NONLINEAR MICROSCOPY SYSTEM AND PROTOCOL FOR RAPID EVALUATION OF FRESHLY EXCISED HUMAN TISSUE

by

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Abstract

Histopathology determines disease type/condition by microscopic evaluation of biopsy and surgically excised tissue, playing a critical role in medicine. The clinical protocol for histology requires physical sectioning of tissue, either with intense chemical processing and paraffin embedding or freezing of tissue, limiting the rapid evaluation applications. Nonlinear microscopy (NLM) is an emerging optical sectioning microscopy technology that enables histological visualization of fresh and intact human tissue without requiring physical sectioning.

In this thesis, we developed high-throughput, real-time NLM imaging system and protocol for intraoperative NLM evaluation of surgically excised tissue. We demonstrated the versatile imaging capability of NLM with comparative studies between NLM and other optical sectioning microscopy techniques. Interventional randomized clinical trials are designed and conducted to demonstrate the feasibility of intraoperative NLM evaluation (breast lumpectomy and radical prostatectomy). A pilot study demonstrates the feasibility of NLM imaging of bone. The studies in this thesis were performed in close collaboration with the Beth Israel Deaconess Medical Center. This thesis aims to develop NLM system and protocol for rapid evaluation of fresh human tissue and to design and perform clinical trials for validation of the efficacy of intraoperative NLM evaluation. The results suggest the practical potential of NLM as a modality to improve/transform clinical/surgical procedures.

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Title: Elihu Thomson Professor of Electrical Engineering
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Throughout my days at MIT, I’ve been incredibly blessed with exciting research topics, an amazing environment, and above all wonderful and thoughtful colleagues and collaborators. Without their help, I would never have been able to achieve this thesis work.

I would first like to thank Prof. James Fujimoto for the opportunity and mentorship. The application of NLM for pathology has incredible potential. I strongly believe that NLM can make a significant impact and positive changes in clinical practice and medical care in the future. I am grateful that Prof. Fujimoto assigned me to this great project and let me devote my Ph.D. to it. He also taught me high-level scientific and critical thinking, rigorous clinical study designing and grant writings, and management of the multi-disciplinary team. His training will lay a sound foundation for my future career.

I would like to thank everyone at Beth Israel Deaconess Medical Center (BIDMC). First, our collaboration with BIDMC would not have happened without Dr. James Connolly. He leads the NLM breast project, supported the development of surgical NLM protocol and systems, and the complex interventional clinical trial. His knowledge and contribution were critical for the work described in this thesis. Dr. Liza Quintana provided deep knowledge and experience to improve the NLM breast project. She also has been performing a lot of intraoperative NLM evaluation and being an expert in surgical breast specimen imaging. Dr. Tejas Mehta was a key member of the initiation of the breast project and has been contributing to the project with her knowledge and experience, organization and team management skills, and passionate enthusiasm. Dr. Mary Jane Houlihan was an enthusiastic collaborator who recruited many patients and performed the NLM intraoperative evaluation protocol. Her trust in NLM and strong intent to challenge improving breast cancer clinical care was inevitable for the project to proceed. Dr. Monica Valero is also enthusiastic about the research and recruited many patients. Without her help, the intraoperative breast study will not be able to be completed. I would like to thank Dr. Yaileen Guzman Arocho for her contribution to develop NLM protocols, conduct imaging studies, and perform incredibly accurate and consistent tissue processing during the intraoperative study. She has been always close to me. We always had fruitful discussions. And her great work ethic was a tremendous help to conduct the complex clinical study with limited personnel.

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developing the feasible protocol for rapid evaluation. I believe this bone and biopsy application has huge potential to improve clinical practice/medical care.

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Chapter 1

Introduction

1.1 Histopathology

Histopathology is a branch of medicine that examines human tissue microscopically to determine disease type/condition. Biopsies and surgically excised specimens are chemically processed, embedded into paraffin blocks, physically thinly sectioned by a microtome, placed on glass slides, and stained with specific dyes. Pathologists examine the glass slides under a conventional transmission light microscope to assess cellular/sub-cellular details of pathologic features. Histopathological examination is a gold standard and the final step of tissue assessment in medicine, and therefore has a critical impact on surgical/treatment plans and patient care. In the US, each histology laboratory generates an average of 100,000 slides annually\(^1\), estimated \(~1\) billion slides per year throughout the country.

Standard preparation processes of histology glass slides (FFPE: formalin-fixed paraffin-embedded) involve multiple chemical processing such as fixation, clearing, paraffinization, and paraffin embedding to enable physical sectioning with a microtome.\(^2\) This cumbersome and labor-intensive protocol requires a long processing time, complicating rapid pathological evaluation such as intraoperative histopathological evaluation. Frozen section (FS) technique, which freezes tissue using cryostats to physically section tissue with a microtome, can provide timely manner pathological evaluation (~15-20 mins). However, freezing poses several limitations: the image quality is compromised from freezing artifact, tissue damages compromise post-processing FFPE
slides, throughput is limited and only small numbers of slides can be evaluated in a timely manner, and trained histotechnicians and histology lab equipment is necessary near operation rooms to provide timely service.\(^3\) As a result intraoperative histopathological evaluation with FS is not available/not commonly used in some scenarios, such as lumpectomy for breast cancer, radical prostatectomy for prostate cancer, and bone tissue excision.

**1.2 Novel imaging techniques for histopathology**

Recently, several optical imaging technologies have been demonstrated to enable rapid evaluation of human tissue by using optical sectioning rather than physical sectioning, including two-photon excitation nonlinear microscopy (NLM)\(^4,5\), confocal fluorescence microscopy (CFM)\(^6-9\), structured illumination microscopy (SIM)\(^10,11\), optical coherence tomography (OCT)\(^12-14\), light sheet microscopy\(^15,16\), and microscopy with ultraviolet surface excitation (MUSE).\(^17,18\) These techniques enable rapid evaluation of pathology with high correspondence to standard histological techniques such as FFPE histology while forgoing labor/time-intensive physical sectioning process.

NLM can image tens to hundreds of microns below the tissue surface with high resolution and strong rejection of out-of-focus light, but it requires costly femtosecond lasers and scanning optics. CFM avoids the use of femtosecond lasers, but still requires relatively expensive and complex optical instruments for physically rejecting out-of-focus light. SIM requires only inexpensive light emitting diodes (LEDs) and spatial light modulators, but rejects out-of-focus light computationally rather than physically, resulting in a tradeoff between available detector dynamic range and optical sectioning thickness. OCT provides high imaging rates and deep penetration into tissue, but is incompatible with fluorescent stains and has limited ability to
visualize cell nuclei which are important diagnostic features. Light sheet microscopy can generate a large 3D volume of images rapidly. However, the huge data size from the large volume acquisition requires time-costly image processing and is inefficient to generate a single layer image of the optical section. MUSE is a simple modality, which only requires deep UV illumination on a conventional light microscope setup, but the imaging is strictly limited to the surface of an object and susceptible to surface contamination. Currently, none of the technologies mentioned above have been adopted in regular clinical practice. Clinical studies of those optical sectioning microscopies for histology application have been limited in sample size and none of the results have been conclusive to drive the clinical community to include it into daily clinical practice.

1.3 Preliminary study

NLM utilizes nonlinear phenomena such as multi-photon excitation and second harmonic generations to generate images of thin layers (optical sections) within a thick object (human tissue). NLM is compatible with both intrinsic and exogenous contrasts, enabling the visualization of nuclear details as well as cytoplasmic/stromal morphologic features by using fluorescent stains. A prior study performed a prospective study of imaging 179 discarded surgical breast specimens from 50 patients at BIDMC to evaluate the diagnostic accuracy of NLM relative to standard FFPE hematoxylin and eosin (H&E) histology. A sensitivity of 95.4% and specificity of 93.3% were achieved in the blinded reading of NLM images of breast specimens for distinguishing benign breast tissue versus invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), and ductal carcinoma in situ (DCIS) (Table 1.1). Representative NLM images and corresponding H&E were shown in Fig 1.1 and 1.2. This study suggests that NLM is a promising modality for the rapid evaluation of pathology during breast cancer excision.

<table>
<thead>
<tr>
<th>Benign v DCIS/IDC/ILC</th>
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Fig. 1.1. Human breast specimen with regions of DCIS and IDC adjacent to normal tissue. (a) NLM and (b) corresponding paraffin-embedded histology showing DCIS and IDC (right) vs benign breast tissue (left) (scale bar: 2 mm). Sieve-like appearance is associated with cribriform DCIS, consistent with low- to intermediate-grade DCIS (red box, scale bar: 500 µm). IDC region shows neoplastic cells infiltrating breast stroma (green box, scale bar: 500 µm). Magnified view of abnormal cellular regions show features consistent with columnar cell change (blue box, scale bar: 500 µm). Image shows maximum intensity
projection through 8 depth-sections acquired at 15 μm increments from the tissue surface. Field of view: 18.25 mm x 12 mm. Adapted from "Assessment of breast pathologies using nonlinear microscopy," by Y.K. Tao et al., PNAS October 28, 2014 111 (43) 15304-15309. Copyright 2014 by the National Academy of Sciences.

**Fig. 1.2.** Human breast specimen with DCIS in various histologic patterns. (a) NLM and (b) corresponding paraffin-embedded histology showing different DCIS subtypes (scale bar: 1 mm). Finger-like projections of neoplastic cells invading the duct lumen are indicative of micropapillary DCIS (red box, scale bar: 250 μm). Irregularly shaped cavities with cellular debris and comedo plug are indicative of comedo DCIS (green box, scale bar: 250 μm). Individual cell nuclei at the periphery of the comedo DCIS exhibit prominent nucleoli consistent with high-grade DCIS (blue box, scale bar: 50 μm). The image shows maximum intensity projection through 6 depth-sections acquired at 10 μm increments from the tissue surface. Field of view: 8.25 mm x 5 mm. Adapted from "Assessment of breast pathologies using nonlinear microscopy," by Y.K. Tao et al., PNAS October 28, 2014 111 (43) 15304-15309. Copyright 2014 by the National Academy of Sciences.
1.4 Scope of this study

In this thesis, we develop rapid NLM evaluation system and protocol, compare the performance of NLM with that of other imaging modalities, and validate it for several clinical scenarios. The studies in this thesis are performed in close collaboration with the Departments of Pathology, Surgery, Radiology, Urology, and Dermatology at Beth Israel Deaconess Center (BIDMC). This thesis proposes a novel technique for intraoperative/intraprocedural histological evaluation of tissue using NLM and studies to show the potential of improving clinical care.

In Chapter 2, we develop NLM technology and methods for the assessment of surgical specimens at rates sufficient to comprehensively assess surgical margins during breast conserving therapy and other intraoperative imaging scenarios.

In Chapter 3, we report a direct comparison between CFM and NLM with dual agent staining and rendering of virtual H&E images for rapid histopathological evaluation of unfixed human breast tissue.

In Chapter 4, we develop a video-rate MUSE system and performed a preliminary evaluation of surgical specimens for two potential applications where surgical margins are assessed intraoperatively: Mohs surgery for basal cell carcinoma of skin and lumpectomy for breast cancer.

In Chapter 5, we design and perform a clinical study for a prospective 98 patient interventional randomized controlled trial at BIDMC on patients who will receive a lumpectomy for breast cancer. Intraoperative NLM real-time surgical feedback is provided to guide re-excision. The hypothesis is that NLM evaluation of surgical margins will reduce the rate of indication for repeat surgeries. We are currently recruiting patients to participate in this study at BIDMC.
In Chapter 6, we demonstrate NLM imaging of simulated nerve-sparing radical prostatectomy specimens. Simulated surgical margins enabled observation of realistic surgical margins of nerve-sparing specimens and optimization of NLM workflow for the future intraoperative radical prostatectomy NLM imaging project.

In Chapter 7, we demonstrate that NLM can provide rapid histological visualization of pathology in bone tissue, and may enable rapid diagnosis and intraoperative/procedural consultation. Rapid histological evaluation of bone may have applications in orthopedic surgery, bone biopsy, and cancer management.
Chapter 2

Wide-field, real-time NLM system development for intraoperative rapid evaluation of surgical margins

2.1 Introduction

Rapid histological assessment of surgical specimen margin status would have applications in many types of cancer surgery, including cancers of the breast, prostate, kidney, pulmonary tract, gastrointestinal tract, and biliary system. Breast cancer, which is responsible for almost 30% of all cancers in American women, is a classic example where intraoperative assessment would be beneficial for patient care and reduce healthcare costs.\(^{19}\) For early-stage breast cancer, breast conserving therapy (BCT) and radiation is the standard of care because it achieves survival and local recurrence rates equivalent to those of a mastectomy while providing superior cosmetic outcomes, reduced morbidity, and improved post-operative quality of life.\(^{20-25}\) To minimize morbidity and improve cosmetic outcomes, it is important to minimize the resection of normal tissue around the tumor margin.\(^{25}\) However, local recurrence following BCT is strongly correlated with margin status as determined by postoperative histopathology, making complete resection of cancerous tissue essential.\(^{26,27}\) Similar challenges occur in surgical scenarios such as prostate cancer where positive surgical margins on postoperative histopathology are associated with an increased risk of biochemical recurrence (increasing prostate-specific antigen) as well as the need for postoperative adjuvant radiation therapy.\(^{28}\)
Standard post-operative formalin-fixed paraffin-embedded (FFPE) histology requires fixation, dehydration, embedding, and microtome sectioning into thin layers. This process is time- and labor-intensive which precludes its use for intraoperative evaluation. Owing to the limited sensitivity of gross examination, a residual tumor can be present near the surgical margin in up to 40% of BCT procedures, typically necessitating a second surgery to eliminate the residual tumor.\textsuperscript{29–31} Frozen section (FS) is an alternative to FFPE histology which allows intraoperative evaluation by freezing tissue to enable cryosectioning without fixation and paraffin embedding, but it has limited acceptance due to low throughput as well as poor sensitivity and specificity as compared to conventional histology of the breast.\textsuperscript{32–34} Consequently, a majority of surgeons do not utilize any form of intraoperative histology to evaluate breast excisions and instead depend on post-operative assessment.\textsuperscript{35}

An alternative to FFPE and FS histology is to image tissue that has not been physically sectioned using optical sectioning microscopy to evaluate two-dimensional planes or three-dimensional volumes within a larger tissue specimen. Because physical sectioning accounts for the vast majority of processing delay for both FFPE and FS histology, optical sectioning can dramatically reduce the processing time and enable intraoperative histological examination in scenarios where FFPE and FS histology are too time-consuming. Various methods have been proposed for imaging breast, prostate, and other surgical margins without physical sectioning, including optical coherence tomography (OCT)\textsuperscript{12,14,36–38}, reflectance confocal microscopy (RCM)\textsuperscript{39,40}, confocal fluorescence microscopy (CFM)\textsuperscript{8,41–44}, structured illumination microscopy (SIM)\textsuperscript{45}, light sheet microscopy\textsuperscript{46}, microscopy with ultraviolet surface excitation (MUSE)\textsuperscript{17,18}, and nonlinear microscopy (NLM).\textsuperscript{4,47–50} Stimulated Raman scattering (SRS) has also been demonstrated for surgical histology\textsuperscript{51}, but it typically has appreciably lower imaging speed or
signal-to-noise ratio when performed without physical sectioning in reflectance mode. Similar to SRS, mid-IR spectroscopy can provide histological imaging based on intrinsic molecular contrast, but it has limited ability to perform reflectance-mode imaging of live tissue, and therefore typically requires time-consuming dehydration and/or physical sectioning of tissue.\textsuperscript{52}

Diffuse optical techniques such as diffuse reflectance spectroscopy can quantitatively measure tissue optical properties associated with malignancy, but do not enable direct histological assessment of pathology.\textsuperscript{53} Extremely rapid molecular identification of breast margin composition has been demonstrated using depth-resolved Raman scattering combined with rotational specimen scanning, although the sensitivity of this technique to different pathologies is still under investigation.\textsuperscript{54} Techniques such as OCT and RCM can perform label-free, structural imaging at extremely high speed but the accurate histological evaluation is challenging because there is low nuclear contrast from scattering.\textsuperscript{42} These and other label-free techniques are complementary to methods that perform histological imaging of the surgical specimen using exogenous labels and have the advantage that they can be used \textit{in vivo} to directly assess the surgical cavity.

