each image in the validation dataset was divided into the 50x50 pixel bins and the model predictor value $g(x)$ was calculated for each bin. The ROC curve for the validation dataset is shown in right side of Figure 31.

**4.2.4 Application to Full Margin Images**

The final step of the analysis was to take site level predictive model and apply it to the full margin images. The margin size from each mouse varied based on the tumor size, but most margins were covered using 4 to 6 separate images. The typical image configuration of the margins was either 2x2 or 2x3. In order to fully visualize each margin, multi-image mosaics were generated by placing each image site in its proper physical location, while also accounting for the overlap among adjacent images. Selected examples of one positive and one negative margin are shown in Figure 32.
Figure 32: Example of a positive and negative margin imaged with the SIM system. These images were viewed by a pathologist who labeled likely regions of tumor (T) and muscle (M).

Following the procedure of the site level images, MSER was then applied to these two full margin images to segment AO-stained features. The segmentation results of the
sectioned images are shown in Figure 33. Again, segmented nuclei with diameters less than 10 µm were false colored in green, while those greater were colored in red.

Figure 33: These are the same positive and negative margin examples from the previous figure. The images have been analyzed with MSER and the segmented AO-
stained features are highlighted in green (diameter < 10 μm) and red (diameter > 10 μm).

Finally, the segmented images from the whole margins were divided into the smaller 50x50 pixel bins used as input for the site level predictive model. This analysis was performed on the two margins shown in Figure 33 and four additional margins (a total of 3 positive and 3 negative). The area fraction, average diameter, and average shape were calculated for each bin within the margin and the predictor value, $g(x)$, was determined. This data is shown in Figure 34 as a cumulative distribution function (CDF) of all bins from the respective positive and negative margins. A boxplot of the same data is also shown on the right side of this figure.

![Figure 34: Each margin was divided into smaller 50x50 pixel bins and each bin was analyzed using the site level predictive model. Based on the output of the model, a tumor probability (%) was assigned to each bin. As expected, the CDFs from the sectioned images (left) show a larger portion of the positive margin bins were assigned a higher tumor probability resulting in clear separation between the positive and negative margin CDF. However, the CDFs from uniform illumination images (right) did not exhibit as much separation, indicating the importance of the contrast enhancement using SIM.](image-url)
4.3 Discussion

Without question, intraoperative diagnosis of surgical margins continues to be a challenging prospect. The presence of a positive surgical margin requires a secondary procedure that impacts the patients, physicians, and healthcare providers. Most importantly, the patient will undergo continued emotional stress and potential financial burdens from the additional operations. In the study presented here, it was demonstrated that SIM microscopy with AO staining is a feasible solution to intraoperative margin assessment. This conclusion was drawn from using the SIM system to image surgical margins from a sample size of 23 spontaneous mouse sarcomas.

The accurate and specific identification of cell nuclei is a vital step to determining the presence of tumor tissue. Pathologists heavily base their classification decision on qualitative visual analysis of nuclear, size, shape, and density. Similarly, in order for SIM microscopy to be clinically viable, it would not only need the ability to visualize, but also segment nuclei, preferably through an automated algorithm. Visual inspection of SIM images acquired on AO-stained tissue clearly revealed that nuclei appeared as small local intensity maxima. The decision to incorporate MSER as the method of automatically segmenting nuclei was based on the expected morphological appearance of nuclei in different tissue types. The goal of applying a segmentation algorithm was to
identify the smaller AO-stained features that likely represented cell nuclei, rather than the larger AO-stained features, such as muscle fibers.

Due to the relatively large field of view (>3 mm²) compared to confocal sectioning microscopy, a typical SIM image from the sarcoma margin would commonly contain more than a single type of tissue. Pure tumor tissue consists mostly of tumor cells with very little or no other organizational structure. However, the density of the tumor tissue can vary, so tumor nuclei can potentially appear with a dark or bright background. Muscle nuclei are located on the periphery of muscle fiber bundles, which are also brightly stained with AO. As a result, nuclei associated with muscle typically appear on a bright background. In adipose tissue, the nuclei are also located on the periphery of an adipocyte, but lipid droplet that occupies the majority of adipocyte does not emit fluorescence from AO. The adipose nuclei would appear on a mostly dark background, with fine streaks of background fluorescence from the connective tissue.