In contrast to optical modalities using scattering or intrinsic contrast to infer tissue properties, techniques such as CFM, SIM, light sheet, MUSE, and NLM can use exogenous labels to generate images that can be evaluated analogously to conventional histology. Techniques that produce histology-like images of surgical specimens involve fewer changes to clinical standards and can also be applied in multiple intraoperative applications, analogously to FS. By reproducing classic histological features, these techniques enable direct histological evaluation of tissue, but without the delay that makes paraffin embedding or cryosectioning prohibitively time-consuming in many surgeries. Furthermore, several groups have demonstrated extraction of exogenous labels following fluorescent imaging, greatly reducing the possible risk of interference with postoperative
immunohistochemistry or DNA assays, while retaining the high contrast enabled by molecularly specific labels. Of these techniques, NLM is particularly attractive because of its very high imaging speed and ability to image below the tissue surface through blood and surgical debris. NLM has been evaluated in a blinded study of breast surgical specimens using a fluorescent nuclear stain and second harmonic generation with virtual H&E rendering (VHE) to evaluate surgical specimens from 50 women, obtaining 95.4% sensitivity and 93.3% specificity for NLM assessment of benign vs malignant pathologies compared with standard FFPE H&E histology. These results suggest that NLM can achieve sufficient performance to evaluate breast surgical pathology.

Despite the large number of optical imaging modalities that have been demonstrated in fresh human surgical specimens, translation of advanced imaging techniques into BCT has been limited due to the extremely large size of typical surgical excisions and the limited time available for assessment of margin status. In BCT, a volume of breast tissue is removed which may be more than 100 cm³. Following excision, the specimen is inked for orientation with a unique color per aspect, transected transversely to the tissue face into ‘bread loaf’ specimens, fixed and embedded, with typically a single paraffin section evaluated per bread loaf. For patients with ductal carcinoma in situ (DCIS), guidelines recommend histological processing of bread loaf specimens at 3-5 mm intervals. For example, in a 3 x 2 x 5 cm lumpectomy specimen (30 cm³) this corresponds to evaluating approximately 10-15 bread loaf specimens with an average surface area of 3-6 cm². The total transected tissue area is therefore on the order of 50 cm² to more than 200 cm² depending on excision size and section thickness. The large area and limited imaging time during surgery pose a substantial barrier for clinical translation, and in the case of FS, typically limits evaluation of the excised tissue to a small subset of what would normally be evaluated with
post-operative FFPE histology.\textsuperscript{58} Previous work developing high-speed strip scanning confocal microscopy has demonstrated microscopic resolution and nuclear contrast with imaging rates as fast as 1.5-3 minutes per cm\textsuperscript{2}.\textsuperscript{59,60} However, even at this rapid imaging rate, it would still take several hours to comprehensively image the face of a large lumpectomy cut into bread loaf specimens, which would represent an unacceptable delay of surgery.

Examination times can be dramatically reduced by limiting imaging to areas of the specimen that are relevant for surgical management. This requires high frame rates and operator guidance but is analogous to the way that pathologists examine histology under light microscopy. For breast cancer evaluation, the Society of Surgical Oncology, American Society for Radiation Oncology and American Society of Clinical Oncology Consensus Guidelines for Breast Conserving Therapy (SSO-ASTRO-ASCO) recommends obtaining a negative inked margin during excision for invasive breast cancer\textsuperscript{61}, and a negative margin width of 2 mm from ink for DCIS.\textsuperscript{62} Therefore, comprehensive intraoperative imaging of the entire bread loaf specimen surface is inefficient because most of the surface contains tissue far from an inked margin and would not affect surgical management. Furthermore, in standard histological evaluation, tissue is first evaluated at low magnification, and areas suspicious for pathology are imaged at high magnification. However, with mosaic imaging, the process of imaging and evaluation are performed in reverse order, and so the entire specimen must be imaged at high magnification, resulting in inefficient use of imaging time.

To address these limitations, we have developed an NLM system for the assessment of surgical specimens that incorporates real-time multiscale imaging using both white light imaging for gross evaluation and variable magnification (5x-40x) NLM imaging. In contrast to previous work using mosaic imaging, real-time operator control is used to reproduce the standard process
of histological evaluation, where suspicious regions are located using low magnification and then evaluated at higher magnification. We demonstrate that real-time, multiscale imaging reduces the time required to image inked margins by more than an order of magnitude as compared to high-speed comprehensive mosaic imaging, facilitating intraoperative assessment of margin status without excessive delay of surgical procedures.

The work in this chapter was first published in Biomedical Optics Express. This work was done in collaboration with Michael G. Giacomelli, Lucas C. Cahill, and James G. Fujimoto at Massachusetts Institute of Technology, and Hilde Vardeh, Liza M. Quintana, Beverly E. Faulkner-Jones, and James L. Connolly at Beth Israel Deaconess Medical Center and Harvard Medical School, and Jeff Brooker at Thorlabs. The work was supported in part by the National Institutes of Health R01-CA178636, R01-CA075289, F32-CA183400, and Air Force Office of Scientific Research AFOSR contracts FA9550-12-1-0551 and FA9550-15-1-0473.

2.2 Methods

Human breast tissue not required for diagnostic purposes that would otherwise have been discarded was acquired during specimen grossing at Beth Israel Deaconess Medical Center (BIDMC). All tissue selection and imaging were performed under protocols approved by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects and the BIDMC Committee on Clinical Investigations and Institutional Review Board. Tissue specimens were inked to simulate surgical margins and then grossed into ‘bread loaf’ specimens. Following grossing, specimens were fluorescently labeled using a protocol described previously that consists of 2 minutes immersion in 40 μg/ml acridine orange (AO) combined with 40 μg/ml
sulforhodamine 101 (SR101) dissolved in 50% ethanol/water solution followed by 20 seconds rinse in buffered saline.

Specimens were imaged at 1030 nm excitation wavelength using a custom, multiscale NLM system (described below) deployed at BIDMC in the Department of Pathology, typically within 3 hours of resection. Bread loaf specimens were mounted in a custom specimen tray on a translating stage and imaged using a line scan camera, generating a widefield white light image with a field of view equivalent to a 0.33x objective, enabling immediate identification of diagnostic features such as inked surgical margins. Next, low magnification 5x NLM was used to rapidly survey inked margins at centimeter-scale, excluding the vast majority of tissue which is typically normal adipose tissue or stroma. When regions of interest were identified at low magnification, a rapid objective translator was used to perform high magnification, cellular resolution imaging. This workflow closely resembles that used to read histology slides on a histology microscope, with the addition of the widefield image that allows navigation to regions of interest on specimens.

Following real-time imaging, mosaic images of each specimen were recorded for comparison to FFPE histology using either a 10x/0.45 NA objective with a pixel pitch of 490 nm or a 5x/0.25 NA objective with a pixel pitch of 830 nm. After mosaic imaging, 10% neutral buffered formalin was added to the specimen imaging tray (described below) and the specimen was fixed for a minimum of 12 hours. Following fixation, specimens were submitted for standard histological processing. Because specimens were fixed before removal from the specimen tray, the orientation and shape of soft tissue were preserved until after fixation is completed, facilitating registration of FFPE histology and NLM imaging.
2.3 Design and construction

2.3.1 Multiscale nonlinear microscopy system

![Diagram of the NLM beam path including laser power controller, beam expander, laser interlock shutter, galvanometer-resonant scanner, scan/tube lens, filter cube, and PMTs.](image)

**Fig. 2.1.** Multiscale NLM system. Diagram of the NLM beam path including laser power controller, beam expander, laser interlock shutter, galvanometer-resonant scanner, scan/tube lens, filter cube, and PMTs (left). Photograph of the instrument deployed at BIDMC (right). All figures in this chapter 2 were adapted from "Multiscale nonlinear microscopy and widefield white light imaging enables rapid histological imaging of surgical specimen margins," by M.G. Giacomelli et al., Biomed Opt Express. 2018 Apr 30;9(5):2457-2475. Copyright 2018 by Optical Society of America.

The multiscale NLM system was constructed on a wheeled, vibration isolated optical table using a 36”x30” optical breadboard (Fig. 2.1). The largest component, a Ti:Sapphire laser (Chameleon Ultra, Coherent, Inc.) occupies the rear (away from an operator) half of the breadboard. The laser output power is controlled using a Glan-Thompson polarizer in a USB-controlled motorized rotation mount followed by an interlock shutter. A diffraction-limited, low dispersion Galilean beam expander expands the beam 3.33x to fill the 4 mm scanner aperture. Beam scanning was performed using a modified commercial scanner (OPX1100; Thorlabs, Inc.) with a resonant mirror scanner (16 kHz bidirectional rate) and a galvanometer mirror scanner for the fast and slow imaging axes, respectively. The OPX1100 includes scanners, relay optics, and a
fold mirror for an eyepiece which was not used in this application. The scan assembly was mounted upside down from the intended orientation, enabling inverted operation. A dichroic mirror and FFV2001 filter cube and photomultiplier tube (PMT) assembly are used to relay the excitation light from the OPX1100 to the back aperture of the imaging objective while collecting emitted fluorescent light.

Assessment of centimeter-scale pathology specimens requires both low magnification imaging for identifying regions of interest and higher magnification imaging for detailed assessment of potential pathological findings. A motorized translation stage (X-LSM050B, Zaber, Inc.) and a custom objective tray (Fig. 2.2) were integrated into the imaging path to rapidly (< 1 second) switch between high magnification imaging using a CFI Plan Apo Lambda 10x 0.45 NA objective (Nikon Instruments, Inc.) with a maximum field size of 2 mm and low magnification using a Fluar 5x/0.25 NA objective (Carl Zeiss Microscopy, LLC) with a 3.4 mm field size. Higher magnifications (20x/40x) were implemented in software using the 0.45 NA objective with a reduced scan angle to more densely sample the field. Z-actuation of the imaging plane was performed using a motorized translator (ZFM2020, Thorlabs, Inc.) to vertically actuate the objective assembly. Excited fluorescence emission was collected through the active objective and relayed via a 680 nm long-pass dichroic beam splitter into the FFV2001 filter cube in a non-descanned geometry. A second, 45-degree dichroic filter (T588lpxr, Chroma Technology, Inc.) was used to split light into AO (DNA) and SR101 (stroma) channels. Each channel was further filtered with an additional emission filter for acridine orange (ET540/40m, Chroma Technology) and sulforhodamine 101 (FF01-650/60, Semrock, Inc.). Filtered fluorescence was detected using H7422PA-40 PMTs (Hamamatsu Photonics, Inc.), amplified using TIA-60 transimpedance amplifiers (Thorlabs, Inc.), and digitized using an ATS9440 PCIe A/D (Alazar Technologies, Inc.).
Fig. 2.2. Objective translator assembly. Shown integrated into the microscope (top left), separate from the microscope (bottom left), as assembled and installed (middle), and a ray diagram of the NLM system and objective translator (right).

2.3.2 Widefield White Light Imaging

Fig. 2.3. Diagram of the widefield imaging system. Line illumination is provided by an LED array projected onto the specimen tray. A line scan camera and a fold mirror are used to image the illuminated line as the specimen tray is translated through the camera focus.

Widefield white light imaging was performed using a line scan camera with specimen translation over a fixed imaging location (Fig. 2.3). Images were acquired using an UNiiQA+ (e2v technologies) color line scan camera with 2048 RGB pixels per line and Asahi Macro-Takumar 50mm macro. The macro lens was configured to give a 62 mm wide field of view along the line
scan axis at a 0.3 NA and a projected pixel size of 30 microns. In conventional bright-field microscopy, this is an equivalent field of view to a 0.33x magnification objective. The quadrature encoder output of the translation stage was used to generate a camera line trigger every 30 microns displacement of the specimen. Both the stage and camera are capable of high line scan imaging rates and the imaging time in this system was limited by illumination power to 1 millisecond per line or 3 seconds for the entire specimen tray.

2.3.3 Interchangeable Specimen Tray

![Image of specimen tray]

**Fig. 2.4.** Specimen tray. Rendering of the interchangeable specimen tray (left) and photograph of the tray with 6 inked, bread loaf breast specimens (right). The tray incorporates a lid with a fill port as well as inset dividers that can be used to separate multiple small specimens.

Interchangeable specimen trays (Fig. 2.4) were designed using a 3x4 inch, 400 μm glass plate that serves as both the mechanical support for the tissue specimens and the imaging window for the microscope objective. A 3D printed specimen divider insert, subdivided the glass window into six cells, each the size of a conventional histology cassette, enabling straightforward
histological processing of specimens after imaging. The specimen tray had an aluminum lid that served both to exclude room light from the imaging system and to provide compression of the tissue specimens against the imaging surface. Open-cell foam similar to conventional histology cassette foam inserts was used on top of the specimens so they are compressed by the lid. Additionally, the lid had a magnetic interlock sensor used to disable the laser via the interlock shutter when the lid is removed, preventing accidental exposure to laser light. Finally, the lid had a rubber gasket and filling port, to enable optionally adding fixative after imaging without having to remove specimens from the tray.

### 2.3.4 Software implementation

Both imaging systems were interfaced with a custom microscopy application written in C++ using the .NET 4.5 APIs (Microsoft, Inc.). Rendering was performed in OpenGL using the OpenTK .NET bindings to embed GPU-accelerated OpenGL surfaces into a conventional Windows 10 application (Fig. 2.5). The application integrated multiple functions, including rendering VHE NLM images, rendering widefield imaging data, initializing and controlling NLM scanner hardware, controlling laser power, switching objectives, recording imaging data for offline analysis or review, and optionally performing mosaic mode imaging for comparison to conventional histology.

Rendering of VHE images was performed using the open-source virtual transillumination microscopy (VTM) algorithm published previously. The implementation of VTM used in this study performs physically realistic per-pixel absorption calculations through a simulated slide to produce transmission images from epifluorescence data. The algorithm rendered epifluorescence from AO and SR101 as virtual hematoxylin and eosin absorption, respectively. The algorithm was
implemented in OpenGL and then rendered on a 4K resolution monitor calibrated to 100% of the sRGB colorspace. OpenGL rendering is essential at 4K resolution because real-time rendering without GPU acceleration was not possible at the data rate required. OpenGL was also used to process the widefield white light images using a flat field correction to compensate for illumination uniformity and a gamma correction for optimal contrast. Finally, a custom vertex shader was used to draw the current NLM stage position on top of the widefield image. On each screen update (16.67 ms period), the current microscope stage position is retrieved from the quadrature encoders, and the vertex shader is updated with the current location. NLM field sizes are also polled and the NLM imaging location icon size is adjusted for the magnification setting.

Fig. 2.5. Software user interface and controls used to image freshly excised, inked bread loafed surgical specimens. The virtual H&E display shows a terminal ductal lobule unit (TDLU) imaged at 20x magnification, while the white light microscopy shows a widefield image with the current imaging location adjacent to a simulated inked margin. The user can rapidly (< 1 second) switch the magnification, while a
joystick and digital focus knob are used for X/Y and Z control, respectively. An LED indicator shows the laser interlock status, enabling the operator to see when the specimen is loaded and the laser is aimed.

![Diagram](image)

**Fig. 2.6.** Analog and digital data flow diagram separated into three phases of system operation.

Figure 2.6 depicts the logical flow of signals during three distinct phases of system operation: (1) prior to NLM imaging when the widefield white light image is acquired (blue), (2) during real-time NLM imaging (red), and (3) in post-processing when the recorded data is analyzed (green). The first phase, prior to the start of real-time NLM imaging lasts 3 seconds, during which the specimen tray is translated over the line scan camera imaging plane, the white light illuminator is activated, and the translation stage quadrature encoders are used to generate uniformly spaced line scan camera triggers at 30 μm intervals. Finally, the line scan image is rendered to the screen and the stage is returned to the initial position.

Following white light image acquisition, the system enters the continuous phase of imaging where NLM images are acquired at 16 frames per second as the operator assesses the surgical margins on the specimen. During this phase, the resonant scanner unidirectional rate (8 kHz) serves as the master clock, synchronizing both the readout of NLM frames at 1 frame every 528 cycles (512 forward lines and 512 backward lines followed by 16 fly back cycles while the slow axis
resets between frames) and the translation stage position is read out by the PCIe-6323 DAQ (National Instruments, Inc.) once per cycle (8 kHz). NLM frames and position encoder data are then sent to the VHE renderer, which compares the stage position to the previous frame’s position, and if the stage is stationary, opportunistically averages frames to improve image quality. If the stage is moving, the previous frame is discarded and no averaging is performed. Following any averaging, the renderer performs vignette correction and absorption calculations for VHE rendering. Opportunistic averaging enables the user to view averaged data when the stage is stationary without distortion or blurring while translating the specimen. During this phase, all acquired data, including the white light image, NLM frames, and position data is recorded, enabling replay of procedures.

After the conclusion of real-time imaging, the logged data is optionally processed to generate a permanent record of the procedure. During this phase, the motion data is combined with the stored NLM frames to perform averaging/data reduction of any stationary frames, and then motion artifacts are removed from frames acquired during motion. Because the fast axis scanner is rapid (62.5 μs per line), a specimen translation speed of 1 cm/s results in an intra-line displacement of 625 nm, less than one resolvable spot. Consequently, individual fast axis lines have no intra-line motion artifacts, and stage motion results only in an additional displacement added between scanlines along the slow axis. Using the position encoder data, specimen motion can be added to the slow axis displacement to reposition individual fast axis lines back to their true, undistorted position, effectively removing the effects of motion. Finally, the undistorted frames are stitched into seamless mosaics as described previously\(^8\) (Fig. 2.7). This enables review of any real-time assessment procedures in a format analogous to a permanent paraffin histological section.
Fig. 2.7. Dewarping and stitching of live data to generate mosaics. By synchronously recording position data with each fast axis cycle, the true geometric position of all pixels in each image can be calculated and the resulting frames stitched into an undistorted mosaic.

2.4 Results

2.4.1 NLM Resolution

Fig. 2.8. Point spread function measurement. Lateral (left) and axial (right) point spread function plots for the 5x (top) and 10x (bottom) objectives measured using sub-resolution fluorescent beads.
Fig. 2.9. Comparison between 5x/0.25 NA and 10x/0.45 NA objectives at 1030 nm excitation wavelength using discarded superficial breast tissue excised during surgery for invasive ductal carcinoma. The biopsy site and skin are present along with large areas of fat and stroma (top left). Mosaic images viewed at low magnification show the relatively limited difference between the two objectives (top center – 5x, top right – 10x), however, zoomed views of the boxed regions show that cellular features are poorly resolved with the 5x/0.25 objective. In contrast, the structure of a blood vessel is readily identifiable with the 10x/0.45 objective. Scale bars: 1 cm (top) and 400 μm (bottom). Total area: 7.9 cm².

To measure the system resolution, 200 nm diameter fluorescent beads (FSDG002, Bangs Laboratories) were embedded in PDMS (n=1.42), deposited onto the specimen tray, and imaged at both 780 nm and 1030 nm to test the system resolution at two commonly used femtosecond
laser excitation wavelengths (Fig. 2.8). To compensate for the limited precision of the axial objective translator, the position encoder was read out synchronously with frame acquisition and z-stacks were resampled to reduce the effects of drift and backlash. The 5x objective lateral resolution was 1.45 μm and 2.10 μm for 780 nm and 1030 nm, respectively while the axial resolution was 20.0 and 27.0 μm, respectively. The 10x objective lateral resolution was 0.93 μm and 1.2 μm for 780 nm and 1030 nm, respectively, while the axial resolution was 7.1 μm and 8.7 μm, respectively. Notably, the 5x objective resolution was more strongly affected by longer wavelength operation than the 10x, likely reflecting the superior NIR achromatization of the 10x objective. The system lateral and axial resolution was degraded because of a substantially thicker imaging glass (400 μm) than the objectives’ design thickness (180 μm), although the effect is modest at 0.45 NA and insignificant at 0.25 NA. In a previous study, we evaluated attenuation at both wavelengths in normal human breast tissue and obtained an e^{-1} depth of ~34 μm at 780 nm and ~52 μm at 1030 nm.  

NLM resolution was further assessed by imaging discarded human breast tissue taken from the superficial aspect of a mastectomy specimen using both 5x/0.25 and 10x/0.45 objectives. Laser power was adjusted so that both objectives produced equal peak fluorescence intensity and 8 cm² mosaic images were acquired using each objective (Fig. 2.9, top row). Both objectives readily resolved areas of fat, stroma, and aggregations of cell nuclei, but cellular details were difficult to discern due to the much thicker axial sectioning of the 5x/0.25 objective (Fig. 2.9, bottom row). In contrast, individual nuclei were well-resolved with the 10x/0.45 objective.
2.4.2 Comparison to histology

Fig. 2.10. Comparison between an NLM mosaic image of the specimen surface using the 10x / 0.45 NA objective and FFPE histology from approximately 300 μm below the surface of a breast surgical specimen with ductal carcinoma in situ. Scale bar: 2 mm. Total area: 2.2 cm².

Mosaics were also acquired to compare NLM imaging to FFPE histology, although it is important to note this would not be performed during clinical procedures due to the long acquisition times. The high speed of the translation stage (~100 ms) and the very high imaging rate of the NLM system enable relatively rapid mosaic mode imaging. Using a 3x image averaging, a 1 mm² field size, and 490 nm sampling density, acquisition of one frame followed by translation and a software delay of ~500 ms requires less than 1 second. Accounting for overlapping of adjacent frames to enable seamless stitching, this is a net equivalent pixel rate of 4 MP/s and a time per area of 2.3 minutes per cm², approaching some of the fastest area-imaging rates reported in the literature.⁶⁰
To validate the NLM imaging and staining protocol, we performed mosaic imaging of 50 discarded breast specimens from 21 patients. Representative NLM VHE images and scanned FFPE H&E slides of a specimen with extensive DCIS are shown in Fig. 2.10. Low magnification images from NLM and the H&E scanned slide show an epithelial proliferation expanding ducts and lobules. Zoomed views of the same images show that the proliferation is composed of monomorphically atypical epithelial cells with low to intermediate grade nuclei consistent with a diagnosis of ductal carcinoma in situ.

Fig. 2.11. Comparison of mosaic and real-time imaging using normal human breast tissue inked to simulate a surgical margin. The widefield white light image (left) enables visualization of the tissue and regions of purple and blue ink on the simulated margins. Mosaic NLM (center) images the entire specimen face but required 487 seconds. In contrast, real-time assessment using the 10x objective required less than 45 seconds because the operator imaged only the margins using opportunistic averaging and did not image non-diagnostic regions such as adipose tissue. The lower scattering and absorption of 1030 nm excitation wavelength enabled imaging below thin layers of ink on the simulated margin. Scale bars: 2 mm. Total area: 3.9 cm².

2.4.3 Comparison between real-time assessment and mosaic imaging

To demonstrate the advantage of real-time margin assessment, inked bread loaf specimens were imaged using both comprehensive mosaic NLM and using real-time assessment where inked
margins were identified using widefield white light microscopy and then imaged under operator control at video rate (Fig. 2.11). Although most surgical specimens have inked margins on only part of the circumference, we tested the most conservative case and imaged the entire circumference. In this example, real-time assessment of the margin using panning at 10x magnification required only 44.5 seconds including the time required to interpret images, compared with 487 seconds to acquire a mosaicked image of the entire specimen. Real-time imaging of the margin at 5x magnification required only 24 seconds due to the larger field of view, although detailed evaluation of pathology identified at low magnification would require changing to 10x magnification in regions of interest. If inked margins were present on only part of the circumference, the assessment time would be proportionally faster.

We further evaluated real-time imaging using mastectomy specimens dissected to expose both invasive ductal carcinoma as well as normal tissue. Figure 2.12 shows a simulated positive margin inked yellow, as well as two simulated negative margins, inked green and orange on an approximately 2 cm² area specimen. Simulated inked margins can be visualized using both the widefield white light image (bottom left frame) as well as at high magnification using NLM, where the inked tissue shows as regions of distinctive fluorescence in the nuclear channel (green ink), stroma channel (orange ink) or both (yellow ink). NLM imaging using the 5x objective (top left frame) enabled all 3 margins to be assessed in less than 30 seconds, while imaging using only the 10x objective required approximately twice as long to survey due to the smaller field of view (top right frame). While cellular details are less well visualized using the 5x objective, normal adipose tissue or stroma and invasive ductal carcinoma can still be differentiated even at low magnification due to the higher density of strongly staining cell nuclei. To confirm this finding, real-time imaging with the 10x objective was used to visualize a region of invasive ductal carcinoma in contact with
the yellow ink (bottom right frame), revealing both a poorly differentiated tumor and an adjacent region of necrosis with few nuclei.

**Fig. 2.12.** Comparison of real-time imaging using 5x/0.25 NA and 10x/0.45 NA objectives to assess an inked tissue specimen containing both normal tissue and invasive ductal carcinoma. Orange, yellow, and green ink are grossly visible in the widefield image (bottom left), while each color is visible as fluorescence in one or both spectral channels in the NLM images. Both 10x and 5x objectives enable surveying the entire simulated margin in less than 1 minute, however, the 5x objective is approximately 2x faster and can assess tissue further from the inked margins. Scale bars: 2 mm (top, left) and 400 μm (bottom right). Total specimen area: 4.0 cm².
2.5 Discussion

Inking, transection, and evaluation of bread loaf specimens is a standard protocol for postoperative surgical margin evaluation in breast, prostate, and other surgeries. In this procedure, specimens are inked to indicate the true surgical margin location and then cut perpendicular to the margin surface. A pathologist evaluates the minimum distance a pathology is present from each of the inked margins on the bread loaf specimens. In contrast to en face margin evaluation, as used in applications such as Mohs surgery, where most or all of the histologically processed tissue surface or aspect is histologically evaluated, in a bread loaf specimen from lumpectomy excision, only the inked regions and an additional zone of tissue adjacent to the ink is relevant to determine margin status. The multiscale imaging approach we have developed is well suited for assessing bread loaf margin status because it follows the standard pathology practice of evaluating tissue grossly followed by evaluating it with increasing magnification while avoiding unnecessary high magnification imaging of non-diagnostic regions. To validate this approach, we used simulated surgical margins dissected from larger breast surgical specimens, and we compared the time required to image and assess inked margins using real-time video-rate imaging versus the time required to acquire mosaic images.

We demonstrated that even for relatively small (2 cm²) bread loaf specimens, comprehensive mosaic imaging of a single depth of an entire tissue aspect was an order of magnitude slower than the combined time required to image and evaluate under real-time operator control. We further found that using a 5x objective for inspection followed by higher 10x magnification for areas of focal pathology was a further factor of ~2 faster. These results were demonstrated using specimens with both simulated negative margins and positive margins with invasive ductal carcinoma. While margins with pathologies such as invasive lobular carcinoma or
DCIS could be more time-consuming to assess than positive margins with uniform invasive ductal carcinoma, we note that the overwhelming majority of surgical margins are ultimately found to contain only normal tissue. Consequently, the time to exclude regions of normal tissue is expected to occupy most of the procedure time, making the ability to rapidly exclude normal tissue critical. The study to determine the time required to evaluate true surgical margins with complex pathologies such as DCIS is described in Chapter 5.

The multiscale NLM system integrates many features that facilitate the rapid histological assessment of unsectioned tissue analogously to a conventional histology microscope viewing FFPE histology slides. First, the maximum field of view is adjustable between 3.4 mm using the 5x / 0.25 NA and 2.0 mm using the 10x / 0.45 NA objective, while higher magnification (20x/40x) is implemented through digital control of the scanner angles. At maximum scan angle, this corresponds to an imaging rate of 145 mm²/s and 50 mm²/s for the 5x and 10x objectives, respectively. The rapid imaging rate, low latency OpenGL VHE rendering, and adjustable magnification reproduce the conventional histological workflow where FFPE H&E slides are evaluated at low magnification to identify suspected areas of pathology prior to high magnification. Changing the objectives takes less than 1 second using the custom-built objective translator, a similar time to changing objectives in a conventional bright-field microscope. Second, to rapidly identify inked margins and grossly apparent features, the system incorporates a widefield white light view that tracks the location of the high magnification NLM image in real-time, enabling the operator to rapidly locate margins on large specimens while avoiding areas that are not relevant to assessment such regions far from any margins. Finally, by synchronously reading out the stage position with each fast axis scan, we implemented opportunistic averaging, a novel feature that enables averaging without motion blur or other artifacts when translating the stage.
This data was post-processed to generate mosaic images from data acquired by real-time user panning, enabling efficient review of procedures in a fraction of the time required to replay the data at real-time speed.