Given the variety of tissue types and the respective differences in nuclear appearance, a simple global threshold was not an appropriate option. MSER was a more robust algorithm with the potential to identify nuclei in complex tissue types. The approach allows the intensity threshold to be selected locally, which is ideal due to the wide range of nuclei appearance. The segmented images shown in Figure 29, clearly demonstrate the ability of the algorithm to highlight nuclei among tumor, muscle, and
adipose tissues. As expected, the area fraction of segmented nuclei was higher in the
tumor image by a factor of 3.51 over the muscle image and 1.70 over the adipose image.

Once it was demonstrated that nuclei were automatically segmented using the
MSER algorithm, the focus was to develop a predictive model to distinguish and classify
different tissue types. Area fraction, average diameter, and average shape were selected
as parameters since they were quantifiable metrics of pleomorphic changes that
pathologists use to identify tumor tissue in H&E. Based on the ROC curves
demonstrating its performance, the model was able to classify tumor and normal tissue
with 77% sensitivity and 81% specificity (Youden’s index). For an unbiased measure of
the model performance, it was applied to a separate validation dataset that resulted in
73% sensitivity and 80% specificity.

It should be noted that the two example margins selected in Figure 32 are very
clear examples of respective positive and negative margins. In the negative margin, the
only visible tissue type was muscle tissue while the positive margin example also
contained muscle, but a clear identifiable tumor in the bottom right. These margins
were originally given their diagnosis by the pathologist who reviewed the post-
operative H&E slides. She accurately re-diagnosed (blinded to her original diagnosis)
each margin using only the SIM images and identified the specific muscle and tumor
tissue regions.
A total of 6 margins (3 positive and 3 negative, including the two in Figure 32) were selected for the CDF plot on Figure 34. It was clear from these plots that the site level logistic regression model was also producing expected results when applied to the full margin images. Specifically, less than 8% of the bins from the selected negative margins were predicated to have a tumor probability of >50%, while over 25% of the bins from the positive margins were assigned a >50% tumor probability. This observation was in agreement with our expectation since a larger portion of bins from the positive margins should be associated with higher tumor probability.

Understandably, the next logical step would be to demonstrate the performance on all 23 mice imaged for this study. However, once the algorithm was applied to all margin-level images, the clear separation between positive and negative margins was no longer present. The increase in sample size revealed the lack of robustness in the site level classification model. As previously explained, the site level tissue classification model was based solely on a subset of images consisting of tumor, muscle, and adipose tissue. While it was true that tumor, muscle, and adipose tissue are the most common occurring tissue types, a closer inspection of all 23 margins indicated the presence of a number of other tissue types. The pathologist confirmed these other tissue types on the H&E sections and also through inspection of the SIM images. The presence of other highly cellular tissues was a source of error, which the site level classification model incorrectly assigned high tumor probabilities. Some examples include, skin, lymph
nodes, nerve bundles, and bone marrow, which are all benign tissue types, but are also dense with cell nuclei. Unfortunately in our 23 margin dataset, these additional tissue types appeared far less frequently than tumor, muscle, and adipose. Due to this limitation, there was an inadequate amount of representative images to include in the training dataset for the site level tissue classification model.

Despite the limitations, it has been demonstrated that SIM imaging combined with MSER nuclei segmentation is a powerful tool for distinguishing tumor from muscle and adipose tissue. Staining tissue with AO was a straightforward procedure that required only topical application and no specialized tissue or contrast agent preparation. This allowed the resected tumor to be rapidly imaged after excision (<15 min), a critical requirement for potential intraoperative use. Finally, we have laid the groundwork for future studies and potential modifications to improve the system.
5. Conclusions

The primary goal of this dissertation was to improve upon existing biophotonic solutions to address the unmet clinical need of surgical tumor margin assessment. Specifically, the ability to quickly and accurately identify residual disease can drastically improve patient care. Although our focus was on breast cancer and pre-clinical sarcoma, the need for intraoperative margin assessment can be extended to virtually any organ site where surgical treatment is needed to de-bulk a tumor. The extensive review of previous works reveals an academic and clinical interest in harnessing optical technologies for this specific healthcare application. The clinical utility of DRS has been clearly demonstrated in not only our group, but many others as well. Its unique ability to quantify biochemical molecules enables detection of physiological changes for differentiating tumor and normal tissue.