As an alternative to real-time imaging, the time required to image margins on bread loaf specimens could be reduced using machine vision algorithms to segment inked margins identified on widefield white light imaging and then guide tissue mosaicking to these regions. However, it is challenging to determine which imaging depths are likely to be diagnostic when mosaicking without the time-consuming process of imaging each depth, as well as what objective and sampling density are optimum to assess a region. In contrast, an experienced pathologist can rapidly identify areas of suspected pathology and perform comprehensive imaging of all depths in seconds while selecting the lowest magnification required. Furthermore, when imaging in mosaic mode, the number of frames to average must be decided prior to imaging, consequently, all areas of the tissue receive the same degree of averaging. In contrast, real-time assessment using the opportunistic averaging method developed here enables less relevant areas of tissue to be imaged without averaging while performing a high degree of averaging over regions that the operator identifies as diagnostically relevant. Finally, while more efficient sampling strategies may enable more rapid acquisition of mosaic images, they do not overcome the fundamental disadvantage posed by the need to sequentially image, stitch frames and then assess histological images.

The emphasis of this study was to develop a technique that is consistent with existing clinical workflows based on bread loaf evaluation of surgical margins, the current standard for postoperative histological evaluation of invasive breast cancer, and DCIS. As of 2018, a large majority of pathologists exclusively use bread loafing for breast margin assessment, a minority use a combination of bread loaves and en face histology, while en face evaluation alone is rarely
utilized due to the difficulty in sectioning the surface aspect of breast specimens. Consequently, the approach presented in this study has the advantage of maintaining the current standard of care. However, bread loaf evaluation images only a very small fraction of the total surgical margin, typically less than 1%. Further studies of the correlation between en face and bread loaf margin assessment may be required to understand if en face evaluation using fluorescent imaging modalities can improve the evaluation of breast surgical margins.

Intraoperative imaging of surgical specimens using NLM reproduces some aspects of existing FS workflows but with dramatically reduced processing times, enabling evaluation of a much larger number of bread loaf specimens in a shorter amount of time. Turnaround times for FS processing are typically about 20 minutes to section a single specimen, necessitating selective sampling of a subset of the tissue that would be evaluated postoperatively. In contrast, using NLM an unlimited number of bread loaf specimens can be stained in less than 3 minutes. This improvement in throughput suggests that the entirety of the tissue postoperatively evaluated with FFPE under the SSO-ASTRO-ASCO guidelines could be evaluated in the time required to process a single FS slide. However, it is important to note that it is possible to perform FS in parallel on multiple specimens to more comprehensively evaluate margins but this requires additional pathology infrastructure which may not be available or cost-effective. One study at the Mayo Clinic using FS during BCT demonstrated a reduction of repeat surgeries from 55% to 19.3% with an average of 6 frozen sections per patient, and 27 minutes average processing time with 2 minutes additional time per specimen using multiple histotechs to process specimens in parallel. However, times increased appreciably for a larger the number of specimens and in one extreme case, evaluation of 15 specimens required 1 hour. This study further used thicker frozen sections (20 µm, analogous to 5x data in Fig. 2.9), resulting in a tradeoff between processing time and axial
section thickness. In contrast, no such tradeoff is required with the multiscale NLM approach presented here because NLM enables thin axial sectioning of fresh tissue. Furthermore, a large study of FS margin evaluation in nerve-sparing prostatectomy reported substantial improvements in rates of nerve-sparing surgeries, but the protocol required 5 cryostats and 4 histotechs working in parallel to evaluate 10 to 24 specimens per patient in an average of 35 minutes. In contrast, multiscale NLM scales much more favorably with increasing numbers of bread loaf specimens, because after inking and grossing, multiple bread loaf specimens can be stained simultaneously and then examined without physical sectioning as shown in Figs. 2.11 and 2.12. Furthermore, although bread loaf specimens were dissected to fit standard histological processing cassettes, in future applications NLM could image a large several centimeter bread loaf specimen without dissection, further reducing imaging times and enabling improved coverage.

While we imaged at 1030 nm, typical for ytterbium fiber lasers, we also evaluated the resolution of the NLM system using 780 nm excitation wavelength, typical for titanium sapphire or frequency-doubled erbium fiber lasers (Fig. 2.8). Both 780 and 1030 nm efficiently excite AO/SR101 labeled tissue, but 1030 nm enables >50% deeper penetration through tissue. At both wavelengths, the maximum lateral resolution was comparable to a typical 20x/0.5 NA bright-field histology microscope, while axial sectioning was ~1.5 to ~2x lower than a typical paraffin section. The 5x/0.25 NA objective had reduced lateral and axial resolution as compared to the 10x objective, however, discrimination of adipose tissue and normal stroma from regions of abnormal stroma or tumor was still possible because of the high contrast of the fluorescent labels used in this study. Notably, the autofluorescence of surgical marking inks as well as the attenuation from thin layers of ink and other surgical debris deposited onto tissue specimens during excision or grossing were reduced at longer wavelengths, enabling a more comprehensive assessment of specimen
margins. While evaluation of multiple depth serial sections per bread loaf is not typically performed for breast margin assessment, the ability to image through surgical debris is likely to be important. Combined with our recent demonstration that inexpensive and compact ytterbium fiber lasers are effective for NLM imaging of surgical specimens\textsuperscript{55}, the ability to operate at long wavelengths may be important for clinical translation of compact, affordable real-time surgical equipment that can image through surgical debris and tissue marking ink.

The multiscale imaging approach demonstrated here may be applicable to other less costly imaging modalities such as CFM, SIM, MUSE, or OCT that can perform high-speed imaging using multiple objectives or variable magnification with a single objective. However, the ability to image through surgical debris, blood, and tissue marking ink is an important advantage of NLM that enables imaging of tissue aspects that have surface contamination typical of breast surgical specimens. In chapter 3, we discuss CFM and its limited ability to image subsurface features\textsuperscript{8}, and in chapter 4 we demonstrate that MUSE can only image a single plane on the tissue surface and so cannot perform a subsurface assessment.\textsuperscript{17} OCT provides excellent subsurface imaging but has limited nuclear contrast. The extent to which these modalities could be used to image specimens with ink surfaces and other contamination requires further investigation.

\textbf{2.6 Conclusion}

We have demonstrated an instrument enabling the assessment of surgical specimens at rates sufficient to comprehensively assess surgical margins during breast conserving therapy and other intraoperative imaging scenarios. In contrast to previous work using image mosaicking, we introduce a multiscale imaging approach combining widefield white light imaging for gross specimen evaluation and identification of inked margins, low magnification NLM for rapid tissue
surveying, and high magnification NLM for detailed evaluation at cellular resolution. GPU-accelerated processing is used to provide a video-rate rendering of fluorescent data as virtual H&E slides. We demonstrate that this approach enables rapid assessment of centimeter-scale surgical specimens in a workflow analogous to FS processing of tissue, but with dramatically faster tissue processing times, no freezing artifacts, and compatibility with subsequent histological processing. Clinical studies are necessary to demonstrate the sensitivity and specificity of intraoperative NLM compared with the clinical standard of post-operative FFPE histology. However, the technology and methods described here could enable comprehensive intraoperative margin assessment in breast surgeries as well as a wide range of other cancer surgeries.
Chapter 3

Direct comparison between confocal and nonlinear microscopy for rapid histological evaluation of fresh human tissue

3.1 Introduction

Histopathological assessment of stained thinly sectioned tissue specimens under bright-field microscopy is the gold standard for the diagnosis of most neoplasms, including breast cancer. In conventional histopathological protocols, specimens are processed by fixation, dehydration, paraffin embedding, sectioning into thin slices, and staining, which typically delays evaluation of pathology by at least one day. Techniques that enable real-time tissue evaluation are of interest for highly time-sensitive procedures, such as intraoperative assessment of surgical margins.

Breast conservation therapy (BCT), which includes lumpectomy to remove cancerous tissue along with a margin of surrounding healthy tissue, followed by radiation and/or chemotherapy, is a standard of care for early-stage breast cancer and ductal carcinoma in situ (DCIS). To verify complete surgical resection of cancer, the surgical margins of the lumpectomy are assessed by postoperative histopathological examination. If postoperative histology finds cancer close to, or on, the surgical margins of excised tissue specimens, re-excision is usually recommended. The rate of second surgeries in BCT due to inadequate resection is up to 40%, resulting in an increased financial burden from repeated surgeries, and worsened morbidity and
cosmetic outcomes.\textsuperscript{29,68,69} Therefore methods for performing an intraoperative assessment of breast pathology are needed.

One established means of performing intraoperative histology to reduce re-excision rates is frozen section (FS). In FS, specimens are frozen, sectioned into thin slices, stained, and examined under bright-field microscopy within 15-30 minutes of excision.\textsuperscript{70,71} However, the freezing process deforms the tissue structure, limits how thin the tissue can be sectioned, and is challenging to perform on fatty tissues, such as breast tissue, that do not freeze well.\textsuperscript{72,73} Furthermore, sensitivity is controversial, with values reported between 65\% and 91\%.\textsuperscript{34,74} with small tumors and DCIS being the most difficult to assess.\textsuperscript{75} Consequently, FS for BCT is not utilized at a majority of clinical centers.\textsuperscript{35}

An alternative to time-consuming embedding and physical sectioning of fixed tissue is optical sectioning of unfixed tissue. Imaging unfixed tissue specimens have been demonstrated using techniques such as reflectance confocal microscopy (RCM)\textsuperscript{76–79}, confocal fluorescence microscopy (CFM)\textsuperscript{6,7,41,80–82}, nonlinear microscopy (NLM)\textsuperscript{4,83,84}, structured illumination microscopy (SIM)\textsuperscript{85}, spectrally encoded confocal microscopy\textsuperscript{40}, and optical coherence tomography (OCT).\textsuperscript{86,87} Furthermore, recently it has been demonstrated that fluorescent contrast agents can be rapidly diffused through viable human tissue, enabling fluorescent labeling without fixation.\textsuperscript{4,81,85}

NLM and CFM enable strong rejection of out-of-focus light, high axial resolution imaging, rapid imaging speed, and compatibility with fluorescent contrast agents. NLM has been comprehensively evaluated for breast pathology assessment.\textsuperscript{4} This study performed a blinded reading of NLM mosaic images color remapped for an H&E-like appearance from 179 unfixed, discarded, and randomly selected breast tissue specimens from 50 patients. Specimens were
mosaic imaged over large fields, fixed, and then corresponding paraffin-embedded H&E slides were obtained. A blinded reading showed a 95.4% sensitivity and 93.3% specificity for identifying invasive cancer and DCIS versus benign breast tissue compared to paraffin-embedded H&E histology of the same specimens. The high sensitivity and specificity obtained in this study were in part due to the use of exogenous fluorescent stains to generate nuclear contrast, the high resolution of NLM, and remapping fluorescent and second harmonic generation signals to resemble H&E histopathology.

Unfortunately, while NLM has demonstrated high diagnostic agreement with paraffin-embedded histology for breast surgical specimens, compared to CFM, it is complex and extremely costly because of the need for femtosecond lasers. This high cost may be a barrier to clinical acceptance. Alternatively, extensive literature supports the efficacy of RCM for histological evaluation of skin cancer in vivo and intraoperative assessment. Further work has demonstrated that fluorescent labels combined with CFM greatly improve diagnostic accuracy for skin lesions. In spite of the extensive work investigating CFM for skin pathology and NLM for breast pathology, studies of CFM for breast pathology have been limited to qualitative evaluations or comparisons of selected, high magnification image fields that are difficult to generalize to readings of intact surgical specimens. Furthermore, to date, protocols for H&E-like rendering of dual-channel CFM for breast surgical specimens have not been demonstrated, further complicating comparison to NLM using virtual H&E (VHE) rendering and sources of contrast such as second harmonic generation that are unavailable to CFM. Consequently, it remains unclear if lower-cost CFM can perform equivalently to NLM for evaluation of breast surgical specimens using H&E-like color rendering applied to large surgical specimens.
In this chapter, we present a direct comparison of CFM, NLM, and paraffin-embedded H&E histopathology using freshly-excised discarded human breast surgical specimens. We demonstrate a new staining protocol using dual contrast agents that can be rapidly applied to tissue specimens for both NLM and CFM imaging and that enable accurate VHE rendering of histopathology. VHE images were generated by using the virtual transillumination microscopy (VTM) algorithm, which enables physically realistic modeling of virtual transillumination white light microscopy images using epi-fluorescence measurements.\textsuperscript{48} The image quality of both CFM and NLM are compared at 20X magnification, for high-resolution evaluation of cellular features, and 10X magnification, for examination of architectural morphology of specimens. Photobleaching effects are assessed for the compatibility of the staining protocol with higher-speed imaging. Degradation of signal-to-background ratio with imaging depth is also compared between CFM and NLM. These results indicate that CFM can be used to produce high-quality VHE images of unfixed breast tissue with performance similar to NLM when imaging near the tissue surface.

The work in this chapter was first published in Journal of Biomedical Optics.\textsuperscript{8} This work was done in collaboration with Michael G. Giacomelli, Lucas C. Cahill, and James G. Fujimoto at Massachusetts Institute of Technology, and Daniel B. Schmolze, Hilde Vardeh, Beverly E. Faulkner-Jones, and James L. Connolly at Beth Israel Deaconess Medical Center and Harvard Medical School. This work was supported in part by the National Institutes of Health (R01-CA178636, R01-CA075289, and F32-CA183400), and Air Force Office of Scientific Research (FA9550-12-1-0551 and FA9550-15-1-0473).
3.2. Methods

3.2.1 Specimen preparation

Unfixed breast tissues were acquired under protocols approved by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects (COUHES) and the Beth Israel Deaconess Medical Center (BIDMC) Committee on Clinical Investigations (CCI). Only discarded and deidentified human tissue specimens which were not required for clinical diagnosis were acquired, and therefore, the protocol was exempt from informed consent. The discarded tissue specimens were kept in a chilled Roswell Park Memorial Institute (RPMI) medium to maintain hydration and imaged within 8 hours of excision. Before imaging, excess fat was removed and representative regions (3~5 mm width) of stroma or pathology were exposed. Propidium iodide (PI) and eosin yellow (EY) were chosen as the nuclear and stromal stains respectively because of their excellent penetration into unfixed tissue, low cost, rapid binding, and separated emission spectra. Dissected tissue specimens were stained for 2 mins in a solution of distilled water, 40 µg/ml PI, and 200 µg/ml EY. The stained specimens were rinsed in saline and placed on a #1.5 glass coverslip attached to a specimen holder. The specimen holder was filled with saline-soaked biopsy foam pads so that the surface of the tissue specimens maintained contact with the glass coverslip without dehydration. This method allows for repeated imaging of the same specimen for several hours without noticeable alteration of a cellular structure or fluorescent signal.
3.2.2 Confocal microscope

A commercial inverted confocal laser scanning microscope (Zeiss LSM510) was used for CFM imaging. Fig. 3.1(a) shows the schematic of the confocal microscope. A diode laser (405 nm) simultaneously excites both contrast agents with 0.5 mW power incident on the sample. The laser was introduced into the optical path using a multiband dichroic beam splitter (Zeiss HFT 405/514/633 nm). The LSM510 uses dual galvanometer scanners with a dwell time of 3 µs per pixel. Fluorescent signals from PI and EY were detected by dual photomultipliers (R6357; Hamamatsu, Inc.) using a dichroic beam splitter (cutoff of 565 nm). An additional long-pass filter at 650 nm (PI) was used to reduce spectral crosstalk to a negligible level. In the EY channel, a long-pass filter at 505 nm was used to reject autofluorescence and scattered excitation light. The combination of beam splitters and filters above produced a detection range for EY from 525 to 565 nm, and for PI from 650 nm to the PMT responsivity cutoff near 800 nm. A Plan-APOCHROMAT 0.8NA 20X dry objective (Zeiss) and a Plan-NEOFLUAR 0.3NA 10X dry objective (Zeiss) were chosen as representative objectives for CFM. The use of a dry objective slightly degrades resolution when imaging through thick tissue, but greatly simplified imaging in an inverted geometry, which may be advantageous for intraoperative scenarios. Each image frame was acquired at 1024 x 1024 pixels. Due to software limitations, the frame time in mosaic mode was significantly longer than the sum of the pixel dwell time, at about 6 seconds per frame, or 24 seconds with 4-fold averaging. With the 20X and 10X objectives, the fields of view were 0.45 mm and 0.9 mm, with pixel sizes of 0.44 µm and 0.88 µm square, respectively. 20X and 10X CFM mosaic images were therefore generated at a rate of 2 and 0.5 minutes per mm², respectively. The detector pinhole sizes were set to 1 airy unit for each objective at the detection wavelength range.
3.2.3 Multiphoton microscope

A commercial nonlinear microscope (Thorlabs, Inc.) was used with a ~150-fs tunable Ti:Sapphire laser (Mira Optima 900-F; Coherent) at ~780 nm with a 76-MHz repetition rate. Fig. 3.1(b) shows the multiphoton microscope schematic. The excitation laser was scanned by a nonresonant galvanometer scanner and an 8 kHz (16 kHz bidirectional) resonant galvanometer scanner. The laser power on samples was <30 mW. Because the fast scan axis moves resonantly, the dwell time per pixel is not uniform along the fast scan axis. The average dwell time per pixel was 60 ns, and the minimum dwell time per pixel at the center of the scan line was 40 ns. PI and EY were simultaneously excited, and the fluorescent signals were detected by dual photomultipliers (H7422; Hamamatsu, Inc.) using a dichroic beam splitter (cutoff of 590 nm). Additional band-pass filters from 520 to 560 nm (EY) and from 620 to 680 nm (PI) were used to reduce spectral crosstalk. A XLUMPFL20XW 1.0 NA 20X water immersion objective (Olympus) and a Plan Apo Lambda 0.45NA 10X dry objective (Nikon) were used. A water immersion objective was selected for 20X imaging to be consistent with our previous study. Each image frame was acquired at 1024 x 1024 pixels. With the 20X and 10X objectives, the fields of view were 0.49 mm and 1.1 mm, with pixel sizes of 0.48 µm and 1.05 µm, respectively. The NLM system operated at 16 frames per second with real-time VHE rendering, however, due to software limitations, approximately 2 seconds were required for mosaic frame acquisition and images were acquired with 50% overlap, for an imaging rate at 20X and 10X magnification of 32 and 8 seconds per mm², respectively. No such delay is present in live panning mode.
Fig. 3.1. Schematic of a) the confocal fluorescence microscope system and b) the multiphoton microscope system. Both systems used dual-channel detectors for nuclear and stromal contrasts. The specimen holder was designed for both inverted and conventional microscope configurations without touching the tissue so that registration could be maintained. (DBS: dichroic beam splitter, VP: variable pinhole, PMT: photomultiplier tube). All figures in this chapter were adapted from "Direct comparison between confocal and multiphoton microscopy for rapid histopathological evaluation of unfixed human breast tissue," by T. Yoshitake et al., J.Biomed. Opt. 21(12), 126021 (2016). Copyright 2016 by Society of Photo-Optical Instrumentation Engineers.

3.2.4 Lateral and axial resolutions

In both CFM and NLM systems, images are laterally undersampled compared with the optical resolution. Therefore, the lateral image resolutions were equal to the sampling interval. Conversely, the axial sectioning of each modality is independent of sampling density. To characterize the axial resolution of each system, the axial edge was measured using a dilute, homogenous ‘fluorescent sea’ of fluorescein underneath a coverslip (Table. 3.1)\(^{90}\) using the EY channel for detection. The experimentally measured 80% to 20% responses at 20X magnification for both CFM and NLM are comparable to the typical thickness of H&E histology slides (~5 µm). The 10X NLM system has about 2X larger optical sectioning thickness as compared with H&E
histology slides, while the 10X CFM has about 4X larger optical sectioning thickness than H&E histology slides. The imaging results and qualitative comparison between H&E histology slides are presented in the results and discussion sections.

**Table. 3.1.** Experimental measurement of axial resolution. Axial step response was measured by using the infinite sea method.

<table>
<thead>
<tr>
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<th>20X CFM</th>
<th>20X NLM</th>
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<th>10X NLM</th>
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<tr>
<td>Axial resolution (20%-80% response)</td>
<td>5.5 µm</td>
<td>4 µm</td>
<td>19 µm</td>
<td>8.5 µm</td>
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**3.2.5 Imaging procedure**

CFM and NLM imaging were conducted within one hour of one another, with no additional preparation done between imaging, to minimize any changes in the unfixed samples. Neither the CFM imaging nor the NLM imaging produced a noticeable effect on the subsequent imaging of the other modality. Single plane, cross-sectional images were acquired by translating the specimen with xyz stages and capturing frames at multiple transverse positions. Sequential images from the surface of each specimen (about 5 µm into the specimen from the glass coverslip surface) and at increasing depth into the specimen (25 µm, 50 µm, and 75 µm from the surface of specimens) were acquired. Because of the low excitation energy per pixel required when imaging highly fluorescent labels, and the long collection wavelengths, no autofluorescence was detected from unlabeled tissue on either system. The effect of photobleaching was assessed by examining the signal intensity change from consecutively acquired frames using the 20X objective at the same position on the specimen with distinct regions used for NLM and CFM. After imaging with both CFM and NLM, specimens were fixed using buffered formalin (10%) while still immobilized against the specimen holder coverslip to retain the image plane orientation. After a minimum of 2
days of fixation, the tissue specimens were processed using the standard H&E histology procedure taking care to maintain the specimen orientation during embedding and sectioning and then digitally scanned (Aperio AT2 slide scanner, 20X magnification; Leica Biosystems GmbH). This procedure enables straightforward registration of NLM, CFM, and histology images by minimizing distortion of the tissue during processing.

VHE rendering was performed using the virtual transillumination microscopy (VTM) algorithm. VTM is a physically realistic light absorption model that enables the high-quality rendering of absorption stains such as H&E using fluorescent data by simulating the transmission of white light through virtual slides, computing a per-dye optical attenuation, and then displaying the remaining transmitted light. The use of a physically realistic model for light absorption enables rendering H&E-like images with a high dynamic range and more accurate colors than simple color remapping. Histogram brightness normalization was used to equalize the brightness of both fluorescent channels such that 0.01% of pixels were overexposed. While VTM enables real-time, live histological evaluation of specimens, wide-area mosaics were generated by stitching rendered VH&E frames using Image Composite Editor (Microsoft Research) to compare to histology slides.

3.3. Results

3.3.1 Imaging results (surface)

The surfaces of unfixed breast tissue specimens were evaluated using CFM and NLM, and compared with standard formalin-fixed, paraffin-embedded, H&E histology. The surface was defined as the plane approximately 5 µm into the specimen from the glass coverslip surface. CFM and NLM images were obtained using both the 20X and 10X objectives. Four frame averaging was conducted for the image acquisition in both CFM and NLM. For purposes of comparing
imaging modalities to H&E histology, we present wide-area mosaic images performed sequentially at each magnification, however, for actual clinical imaging, live panning imaging is preferred due to the time required to mosaic images. More details of real time imaging are discussed in chapter 2. Example images of normal breast tissue specimens are shown in Figs. 3.2 and 3.3, and images of invasive breast cancer specimens are shown in Figs. 3.4 and 3.5.

Figure 3.2 shows 20X magnification stitched images of normal breast tissue (5 mm x 5 mm) from CFM, NLM, and H&E histology. CFM, NLM, and H&E histology all show normal terminal duct lobular units (TDLUs) at low magnification (Fig. 3.2). Magnified images of a 500 µm wide area show a single normal TDLU (Fig. 3.2, blue box). Higher magnification images of a 100 µm wide area demonstrate cellular level constituents, including luminal epithelial cells and surrounding myoepithelial cells, with good concordance between the three modalities. The H&E histology images (Fig. 3.2.c, 3.3.c, 3.4.c, and 3.5.c) show that the specimen deformed and shrank by approximately 10% during formalin fixation and paraffin embedding.

Figure 3.3 shows 10X images of normal breast tissue (5 mm x 5 mm) from CFM, NLM, and H&E histology. Magnified images show the same area size (blue: 500 µm wide, black: 100 µm wide) as magnified images in Fig. 3.2. Similar to the 20X images (Fig. 3.2), normal TDLUs are seen in the 10X images of the three modalities (Fig. 3.3, black box, and blue box). Further magnified views of CFM and NLM images (Fig. 3.3. g and h) show blurred images, but still, allow identification of individual nuclei.

Figure 3.4 shows 20X images of breast tissue with invasive ductal carcinoma (IDC) (2.5 mm x 2.5 mm) from CFM, NLM, and H&E histology. CFM, NLM, and H&E histology all show cellular areas of viable tumor as well as broad zones of geographic necrosis at low magnification (Fig. 3.4, black box). Magnified images of a 500 µm x 500 µm area show a similar view of invasive
carcinoma and surrounding tumor stroma revealed by the three modalities (Fig. 3.4, blue box). The higher magnification view of the NLM image (Fig. 3.4h) demonstrates individual nuclei with malignant features, including varying size and shape and increased nuclear/cytoplasmic ratios. The higher magnification view of the CFM image (Fig. 3.4g) shows a noticeable increase of background signal because of the fluorescence from densely aggregated nuclei out of the focal plane. However, most nuclei identifiable in the NLM image are also identifiable in the CFM image.

Figure 3.5 shows 10X images of breast tissue with IDC (2.5 mm x 2.5 mm) from CFM, NLM, and H&E histology. The features identified at 20X (Fig. 3.4) are also seen in the 10X images from the three modalities (Fig. 3.5, black box). Magnified images of a 500 µm x 500 µm area show invasive carcinoma and associated tumor stroma (Fig. 3.5, blue box). A higher magnification view of the NLM image (Fig. 3.5h) shows blurring and a small increase of background signal but retains sufficient resolution to identify individual nuclei. By contrast, the high magnification view of the CFM image (Fig. 3.5g) shows a significant increase of background signal in addition to blurring and loss of differentiation of individual nuclei.
Fig. 3.2. Comparison of images of normal breast tissue between CFM (20X), NLM (20X), and H&E histology. a) CFM image of unfixed breast specimen with terminal duct lobular units (TDLUs), b) corresponding NLM image, and c) corresponding H&E histology image showing equivalent low magnification view of the specimen. d) Magnified CFM image of lobules and a terminal duct, e) a corresponding NLM image, and f) corresponding H&E histology. g) higher magnification CFM image of several lobules, h) a corresponding NLM image, and i) corresponding H&E histology all show cellular level structures, including luminal epithelial cells (LEC) and surrounding myoepithelial cells (MEC).
Fig. 3.3. Comparison of images of normal breast tissue between CFM (10X), NLM (10X), and H&E histology. a) CFM image of unfixed breast specimen showing terminal duct lobular units (TDLUs), b) corresponding NLM image, and c) corresponding H&E histology image. d) Magnified CFM image showing a single TDLU, e) a corresponding NLM image, f) magnified view of H&E histology. Higher magnification images of CFM and NLM (g, h) show blurring, but cellular structures are still identifiable. i) Further magnified view of H&E histology. The histology images are identical to those in Fig.3.2.
Fig. 3.4. Comparison of images of an invasive ductal carcinoma specimen between CFM (20X), NLM (20X), and H&E histology. a) CFM image of unfixed breast specimen with areas of viable tumor and zones of geographic necrosis, b) corresponding NLM image, and c) corresponding H&E histology image. d) Magnified CFM image of carcinoma cells and surrounding tumor stroma, e) a corresponding NLM image, and f) corresponding H&E histology image. Higher magnification images of NLM (h) show image quality comparable to H&E histology and identify individual nuclei with malignant features. Higher magnification images of CFM (g) shows higher background signal than NLM, but individual nuclei are still identifiable.
Fig. 3.5. Comparison of images of an invasive ductal carcinoma specimen between CFM (10X), NLM (10X), and H&E histology. a) CFM image of unfixed breast specimen with viable tumor and geographic necrosis, b) corresponding NLM image, and c) corresponding H&E histology image. d) Magnified CFM image of carcinoma cells and surrounding tumor stroma, e) a corresponding NLM image, and f) corresponding H&E histology image. A higher magnification image of CFM (g) shows a significant increase of background signal, with resultant difficulty in resolving cellular detail. Higher magnification images of NLM (h) show a blurrier image than 20X, but individual nuclei are identifiable. The histology images are identical to those in Fig.3.4.
3.3.2 Photobleaching assessment

Photobleaching limits the amount of energy that can be applied to any fluorescently labeled specimen as well as the number of times any individual area can be imaged, and it is of particular concern for confocal imaging because all planes within a specimen are bleached during imaging. To enable intraoperative imaging of surgical specimens, the staining protocol must enable repeatable imaging without substantial photobleaching. Photobleaching effects were assessed for both CFM and NLM using 20X objectives. 100 frames were sequentially acquired with the imaging protocol detailed in section 3.2. Figure 3.6 shows the normalized fluorescence signals from each contrast agent over 100 frames acquisition. After 100 frames of exposure, the signal magnitudes decayed by 8% for PI, 11% for EY in CFM, and 10% for PI, 7% for EY in NLM. Because the effects were small for 100 consecutive frames, we conclude that photobleaching is not a significant limitation for either modality using the staining protocol presented.