As presented in chapter 2, the objective was to reduce the overall cost and physical footprint of a clinical DRS system. Specifically, I proposed to construct a new compact DRS system where the detector in the clinical system, a cooled CCD, was replaced with low-cost silicon photodiodes. This change to the system immediately reduced the overall cost of the system by over $15,000. In addition to the changes on the detection path, the method of illumination was also modified to include an 8-channel filter wheel. By sequentially launching discrete wavelengths into the tissue, the
expensive and physically cumbersome grating-based spectrometer was no longer required. Again, with this change, the cost of the system was reduced another $10,000.

Once redesigning the system and replacing these key components, it was important to determine the impact on system performance. The sensitivity of a scientific grade cooled CCD was far superior to that of an off-the-shelf silicon photodiode. Additionally, in opting to use the filter wheel for illumination, the number of wavelengths to interrogate the tissue was reduced from a continuous spectrum (over the range of 400-600 nm) to 8 discrete wavelengths. The latter goal of this chapter was demonstrating the new compact system performed equivalently to the original clinical system despite the loss in detection sensitivity and reduction in wavelengths in reconstructing the spectra.

It was experimentally determined that the compact DRS system yielded an SNR (40 dB), system drift (±3%) and inter-pixel cross talk (1-9%) which were all on par the original clinical system. Most importantly, the ability to accurately extract optical properties was shown on a set of tissue mimicking liquid phantoms. The compact DRS system yielded an optical property extraction accuracy of 6.40 ± 7.78% for the absorption coefficient (µ Tình) and 11.37 ± 19.62% for the wavelength-averaged reduced scattering coefficient (µ' Tình) as compared to 9.03% for the absorption coefficient (µ Tình) and 7.3% for the wavelength-averaged reduced scattering coefficient (µ' Tình) in the original clinical system.
As shown in previous work, DRS relied on accurately quantifying the scattering coefficient, $\mu_s'$, to distinguish tumor and normal tissue types. Tumor tissue is expected to have a higher scattering coefficient, but unfortunately, certain benign tissue types, such as fibro-glandular tissues, are also highly scattering. The inherent lack of spatial resolution in diffuse reflectance spectroscopy (on the scale of mm, rather than \( \mu m \)) led us to explore other optical technologies. In order to distinguish fibro-glandular tissue from malignant tissue, a new optical technology with resolution on the micron scale was required to identify the cellular structures. Fluorescence microscopy was selected due to its ability to generate high-resolution images for visualizing tissue microstructure. In combination with the appropriate contrast agent, fluorescence microscopy was capable of visualizing cell nuclei and other micro-morphological structures without requiring extensive sample preparation.

Though the approach was not without drawbacks, as the resected tumor mass removed from a patient is relatively thick in terms of optical path length. In the realm of optical microscopy, sample thicknesses greater than a 1-2 mm pose a unique challenge. For fluorescence microscopy, a physically thick tissue sample would be difficult to image due to the large amount of unwanted background fluorescence. To address this issue, we chose to design the system with SIM capabilities to reject the unwanted background fluorescence.
In Chapter 3, I presented a design and carried out a procedure to characterize a wide field fluorescence SIM system. The measured basic imaging parameters were a single-shot FOV of 2.1×1.6 mm (3.4 mm²) and sub-cellular lateral resolution (4.4 µm). While SIM has been used for background rejection for nearly two decades, there has been limited application to thick intact biological samples. The objectives of this chapter were to measure the relationship between illumination pattern frequency and optical sectioning strength and signal-to-noise ratio in turbid (i.e. thick) samples for selection of the optimum frequency, and to determine feasibility for detecting residual cancer on tumor resection margins, using a genetically engineered primary mouse model of sarcoma. The SIM system was tested in tissue mimicking solid phantoms with various scattering levels to determine impact of both turbidity and illumination frequency on two SIM metrics, optical section thickness and modulation depth. To demonstrate preclinical feasibility, \textit{ex vivo} 50 µm frozen sections and fresh intact thick tissue samples excised from a primary mouse model of sarcoma were stained with AO, which stains cell nuclei, skeletal muscle, and collagenous stroma. The cell nuclei were segmented using a high-pass filter algorithm, which allowed quantification of nuclear density. The results showed that the optimal illumination frequency was 31.7 µm⁻¹ used in conjunction with a 4×0.1 NA objective (\(\nu = 0.165\)). This yielded an optical section thickness of 128 µm and an 8.9x contrast enhancement over uniform illumination. I successfully demonstrated the ability to resolve cell nuclei \textit{in situ} achieved via SIM,
which allowed segmentation of nuclei from heterogeneous tissues in the presence of considerable background fluorescence. Specifically, it was demonstrated that optical sectioning of fresh intact thick tissues performed equivalently in regards to nuclear density quantification, to physical frozen sectioning and standard microscopy.