Fig. 3.6. Photobleaching assessment. The decays of fluorescent signal intensity at the same position were measured in CFM and NLM using 20X objective until 100 frames of exposure. The maximum signal decay was 11% at most after 100 frames of exposure. EY had a small, but reproducible, increase in fluorescent emission after exposure to 405 nm illumination, possibly due to photochemical reactions in fresh tissue.
### 3.3.3 Imaging results (depth)

**Fig. 3.7.** Nuclear channels (PI) of 20X CFM images of normal breast tissue at different depths ((a). surface, (b). +25 µm, (c). +50 µm, and (d) +75 µm), and corresponding 20X NLM images ((e). surface, (f). +25 µm, (g). +50 µm, and (h) +75 µm). 20X CFM images of the invasive ductal carcinoma specimen at different depths ((i). surface, (j). +25 µm, (k). +50 µm, and (l) +75 µm), and corresponding 20X NLM images ((m). surface, (n). +25 µm, (o). +50 µm, and (p) +75 µm). Degradation of signal-to-background ratio is more significant in the nuclei rich invasive specimen than in the normal specimen. CFM images show faster degradation of signal-to-background ratio with depth. (scale bar: 20 µm)
Fig. 3.8. Nuclear channels (PI) of 10X CFM images of the normal breast tissue at different depths ((a). surface, (b). +25 µm, (c). +50 µm, and (d) +75 µm), and corresponding 10X NLM images ((e). surface, (f). +25 µm, (g). +50 µm, and (h) 75 µm). 10X CFM images of the invasive ductal carcinoma specimen at different depths ((i). surface, (j). +25 µm, (k). +50 µm, and (l) +75 µm), and corresponding 10X NLM images ((m). surface, (n). +25 µm, (o). +50 µm, and (p) +75 µm). Degradation of signal-to-background ratio is more significant in the nuclei rich invasive specimen than in the normal specimen. (scale bar: 40 µm)
The degradation of signal-to-background ratio with increasing image depth in breast specimens was qualitatively evaluated for CFM and NLM using both the 20X and 10X objectives. To mitigate the effect of attenuation, PMT gains were adjusted to the optimal value for each depth. The nuclear channels are used for evaluation to see the degradation of image quality of individual nuclei clearly. Areas with uniform density of nuclei axially were selected to evaluate the effect of depth.

Figure 3.7 shows CFM and NLM images of normal breast tissue and IDC taken with the 20X objective at different depths from the surface. Comparing the images of normal and IDC, significant degradation of signal-to-background ratio is seen in the images of IDC in-depth (Fig. 3.7k, l, o and p). Comparing CFM and NLM, degradation of image quality is more evident in CFM images than NLM images (Fig. 3.7d and h, or k and o). Fig. 3.8 shows the 10X images of normal breast tissue and IDC specimens at different depths from CFM and NLM. Similar to 20X images, the degradation of the signal-to-background ratio is significant in IDC images. For both CFM and NLM, individual nuclei are hard to identify in the images at 25 µm depth in IDC images. Comparing Fig. 3.7 and 3.8, the 20X images have better image quality with increasing depth than 10X.

3.4. Discussion

In this study, we directly compare CFM, NLM, and paraffin-embedded H&E histology using unfixed human breast tissue, which is representative of what would be encountered in intraoperative histopathological evaluation during BCT. To improve clinical acceptance by pathologists trained on H&E histopathology, we introduced an imaging protocol using dual-channel detection, two contrast agents with rapid penetration into unfixed human tissue (PI and
EY), and VHE rendering of fluorescent data. Although originally used for NLM imaging, the staining protocol was highly effective for CFM when excited at 405 nm. To evaluate the suitability of CFM and NLM for breast histopathological assessment, images of unfixed breast tissue specimens were acquired using both modalities and compared with corresponding H&E histology. Photobleaching effects were measured, and the staining protocol was shown to be robust against photobleaching using both NLM and CFM. The degradation of signal-to-background ratio with depth was assessed for both CFM and NLM, and subsurface tissue imaging capability on various tissue types was demonstrated. Although higher magnification objectives were investigated, it was especially important to assess the performance of 10X magnification objectives, because a large field of view is important for rapid assessment of large tissue specimens in live panning mode.

3.4.1 Comparison between CFM and NLM for high and low magnification imaging

In typical histopathology workflows, the evaluation of large surgical specimens usually requires both low magnification imaging for assessing broad architectural features and locating diagnostically relevant regions, and higher magnification imaging for analyzing foci of pathologically relevant features identified at lower magnification. While mosaic mode imaging can be performed using a single magnification, this approach may be too slow for intraoperative applications. Therefore, both 10X and 20X magnification images, which would be required for live, non-mosaic imaging, were evaluated for each modality and both normal and cancerous tissue.

The comparison of the surface images of normal breast tissue from 20X CFM, 20X NLM, and paraffin-embedded H&E histology indicate that both CFM and NLM can provide sufficient resolution and axial sectioning to identify individual nuclei in unfixed breast tissues, analogously
to formalin-fixed paraffin-embedded histology (Fig. 3.2). Low magnification CFM and NLM imaging of normal breast tissue with 10X objectives show that 10X images were sufficient to allow identification of individual nuclei, although the images have blurring compared with 20X images because of larger lateral sampling interval (Fig. 3.3).

High magnification CFM and NLM imaging of IDC breast tissue with 20X objectives show sufficient resolution and axial sectioning to resolve individual nuclei in nuclei-dense malignant tissue (Fig. 3.4). Although individual nuclei are resolved, the 20X CFM image of IDC shows a noticeable increase of background signal compared with the 20X NLM image. Because the axial 20%-80% sectioning of both 20X CFM and 20X NLM are comparable, the difference in background signal is due to fluorescence originating significantly out of plane that is incompletely rejected. The high density of nuclei in an invasive carcinoma specimen generates a strong fluorescent signal above and below the image plane for CFM, and while the confocal pinhole rejects most out of plane light, in highly fluorescent samples, a substantial background signal is still present. This effect is particularly pronounced in Fig. 3.8 where features at 25 µm are directly visible in the underlying depths at 50 and 75 µm. In contrast, the majority of fluorophore excitation occurs only in the focal plane for NLM, resulting in a noticeably lower background signal and consequently better imaging of nuclei-dense invasive cancer.

In spite of blurring from the coarser lateral sampling and thicker axial sectioning of the 10X objective, low magnification 10X NLM imaging of IDC demonstrates that individual cellular features including individual nuclei are readily identifiable over a wider field of view (Fig. 3.5). Conversely, the 10X CFM image of IDC shows a substantial reduction in image quality, and most individual nuclei are poorly distinguished from the background (Fig. 3.5). As indicated by the 10X CFM images of normal tissue specimens, 10X CFM can clearly visualize isolated individual nuclei
in less densely packed specimens despite the large optical section thickness. Therefore, the reduction in image quality seen in the IDC image at 10X is likely due to stronger scattering in IDC tissue, the weaker axial sectioning, and incomplete rejection of intense out of plane fluorescence from aggregated nuclei. As discussed in Sec. 3.2.4, the 10X CFM system has an axial section thickness greater than the size of typical cells. Therefore, multiple axially separated nuclei may contribute concurrently to a single voxel, resulting in decreased contrast.

The reduction in signal to background ratio observed in the 10X CFM images of IDC could be reduced by using higher NA objectives. However, lower magnification objectives are frequently used to survey large areas or assess broad architectural features, for which identification of individual sub-cellular features is not required. In this case, the use of a 0.3 NA objective may be sufficient if a higher magnification objective is used to evaluate small areas of pathology. Conversely, the relatively good image quality achieved by 0.3 NA confocal imaging suggests that even lower magnification (e.g. 5X) NLM imaging may be suitable for evaluating some pathologies over very wide fields of view.

### 3.4.2 Imaging speed

The data presented in this chapter use mosaic mode images that are stitched together after acquisition to provide a comprehensive image of a specimen for comparison to histology slides. However, in clinical applications, this approach may be too inefficient to evaluate large surgical specimens as in BCT. Instead, video-rate, user-directed imaging of areas of interest, such as the tumor margins, may be preferred because this avoids imaging areas of tissue that are irrelevant to tissue evaluation, such as the tissue at the tumor center in margin evaluation, and enables evaluation in parallel with imaging. The finding that both 10X and 20X objectives enable high-
quality VHE rendering is therefore important because it enables the use of multiple magnifications during user-directed imaging, which may avoid the need to perform time-consuming mosaic imaging entirely.

In this study, spectrally separated dual-channel detectors and two contrast agents were used to improve the readability of images by generating VHE contrast using PI and EY excited by a single illumination laser. The use of spectrally separated fluorophores that can be efficiently excited by a single laser wavelength is important because it enables higher-speed imaging than multiplexing excitation wavelengths. In our NLM system, the use of resonant scanning rate enables video-rate imaging at 16 frames per second, while GPU processing enables latency-free VHE rendering. In the CFM system, the per-pixel dwell time was 50-times-longer than the NLM system because of the non-resonant galvo-galvo scanning. Imaging speed could be improved by using resonant scanning for CFM as was used for NLM, or possibly by parallel pinhole scanning, although we note that techniques such as spinning disk may incur substantial cross-talk between pinholes when imaging highly scattering specimens such as human tissue. Higher imaging speed in CFM is ultimately limited by fluorophore saturation, which occurs when illumination power is sufficiently large such that few unexcited fluorophores are available within a focal volume. However, the NLM system, which had a smaller focal volume than either CFM configurations and inherently less efficient use of pixel time due to the fixed laser pulse rate, did not encounter fluorophore saturation even at much higher illumination powers than were used in this study, indicating that the density of fluorophores in human tissue stained using the current protocol is sufficient to support extremely high imaging speeds. A further concern is that more rapid scanning might decrease the absolute time required to photobleach a specimen, however, we note that there
is negligible photobleaching with our staining protocol even after 100 sequential frames and photobleaching would scale as the number of frames, irrespective of the frame rate.

### 3.4.3 Imaging in depth

As shown in Fig. 3.7 and 3.8, the signal-to-background ratio of CFM and NLM degrades with increasing depth into specimens. NLM has a superior signal-to-background ratio with depth compared to CFM because, for CFM, scattering affects both illumination intensity and fluorescence background from outside of the imaging plane. In NLM, scattering affects illumination intensity, but the scattering of fluorescence from the two-photon absorption does not significantly contribute to the background because excitation is localized to the imaging plane. In addition, the wavelength of illumination of NLM (780 nm in this study) is approximately twice as long as that of CFM (405 nm in this study), greatly reducing the scattering coefficient of excitation light for NLM. Finally, specimen-induced spherical aberration likely degrades image quality to some extent for the high NA objectives. This effect could be reduced by using a water immersion objective for 20X CFM, however, as evidenced by the large difference in imaging depth between normal and IDC specimens, the primary limitation on imaging depth appears to be the scattering of the 405 nm illumination and out of plane fluorescence rather than the spherical aberration.

The image results at different depths indicate that tissue characteristics significantly affect the imaging performance with depth, independent of the objective and modality. As shown in Fig. 3.7 and 3.8, the degradation of image quality in IDC is much more pronounced for both CFM and NLM than in normal breast tissue because IDC is more highly scattering and has a high density of fluorescent nuclei that contribute to the background signal. Consequently, imaging depth in IDC
is more limited for both modalities as compared to normal tissue, although this limitation is more pronounced for CFM than NLM.

Finally, we note that although both NLM and CFM have limited maximum imaging depths due to the high scattering coefficients of breast tissue, it is uncommon in clinical practice to examine serial sections of breast surgical margins. Instead, because of the very large volume of tissue typically extracted during a lumpectomy, specimens are transected (‘breadloafed’) into multiple specimens on the order of several mm to 1 cm thickness to represent the surgical margins, and which are then evaluated on or near the transected surface. Therefore the limited image depths in specimens would not be a limitation in current clinical workflows.

3.5. Conclusion

We report a direct comparison between CFM and NLM with dual agent staining and rendering of VHE images using the VTM algorithm for rapid histopathological evaluation of unfixed human breast tissue. We demonstrate that CFM can image the surface of unfixed breast tissue with comparable quality to NLM. Using the dual staining protocol presented and a light absorption VTM model, both modalities can generate images to correspond closely to H&E histology. Photobleaching effects are shown to be negligible even after 100 sequential images of the same location. Wide area mosaicking of large specimens enables comprehensive comparison between CFM, NLM, and H&E. The images of the surfaces of the specimens demonstrate that 20X CFM and 20X NLM have comparable imaging performance for individual nuclei in both normal breast tissue and IDC. Both 10X CFM and 10X NLM show degraded image quality as compared with 20X due to reduced lateral resolution and axial sectioning, but still enable visualization of individual nuclei in normal breast tissue. The 10X CFM images showed a
significant increase in background signal and loss of individual nuclei identification when imaging IDC because of the reduced optical sectioning and increased contribution of out of focal plane signal, although this effect was partially attributable to the lower objective NA.