Finally, the SIM system was applied to a larger dataset of 23 animals to demonstrate clinical viability and the study design with corresponding results were shown in Chapter 4. The biological samples used in this study were a genetically engineered mouse model of sarcoma, where a spontaneous solid tumor was grown in the hind leg. After the tumor was surgically removed from the animal and the relevant margin was stained with AO and then imaged with the SIM system with the primary goal of visualizing specific morphological changes in cell nuclei. To automatically segment nuclei, rather than using a simple high-pass filter, an algorithm known as maximally stable extremal regions (MSER) was optimized and applied to the images.

As an intermediate step prior to diagnosing whole margins, a tissue-type classification model was developed to differentiate localized regions (75x75 µm) of tumor from skeletal muscle and adipose tissue based on the MSER nuclei segmentation output. A logistic regression model was used which reported a final output in terms of probability (0-100%) the tumor within the localized region. Using this output as a binary classifier, the model performance was tested using an ROC curve analysis that revealed 77% sensitivity and 81% specificity. For margin classification, the whole
margin image was divided into localized regions and the tissue-type classification model was applied. In a subset of 6 margins (3 negative, 3 positive), it was shown that at a tumor probability threshold of 50% only 8% of all regions from a negative margins exceeded this threshold, while over 25% of all regions exceeded the threshold in the positive margins.

In summary, Table 12 shows a comparison of the two optical technologies that were presented in this dissertation. It should be noted that the purpose of this table is not for direct comparison of the two systems, as the technologies are inherently different. However, the table does point out the specific strengths of each technology and the specific gaps that each can fill. DRS is superior in terms of ability to image whole margins (<20 min) due to significantly larger FOV. Additionally, DRS is capable of detecting close margins with a clinically relevant sensing depth (2-3 mm). On the other hand, SIM has the capability of visualizing individual cells and differentiating tissue types at the microscopic level.
Table 12: Comparison of the two optical systems which were develop for surgical tumor margin assessment

<table>
<thead>
<tr>
<th>Optical Technology</th>
<th>Lateral Resolution</th>
<th>Single-Shot FOV</th>
<th>Sensing Depth</th>
<th>Single Site Acquisition Time</th>
<th>Source of Contrast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compact DRS</td>
<td>5.8 mm</td>
<td>~20x20 mm</td>
<td>2-3 mm</td>
<td>10 sec</td>
<td>[Hb] [HbH] [beta-carotene] μs'</td>
</tr>
<tr>
<td>SIM system</td>
<td>4.4 μm</td>
<td>2.1 x 1.5 mm</td>
<td>~100 μm</td>
<td>1-3 sec</td>
<td>From AO stain: Cell Nuclei Fibrous Tissues</td>
</tr>
</tbody>
</table>

5.1 Future Work

5.1.1 Combination of DRS and SIM Imaging

While the focus of my work was a modular approach focusing on improvements to DRS and SIM separately, based on Table 12 the strength of each technique would fill the gaps of the other. On their own, each technology has been systematically validated on both pre-clinical and clinical settings. A combination of the two technologies can potentially yield a system that is sensitive to both changes in biochemical properties and nuclear morphology. However, simply combining the two technologies that I specifically developed is not a feasible solution. In particular, the compact DRS system presented in Chapter 2 requires direct contact with the sample, while the SIM system (Chapters 3 and 4) is noncontact. Fortunately, our group has continued to improve upon the compact photodiode DRS system. The latest iteration of the system involves the photodiode array, but the illumination light is delivered thorough a back-lit
geometry. In addition, a lenslet array aligned to the photodiode apertures is used to focus the light into the tissue. The result of these modifications is a noncontact form of the compact DRS system. Other groups have also presented concepts for non-contact DRS [108].

![System Schematic](image)

**Figure 35:** A system schematic for a combination DRS and SIM imaging system. The primary advantage would be to utilize the fast scanning and coverage ability of DRS, while also maintain the ability to visualize high-risk regions with the high spatial resolution of SIM.

To combine these two technologies, a potential system design is shown in Figure 35. The advantage of implementing this system is that the light source would be used for both system and majority of the optical path would be the same. In this
implementation, the Fianium laser is used due to its broadband output (400-1300 nm) and a filter wheel would be used to select the appropriate wavelengths required for DRS and fluorophore excitation in SIM. A simple flip mirror is added in the optical path to select between the two systems.

Ideally, the first step would be to image sample using DRS to take utilize of the inherent acquisition speed and coverage advantage. If a certain region is identified as high risk, then the SIM system can be used to rescan the region at microscopic resolution to confirm the assessment from DRS. Clearly, there will be some spatial offset between the FOV of the DRS system and the FOV of the SIM system, so the exact same region cannot be imaged simultaneously with both systems. However, these spatial locations can be easily calibrated and accounted for using a programmable automated translation stage.

5.1.2 SIM System Technical Improvements

Though I have demonstrated the ability of the SIM system to image biological samples in mouse sarcomas, a number of technical improvements can be made to increase the system viability. In its current form, the image acquisition time for sarcoma tissue is 1-3 seconds. This is compounded by the fact that SIM requires three images to be captured at each location. For this technique to be truly scalable to larger human surgical margins, the acquisition time must be reduced. There are some potential
technical improvements that can be implemented in the system to reduce the acquisition time.

The current SIM system delivers 1.3 mW of 480 nm light to the sample. Increasing the excitation power is a potential solution to decreasing the acquisition time. The main concerns in raising the optical power are patient safety and photobleaching. Regarding patient safety, because all proposed imaging takes place ex vivo, the optical power limit is not necessarily the same as if the light was applied directly on the patient. The true danger would be in unintentional scattered or reflected light that could pose a threat to the patient, physician, and/or operator. An increase in excitation may cause the contrast agent to photobleach and cease to emit fluorescence. A straightforward study can be carried out to determine to photobleaching limit of AO both in aqueous solution and in biological samples. In order to increase the excitation power, a new source must be explored. Laser diodes or LEDs can provide a feasible option cost significantly less than the Fianium laser source.

Finally, if an increase in excitation power is not a viable option, then the current objective may be replaced with another objective with a higher NA. The NA is generally related to the single image FOV, so it would be important to determine this trade-off. However, a significant reduction in acquisition time can negate the decrease in FOV. If multiple images with a smaller FOV can be acquired faster than a single large FOV, then there is still a decrease in overall imaging time. As an example, if the current 4x
objective is replaced with a 10x objective, the FOV will be decreased by a factor of 2.5. Fortunately, the typical NA of a 10x objective (Nikon PlanFluor) is 0.3, so the collection efficiency will actually increase by a greater factor, 3.

5.1.3 Identification of Additional Tissue Types

During the course of the 23 sarcoma margin study presented in Chapter 4, I presented the results of applying the SIM system to a study of 23 sarcoma margins. A portion of this work focused on developing an algorithm to differentiate tumor, muscle, and adipose tissue. While it was true that tumor, muscle, and adipose tissue are the most common occurring tissue types, a closer inspection of all 23 margins indicated the presence of a number of other tissue types. The pathologist confirmed these other tissue types on the H&E sections and also through inspection of the SIM images. The presence of other highly cellular tissues was a source of error, which the site level classification model incorrectly assigned high tumor probabilities. Some examples include, skin, lymph nodes, nerve bundles, and bone marrow, which are all benign tissue types, but are also dense with cell nuclei. Examples SIM images of a nerve bundle and bone marrow are shown in Figure 36. Unfortunately in our 23 animal dataset, these additional tissue types appeared far less frequently than tumor, muscle, and adipose. Due to this limitation, there was an inadequate amount of representative images to include in the training dataset for the site level tissue classification model.
Figure 36: Images of a nerve bundle, bone, and bone marrow acquired with the SIM system. These tissue types were not as common as tumor, muscle, or adipose, however due to their high cellular contact these were a source of false negatives in the margin images. Scale bars are 200 µm.

Additional studies should be conducted to specifically harvest and image a larger set of normal tissue types. The site level model can then be trained and re-optimized on a larger of tissue types to increase its robustness. Another important consideration is that the introduction of additional tissue types can also impact the MSER nuclei segmentation. The segmentation performance would be important to verify prior re-developing the site level model. Using representative images of these new tissue types, the same MSER optimization procedure outlined in this study should be followed to validate performance on a broader range of tissues.