The main limitations of this comparison are the modest difference in numerical apertures between the CFM system and NLM system and the difference in immersion media. However, even at slightly lower NA, CFM demonstrated excellent correspondence with both NLM and paraffin-embedded histology, and in normal workflows using two objectives, higher NA and immersion media may be unnecessary. These results suggest that CFM may be a promising and cost-effective alternative to NLM for BCT.
Chapter 4

Assessment of microscopy with ultraviolet surface excitation;
feasibility and limitation

4.1 Introduction

Histopathological evaluation of thinly sectioned formalin-fixed, paraffin-embedded (FFPE) tissue specimens is the gold standard in many clinical scenarios including cancer diagnosis and surgical margin evaluation. Standard histological tissue processing, however, involves multiple time-consuming steps including tissue fixation, dehydration, paraffin embedding, physical sectioning, and staining, and therefore typically requires at least one day before specimens can be evaluated. Frozen section (FS) is an alternative to FFPE histology that avoids lengthy fixation and paraffin embedding by freezing fresh tissue prior to physical sectioning, but it still requires 15 to 30 minutes for preparation. While FS is widely used during Mohs surgery for nonmelanoma skin cancer to evaluate surgical margins, the procedure is relatively time-consuming and labor-intensive, especially when multiple excisions must be evaluated. Conversely, FS is less commonly used during breast lumpectomy surgery for early-stage breast cancer, in part because of freezing artifacts, long processing times, and limited areas that can be sampled. The long processing time and expense of both FFPE and FS have motivated the development of alternative technologies that enable rapid histopathological evaluation of tissue without requiring physical sectioning.
Recently, optical sectioning microscopy has attracted interest as an alternative to FS for rapid evaluation of pathology. Optical sectioning microscopy images a thin layer within an intact, thick specimen, greatly simplifying specimen preparation by forgoing physical sectioning. To date, various methods of histological imaging using optical sectioning microscopy have been proposed, including confocal fluorescence microscopy (CFM),\(^8,^{41,43}\) two-photon excitation nonlinear microscopy (NLM),\(^4,^{93,94}\) structured illumination microscopy (SIM),\(^45,95\) optical coherence tomography (OCT),\(^96\) and light sheet microscopy.\(^15\) These techniques enable rapid evaluation of pathology with high correspondence to standard histological techniques.\(^4,41\)

Unfortunately, each of these approaches has substantial limitations. NLM can image tens to hundreds of microns below the tissue surface with high resolution and strong rejection of out of focus light, but it requires costly femtosecond lasers and scanning optics. CFM and light sheet microscopy avoid the use of femtosecond lasers, but still require a relatively expensive and complex optical instrument for physically rejecting out of focus light. SIM requires only inexpensive light emitting diodes (LEDs) and spatial light modulators, but rejects out-of-focus light computationally rather than physically, resulting in a tradeoff between available detector dynamic range and optical sectioning thickness.\(^97,98\) OCT provides high imaging rates and deep penetration into tissue, but is incompatible with fluorescent stains and has limited ability to visualize cell nuclei which are important diagnostic features.

An alternative approach to optical sectioning, called microscopy with UV surface excitation (MUSE), was proposed by Levenson and Demos.\(^99\) In this approach, optical sectioning is achieved by the strong absorption of deep ultraviolet (DUV) light around 280 nm in wavelength by proteins which limits fluorescent excitation primarily to the tissue surface. The optical sectioning thickness depends on the illumination angle, wavelength, and tissue absorption
coefficient, enabling simple imaging system schemes such as ring illuminators and conventional optical microscopy without requiring DUV transmissive microscope optics, beam scanning, spatial light modulators, or complex postprocessing. Furthermore, MUSE benefits from the recent commercialization of AlGaN LEDs, making inexpensive DUV light sources available at wavelengths as short as 200 nm. As a result, the cost and complexity of MUSE are very low compared to alternative imaging techniques.

To date, studies of MUSE imaging have been limited, and to our knowledge, the optical sectioning and contrast of MUSE relative to other modalities have not been explored. We have developed a video-rate MUSE system and performed a preliminary evaluation of surgical specimens for two potential applications where surgical margins are assessed intraoperatively: Mohs surgery for basal cell carcinoma of skin and lumpectomy for breast cancer. We report a water immersion DUV illuminator using surface mount LEDs and DUV illumination guiding optics for high incident angle illumination. Increasing the incident angle of the illumination improves MUSE optical sectioning and contrast. We also present a staining protocol that enables rapid imaging of fresh, unfixed human tissue while minimizing the contribution of tissue autofluorescence by using a red fluorescent nuclear stain. Virtual H&E color rendering with the virtual transillumination microscopy (VTM) algorithm is used to generate images similar to standard H&E histology from two-channel fluorescence detection\textsuperscript{48}. MUSE images of human skin and breast tissue are compared with NLM images and corresponding H&E stained FFPE and frozen section histology by experienced pathologists and a Mohs surgeon. The optical sectioning thickness of MUSE is quantitatively measured by using co-registered NLM volumetric images.

The work in this chapter was first published in Scientific Report\textsuperscript{18}. This work was done in collaboration with Michael G. Giacomelli, Lucas C. Cahill, and James G. Fujimoto at
Massachusetts Institute of Technology, and Hilde Vardeh, Beverly E. Faulkner-Jones, James L. Connolly, and Daihung Do at Beth Israel Deaconess Medical Center and Harvard Medical School. This work was supported by the National Institutes of Health (CA178636, CA075289, and CA183400) and Air Force Office of Scientific Research (FA9550-12-1- 0551 and FA9550-15-1-0473).

4.2 Results

4.2.1 Theory, design, and protocol for immersion MUSE

MUSE achieves optical sectioning by using DUV (around 280 nm wavelength) excitation, which is strongly absorbed by proteins in human tissue, limiting fluorescence emission to the specimen surface. Although the absorption coefficient is an intrinsic tissue property, the illumination optical mean free path length can be increased relative to the absorption depth by illuminating at a high angle, as proposed by Levenson and Demos. However, the improvement in optical sectioning thickness from high angle illumination is limited by refraction at the tissue interface (Fig. 4.1a). Because human tissue has a refractive index (n) similar to or greater than water (tissue: typical n=1.4, water: n=1.353 at 280 nm)\textsuperscript{100,101}, using water immersion (or an even higher index media) will enable a higher angle of illumination relative to the surface normal (Fig. 4.1a). Table 4.1 summarizes the angle of refraction and relative axial penetration depth of DUV light for a 70-degree illumination angle. At this angle, water immersion MUSE will achieve a ~50% reduction in optical sectioning thickness for typical human tissue compared with air immersion MUSE. We integrated dual camera fluorescence imaging into the microscope eyepiece port of a commercial NLM system (Fig. 4.1b) and mounted 3D printed DUV illuminators on the
objective (Fig. 4.1c), enabling water immersion MUSE imaging at video-rate (up to 8 fps) co-registered to NLM.

We developed a 2-minute specimen staining protocol using the red fluorescent nuclear stain propidium iodide (PI) and the standard histology counterstain eosin yellow (EY) dissolved in 70% ethanol or water. While PI staining of freshly excised tissue in water has been demonstrated, some pathologies such as dense ducts in breast tissue specimens show limited uptake of PI. Using 70% ethanol as a solvent improves PI uptake in freshly excised tissue (Fig. 4.8) and leads to more consistent nuclear staining. The microscope, imaging setup, and staining protocol are described in detail under Methods.

Fig. 4.1. Principle of MUSE optical sectioning and MUSE imaging system. (a) Penetration depth of light into tissue decreases with wavelength and with increasing illumination angle. Higher index immersion media enables high incident angle illumination, reducing the optical sectioning thickness. (b) Drawing of the co-registered MUSE/NLM imaging system. MUSE imaging unit was connected to the eyepiece port of a commercial NLM system to compare MUSE images with corresponding high resolution NLM. Dual channel imaging using fluorescence filters is used for both MUSE and NLM, while a precision linear motor translation stage enables mosaic imaging. Spectrally separated fluorescence is detected by CMOS cameras (MUSE) or PMTs (NLM). DUV illuminator is mounted on the microscope objective. (c) Photo of the illuminator with three DUV sapphire lightguides for MUSE and three visible LEDs for alignment. All figures in this chapter 4 were adapted from "Rapid histopathological imaging of skin and breast cancer..."

Table 4.1. Relation of illumination angle and penetration depth of DUV light into human tissue (Tissue n=1.4). The range of geometrically possible illumination angles (viewing angle to the edge of illuminator) for the system used in this chapter is shown along with the central angle.

<table>
<thead>
<tr>
<th></th>
<th>0. normal</th>
<th>1. Air immersion</th>
<th>2. Water immersion</th>
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<tbody>
<tr>
<td>Angle of incidence: θ₀ (degree)</td>
<td>0</td>
<td>70 (58 &lt; θ₀ &lt; 83)</td>
<td>70 (58 &lt; θ₀ &lt; 83)</td>
</tr>
<tr>
<td>Angle of refraction: θᵢ´ (degree)</td>
<td>0</td>
<td>42 (37 &lt; θᵢ´ &lt; 45)</td>
<td>63 (55 &lt; θᵢ´ &lt; 74)</td>
</tr>
<tr>
<td>Normalized axial penetration depth: z=cos θᵢ´</td>
<td>1</td>
<td><strong>0.74</strong> (0.71 &lt; z &lt; 0.80)</td>
<td><strong>0.45</strong> (0.28 &lt; z &lt; 0.57)</td>
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4.2.2 MUSE imaging of Mohs surgical specimens

An experienced pathologist and Mohs surgeon performed an unblinded comparison of MUSE, NLM, and FFPE H&E images of fresh, (not frozen) skin tissue discarded from Mohs surgical procedures. To compare with conventional H&E histology, virtual H&E color rendering was applied to MUSE and NLM images.

Figure 4.2 shows a comparison of air and water immersion MUSE, NLM, and conventional FFPE H&E histology of normal skin excised from the scalp of a patient undergoing Mohs surgery. PI/EY in 70% ethanol was used for MUSE and NLM fluorescent staining. The low magnification images (~60 mm²) show normal skin features such as epidermis, hair, follicles, sebaceous glands, and eccrine glands (Fig. 4.2a-d). Magnified views (Fig. 4.2e-h) show a clearly resolved epidermis layer for all imaging modalities, while the stratum corneum is not well visualized in both air and water immersion MUSE. At higher magnification (Fig. 4.2i-l), hair follicles, sheathes, and sebaceous glands can be seen alongside individual hairs (white line) in all modalities. Although overall correspondence of MUSE and FFPE H&E is good, the thicker optical sectioning of MUSE
compared to FFPE H&E and NLM results in some glands appearing more cellular due to the multiple cell layers at different depths visualized in MUSE images. In contrast, NLM and FFPE H&E enable differentiation between cell layers at different depths in the specimen, thereby resolving the glands without the same artifacts.

To enable a direct cellular level comparison between the fluorescent imaging modalities and diagnostic FS histology slides used during Mohs surgery, thick tissue sections (~50 µm) were taken from frozen Mohs blocks immediately above the diagnostic cutting plane. Because the thick sections are cut while frozen, this protocol enables co-registration of the FS and MUSE / NLM fluorescent imaging planes with minimal distortion. Mosaicked images were evaluated by a pathologist and Mohs surgeon. Figure 4.3 shows a qualitative comparison of air and water immersion MUSE, NLM, and FS H&E histology of a specimen from a patient undergoing Mohs surgery for basal cell carcinoma (BCC) located on the neck. PI/EY in water was used for MUSE and NLM fluorescent staining. The low magnification images (~50 mm²) show multiple large basaloid tumor nests in the papillary and reticular dermis typical for superficial and nodular BCC (Fig. 4.3a-e). Magnified views (Fig. 4.3f-m) show basaloid tumor nests with peripheral palisading and slit-like retraction. Although water immersion MUSE achieves noticeably thinner optical sections than air immersion MUSE, features of BCC are readily identified in both cases.
Fig. 4.2. Imaging of fresh (not-frozen), normal human skin discarded during a Mohs surgical procedure. (a) Air immersion MUSE, (b) water immersion MUSE, (c) NLM and (d) FFPE H&E. The epidermis and multiple hair follicles can be seen using each modality. Magnified images of the epidermis layer (e: air immersion MUSE, f: water immersion MUSE, g: NLM, h: FFPE H&E) demonstrate that both air and water immersion MUSE reproduce the appearance of the normal epidermis layer, while the thinner optical sectioning of water immersion MUSE enables visualization of finer details than air immersion. Magnified images of a hair follicle and glands (i: air immersion MUSE, j: water immersion MUSE, k: NLM, l: FFPE H&E) demonstrate that both air and water immersion MUSE enable visualization of structures of the hair follicle (hair, sheath, and glands), although some glandular structures are less clear in MUSE images because of the stacked appearance of surface and subsurface cell layers. Scale bar 1 mm (a-d), 100 µm (e-h), 200 µm (i-l). Exposure time of MUSE: 0.3 s (water), 0.7 s (air).
**Fig. 4.3.** Comparison of MUSE and NLM to FS using a 50 µm thick tissue section from the surgical margin of a discarded Mohs surgical frozen specimen. (a) Entire specimen imaged with water immersion MUSE. Nests of basal cell carcinoma (red box in (a)) visualized with (b) air immersion MUSE, (c) water immersion MUSE, (d) NLM, and (e) FS H&E. Clusters of tumor cells, reduced stroma, and normal skin structure can be visualized using each modality. Magnified images of large basaloid tumor nests with peripheral palisading (f, j: air immersion MUSE, g, k: water immersion MUSE, h, l: NLM, i, m: FS H&E) suggest that both air and water immersion MUSE can reproduce the features of pathologies required for diagnosing BCC, while the thinner optical sectioning of water immersion MUSE enables visualization of detailed features better than air immersion MUSE. Scale bar 1 mm (a-e), 100 µm (e-m). Exposure time of MUSE: 0.3 s (water), 0.7 s (air).

**4.2.3 MUSE imaging of breast specimens**

Three experienced pathologists performed an unblinded qualitative comparison of air and water immersion MUSE, NLM, and FFPE H&E histology of fresh, unfixed normal human breast tissue imaged shortly after mastectomy (Fig. 4.4). PI/EY in 70% ethanol was used for MUSE and NLM fluorescent staining. The low magnification images of normal breast tissue (~25 mm²) show terminal ductal lobular units (TDLUs), normal adipocytes, and stroma (Fig. 4.4a-d). Magnified images of TDLUs, including the acini and intra-lobular terminal ducts, show high correspondence between NLM and FFPE H&E (Fig. 4.4g,h); however, visualization of the lobular structure is limited in MUSE images (Fig. 4.4e,f). Magnified images of another lobule clearly show the lobular structure on NLM and FFPE H&E (Fig. 4.4k,l), but MUSE images (Fig. 4.4i,j) do not show detailed acinar structures, although the boundary of the lobule is apparent. Water immersion MUSE images show fewer artifacts than air immersion MUSE, but the optical sectioning is still insufficient and image evaluation is difficult.
**Fig. 4.4.** Imaging of normal breast specimen stained with PI/EY dissolved in 70% ethanol. Low magnification views of (a) air immersion MUSE, (b) water immersion MUSE, (c) NLM, and (d) FFPE H&E showing TDLUs and connecting ducts. Magnified images of acini and intralobular terminal ducts with (e) air immersion MUSE, (f) water immersion MUSE, (g) NLM and (h) FFPE H&E, and another lobule with (i) air immersion MUSE, (j) water immersion MUSE, (k) NLM and (l) FFPE H&E. The thicker optical sectioning in MUSE limits accurate visualization of lobular structures. Scale bar: 1 mm (a-d), 100 µm (e-h), 50 µm (i-l). Exposure times of MUSE: 0.125 s (water), 0.300 s (air).