5.1.4 Multi-spectral Contrast in SIM

Through this work, it has been clearly demonstrated that AO is an appropriate contrast agent for visualizing cell nuclei. Its high fluorescence yield generates more than enough signal for the SIM system to detect. An additional feature of AO that has not
been explored is its spectral-dependent features. AO is known to be a metachromatic dye—in monomeric form its fluorescence emission peak is 525 nm, the peak is shifted to 590-630 nm [109]. It has been reported that AO tends to aggregate between myofibrils in skeletal muscle [109]. Thus, imaging the two emission peaks of AO could in identifying where muscle is located within an image.

In order to obtain spectral information from the SIM system, the emission filter must be replaced with a tunable filter, so that a corresponding image can be acquired for each wavelength. A 50 µm tissue section containing both tumor and muscle was imaged at all emission wavelengths (Figure 37A) as well as discrete spectral bins through using a liquid crystal tunable filter (LCTF). A combined image of the monomeric emission peak (530 nm) and the aggregated emission peak (630 nm) is shown in Figure 37B with the corresponding H&E stained section shown in Figure 37C. The skeletal muscle in the upper right hand corner primarily emits fluorescence at the longer red wavelength, while the tumor region in the left half of the image primarily emits fluorescence at the shorter green wavelength.
For multi-spectral imaging, either additional emission spectra from AO can be collected or additional fluorophores could be used to stain different tissue components. Either AO or other fluorophores can be incorporated into the imaging protocol in order to acquire multi-spectral image sets. Additional possibilities for fluorophores that have been identified include AlexaFluor568 Phalloidin or LipidTox Deep Red. AlexaFluor568 Phalloidin selectively stains F-actin, which is present in skeletal muscle, and has an excitation and emission peak of 578 and 600 nm respectively [110]. LipidTox Deep Red Neutral lipid stain has a high affinity for neutral lipid droplets, and has an excitation and emission peak of 577 and 609 nm respectively [111].

5.1.5 Validation of Image Processing Algorithms

A vital portion of margin analysis using the SIM system was the proper segmentation of nuclei within the images. In Chapter 3, the concept was first introduced using a simple high pass filter. A more advanced algorithm called MSER was implemented in Chapter 4 with an extensive procedure to optimize on specific tissue
types. The optimization of the MSER algorithm was based primarily on \textit{a priori} knowledge of tissue physiology and expected location of nuclei. Though cell nuclei are clearly seen, no direct study was carried out to determine if segmented areas definitively corresponded to actual cell nuclei. An additional study has been proposed to use mice expressing the red-fluorescence protein (RFP) gene. By adding the RFP gene to adenovirus transfection sequence, the nuclei corresponding to sarcoma tumor cells will emit peak fluorescence at 584 nm with peak excitation at 555 nm. Alternatively, a secondary contrast agent that stains only nuclei can be used as a counter-stain to verify nuclei location.

Additional studies are also being performed on a larger dataset of frozen tissue sections. Imaging frozen sections provide a good correlation to H&E (as shown in Figure 18) since the slide can be immediately fixed and stained after imaging. However, it would be important to keep in mind that the tissue morphology can be slightly altered between fresh and fixed tissue. Primarily, tissue sections are known to undergo some shrinkage from the dehydrating and cross-linking process when submerged in formalin. If a quantitative comparison is desired, an automated image processing algorithm must be implemented on the images. MSER could potentially be applied to the H&E images, however a similar optimization procedure would need to be followed to ensure accurate segmentation. Co-registering the exact FOV between the SIM and H&E image can be challenging unless prominent features are present in the images.
An additional goal of acquiring the larger sample size of frozen section images was to further optimize the SIM illumination frequency to determine if there is a relationship between contrast enhancement and automated nuclei segmentation. Images were acquired using multiple objectives, 2x, 4x, and 10x, multiple physical tissue thicknesses, and multiple illumination frequencies. MSER will be applied to all images acquired to first understand at what physical section thickness structured illumination + MSER shows improvement over uniform illumination + MSER. Secondly, this information will be used to determine which frequency yields the optimal performance of MSER for each objective.
References


**Biography**

Henry Li-wei Fu was born in Wichita, KS. He grew up in Seattle, WA and attended the University of Washington as an undergraduate. He received his Bachelor of Science in Bioengineering in August 2006. He attended Duke University for his graduate studies and received his PhD in Biomedical Engineering in December 2014.

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- Annual Dean’s list, Recipient, 2002-2003, 2003-2004 (University of Washington)
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