### 4.2.4 Surface contamination in optical sectioning microscopy

MUSE has potential limitations for intraoperative applications because imaging is limited to the tissue surface. Cauterization or cellular debris might contaminate the tissue surface during surgery or grossing and can interfere with evaluation. Conventional FFPE histology and FS do not have surface contamination because the specimen surface is removed by trimming and facing the embedded tissue block during microtoming. Figure 4.5 shows mosaicked images with air and water immersion MUSE, NLM, and FFPE H&E histology of a breast specimen with high-grade invasive ductal carcinoma (IDC) (~20 mm²). PI/EY in water was used for MUSE and NLM fluorescent staining. Loose tumor cells and other debris from grossing are present in the MUSE and NLM images of the tissue surface (Fig. 4.5a-c) and interfere with image evaluation. Magnified images of the tissue surface with both MUSE and NLM (Fig. 4.5e-g) show contamination by aggregated cells that obscure the underlying tissue and may be mistaken for invading tumor cells. Subsurface imaging with NLM (Fig. 4.5h, 10 µm below the surface) demonstrates that the aggregated cells are not present in the underlying tissue. This is confirmed by FFPE H&E histology which has a histological section plane deeper in the tissue, below the NLM image planes (Fig. 4.5d, i).
Fig. 4.5. Imaging of a breast specimen with high-grade invasive ductal carcinoma stained with PI/EY dissolved in water. (a) Air immersion MUSE, (b) water immersion MUSE, (c) NLM and (d) FFPE H&E images show a high density of cells from high-grade invasive ductal carcinoma and surface contamination. Magnified views of (e) air immersion MUSE, (f) water immersion MUSE, (g) NLM at the specimen surface shows aggregated cells displaced from original sites which can be difficult to distinguish from invading cancer cell. A magnified view of (h) NLM at 10 μm below the surface shows the tissue structure without surface contamination artifacts. (i) Magnified view of FFPE H&E shows invasive cancer without surface contamination artifacts. Surface contamination is not present in conventional histology because it is removed during microtoming. Scale bar: 1 mm (a-d), 100 μm (e-i).

4.2.5 Relationship between MUSE optical sectioning and image resolution

Unlike NLM or CFM, MUSE optical sectioning is independent of the imaging numerical aperture (NA). Therefore the NA and magnification can be changed while maintaining a constant
optical sectioning thickness. However, the maximum NA and transverse image resolution is limited because the optical sectioning thickness should not exceed the depth of field. To demonstrate the effect of NA and depth of field on image resolution, water immersion MUSE images of fresh, unfixed normal skin were acquired with a 20x, 1.0 NA objective, a 10x, 0.3 NA objective, and compared with NLM using a 20x, 1.0 NA objective (Fig. 4.6). The 1.0 NA objective has an order of magnitude less depth of field (1.5 µm) than the optical sectioning thickness of water immersion MUSE (10 µm, see the following section), while the 0.3 NA objective has a depth of field (10 µm) comparable to the MUSE optical sectioning thickness. NLM images at (Fig. 4.6c, g) and 10 µm below the surface (Fig. 4.6d, h) show that both MUSE images (10x, 0.3 NA MUSE: Fig. 4.6a, e, 20x, 1.0 NA MUSE: Fig. 4.6b, f) enable visualization of nuclei at (black arrow) and below the tissue surface (yellow arrow). However, the higher, 1.0 NA MUSE images exhibit decreased transverse resolution because fluorescence from depths that are out of focus contributes to the image and causes blurring. In contrast, the 0.3 NA MUSE image has a longer depth of field, maintaining focus over most of the depth range of the fluorescence. Using higher index immersion media should extend the usable NA for MUSE because it will increase the depth of field while further decreasing optical sectioning thickness.
Fig. 4.6. Relationship between MUSE optical sectioning thickness and transverse image resolution / NA of the objective. Comparison of (a, e) 10x, 0.3 NA water immersion and (b, f) 20x, 1.0 NA water immersion MUSE with (c, g) NLM image of the tissue surface, and (d, h) 10 µm below the surface. MUSE at 20x, 1.0 NA shows poorer transverse resolution compared with 10x, 0.3 NA because the MUSE optical sectioning thickness is an order of magnitude larger than the depth of field of the 20x, 1.0 NA objective. Fluorescence from depths that are out of focus blurs the image. Black arrows indicate nuclei on the tissue surface and yellow arrows indicate nuclei 10 µm below the surface. Scale bar 50 µm.

4.2.6 Quantitative assessment of optical sectioning thickness in MUSE

Measuring the optical sectioning thickness in MUSE is challenging because the absorption depth of the DUV illumination depends on the tissue properties and can also have focal variation. Furthermore, in contrast to NLM or CFM, acquiring images at multiple depths (z-stacks) in MUSE only changes the focal plane depth without changing the optical sectioning from DUV absorption, precluding the use of traditional microscopy characterization methods. To determine the optical sectioning thickness of MUSE in human tissue, we developed a method that images individual cell nuclei sparsely located at different depths. In this method, the depths of nuclei are measured precisely using volumetric NLM images acquired at 1.0 NA, and then the same tissue specimens are imaged using MUSE. By correlating the signal intensity measured with MUSE with the depth information obtained from volumetric NLM, the reduction in MUSE signal with depth below the tissue surface can be computed. Furthermore, by measuring an ensemble of nuclei, random variations in local tissue absorption or nuclei brightness can be averaged to determine an effective overall measure of MUSE optical sectioning.

This method was applied to fresh, unfixed benign human breast tissue (Fig. 4.7a). Volumetric NLM images were co-registered with air and water immersion MUSE images from the specimen surface. The NLM and MUSE images were divided into ~260,000 areas (4 µm²), of
which ~77,000 areas containing isolated nuclei were used for analysis (Fig. 4.7b, white area). Areas without nuclei or with nuclei at multiple depths were excluded. Each area was then sorted by depth as measured by NLM, and the MUSE intensity for all areas at each depth was grouped for analysis. Figure 4.7c shows a bar plot of the median and range (25-75 percentile) of signal intensities from nuclei for air and water immersion MUSE as a function of depth. The fits were calculated by modeling the nuclei fluorescence as a combination of a DUV excitation term following Beer’s law and a constant background fluorescence term; \( (1-a) \exp(-d/d_o) + a \) \((a: \text{constant background fluorescence, } d: \text{depth of nuclei, } d_o: e^{-1} \text{ depth})\). The average calculated \( e^{-1} \) depths were 10 µm and 20 µm for water and air immersion MUSE respectively. Although there was considerable variation in fluorescence intensity and attenuation with position, as evident by the large 25-75 percentile range, water immersion improved MUSE optical sectioning at all depths below the surface. The calculated ratio of the DUV excitation term to the constant background term was 1.22 for water immersion MUSE and 0.56 for air immersion MUSE.
Fig. 4.7. Quantitative assessment of optical sectioning thickness in MUSE. (a) Volumetric NLM images of fresh, unfixed benign breast tissue (~1 mm²) were used to measure the depth of nuclei observed in MUSE. Tissue was stained with PI/EY in water, but only the PI channel signal was used for this analysis. Isolated nuclei were segmented ((b), white areas. Total 0.3 mm²) and the signal intensity of nuclei in co-registered water immersion and air immersion MUSE images were calculated as a function of depth measured by NLM. (c) Box plot of the signal intensities for each depth shows that water immersion MUSE suppresses signals from nuclei deep inside tissue substantially more than air immersion MUSE. Boxes show 25-75% range, and solid lines show the median. Fitting curves indicate that the $e^{-1}$ depths are 10 µm (water immersion MUSE) and 20 µm (air immersion MUSE).

4.3 Discussion

MUSE is a promising method for rapid histological evaluation of surgical specimens at a lower cost than other optical sectioning microscopy technologies such as NLM or CFM. We designed and characterized air and water immersion MUSE systems using high incident angle illumination delivered with illumination guiding optics. We developed a tissue staining protocol using PI as a fluorescent nuclear stain and EY as a counterstain. In contrast to other nuclear stains such as acridine orange (AO) which have been used to stain skin and breast tissue⁴,⁴¹, PI is particularly advantageous for MUSE because the red fluorescence emission is at a longer wavelength than the dominant tissue autofluorescence. Images were acquired using low-cost machine vision cameras and an efficient light collection strategy. We demonstrated that MUSE can generate virtual H&E images of fresh, unfixed human tissue at video-rate using a rapid sample preparation protocol.

We performed a preliminary investigation to assess the feasibility of MUSE for surgical pathology of skin and breast cancers. Based on unblinded, qualitative assessment of MUSE, NLM, and FFPE or FS H&E histology by an experienced pathologist and Mohs surgeon, MUSE images reproduced many of the diagnostic features of human skin specimens seen on FFPE and FS H&E.
Features of BCC pathology such as large nests of basaloid tumor cells, clefts, and the atypical appearance of palisading nuclei were apparent on MUSE images, as were features of normal skin such as the epidermis and glands. The high correspondence of skin images between MUSE and conventional H&E histology suggests that MUSE may be a promising method for evaluating skin pathology more rapidly than FS, potentially reducing processing time from 15-30 minutes to a few minutes.

In contrast, we had difficulty interpreting MUSE images of normal breast tissue when compared to FFPE H&E or NLM. The thicker optical sectioning of both air and water immersion MUSE leads to the appearance of acini (∼50 µm diameter) as relatively homogenous structures, instead of segmented glands with open lumens that are seen on FFPE H&E and NLM (Fig. 4.4). Furthermore, MUSE images of breast tissue had low contrast in the EY channel that interpreted some dense stromal features challenging. Improvements in MUSE resolution and contrast may be required for breast pathology applications.

Artifacts from surface contamination may pose limitations for intraoperative applications using MUSE. Unfixed breast tissue specimens exhibited surface contamination from grossing and handling procedures, such as displacement of cells from other areas, smeared or transferred tissue marking inks, and other debris, creating a possible false appearance of pathology and obscuring underlying tissue features. These contamination artifacts can be reduced with careful handling, but may still be present to some degree. Surface contamination can be avoided in NLM by imaging and evaluating 10~20 µm or more below the tissue surface. Controlling surface contamination appears to be much easier for smaller Mohs specimens than for larger breast tumors, for scalpel rather than for electrocautery excisions, and solid rather than for friable specimens.
As expected, high angle illumination with water immersion MUSE improves optical sectioning thickness by a factor of two, while reducing background fluorescence. This was qualitatively assessed using mosaic images and quantitatively confirmed using co-registered volumetric NLM to measure the depth of cell nuclei contributing to MUSE images. Quantitative measurements with fresh, unfixed benign breast tissue indicate that water immersion MUSE obtains a 10 µm e\(^{-1}\) optical sectioning thickness while air immersion MUSE obtains a 20 µm e\(^{-1}\) thickness, although this does not include the contribution from a substantially uniform background fluorescent signal and absolute thicknesses vary within individual specimens. This difference in optical sectioning thickness is consistent with the predicted improvement based on simple geometric arguments (Table. 4.1). Unfortunately, we could not quantitatively measure MUSE optical sectioning thickness in skin specimens due to the high density of nuclei and non-specific staining of PI, but subjectively the optical sectioning thicknesses appeared similar in skin and breast.

The presence of a diffuse, uniform background fluorescent signal that is independent of tissue surface features (Fig. 4.7c) suggests that an additional mechanism excites fluorescence below the surface. Scattering may contribute to the background fluorescence because the scattering mean free path at 280 nm wavelength in many human tissues is estimated to be 30~60 µm\(^{102,103}\), which is only modestly larger than the absorption mean free path of 20~25 µm, calculated from the measured optical sectioning thickness and illumination angle. Another possible mechanism is secondary excitation of exogenous fluorophores (e.g. EY/PI) by tissue near-UV autofluorescence. The optical sectioning in superficial tissue layers is presumed to be governed by the strong attenuation at 280 nm wavelength by amino acids, primarily tyrosine and tryptophan. However, both tyrosine and tryptophan are also fluorescent with quantum yields of 0.12 and 0.13 and
emission maxima of 300 nm and 350 nm respectively. The relatively large stokes shift of the tryptophan emission may cause secondary excitation of deep underlying tissue layers. Likewise, secondary excitation by EY fluorescence may also contribute to a background signal in the PI channel. Finally, we note that primary tissue autofluorescence does not directly contribute to the background because the choice of red-shifted fluorophores and emission filters reduced the autofluorescence of unstained tissue to negligible levels. Further measurements and modeling are required to understand the extent to which scattering and secondary near-UV fluorescence influences optical sectioning and contrast in MUSE, however, the use of fluorophores such as PI that have relatively low absorption in the near-UV may improve performance compared with near-UV excitable agents such as DAPI or Hoechst 33342.

The ultimate resolution and contrast limits of MUSE remain an open question. While MUSE can be performed at low to moderate NA, the requirement that the optical sectioning thickness approximately matches the depth of field means that higher NA imaging will not improve transverse image resolution unless the optical sectioning thickness can be reduced. In fact, our results demonstrate that 0.3 NA is well-matched to the optical sectioning thickness of the water immersion MUSE system, while 1.0 NA has degraded resolution because the optical sectioning thickness is larger than the depth of focus. Although we have shown that water immersion can markedly improve both optical sectioning and contrast, it is likely that further improvements are possible using even higher index immersion, narrower ranges of illumination angles (e.g. from spatially coherent light sources), and staining protocols that minimize contributions from Stokes-shifted secondary autofluorescent emission.

Further experimental and modeling studies are required to understand how MUSE imaging hardware and staining protocols can be optimized. The preliminary findings in this study suggest
that current air and water immersion MUSE may not be amenable for intraoperative breast cancer applications because of limitations in optical sectioning thickness and the ability to image below surface contamination. At the same time, findings suggest that MUSE may be promising for BCC in Mohs surgery because this application exhibited much greater correspondence between MUSE and H&E histology, and because specimens exhibited less surface contamination. Larger scale studies of skin pathology comparing the blinded reading of MUSE to conventional H&E histology are warranted to assess sensitivity and specificity.

4.4 **Methods**

4.4.1 **MUSE illuminator**

Efficient illumination of the tissue surface with DUV LEDs is challenging because LED modules typically do not fit into the short working distance of an immersion objective. To effectively couple DUV light to the field under the microscope objective, we developed a novel illuminator using surface mount high power 280±5 nm wavelength LEDs (Nikkiso, VPS161), sapphire rods (3 mm dia.), and quartz and sapphire hemisphere lenses (3 mm and 2.5 mm dia. respectively). The high refractive index of sapphire produces a total internal reflection of the wide-angle DUV light emission from the surface mount LED (130-degree viewing angle), efficiently guiding light to the region of interest. The use of inexpensive sapphire rods, quartz, and sapphire hemisphere lenses reduces system cost while minimizing the amount of immersion fluid required, enabling surface tension to draw immersion media onto the illuminator and under the objective.

The power from a single LED was 25 mW measured at the LED surface and 6 mW from the lightguide, a 24% efficiency. Losses include reflection loss between air, rod, and lenses (~40 %) and incomplete total internal reflection, mainly between the sapphire rods and diodes, due to
the very large divergence angle of the diodes. Reflection loss could be reduced by index matching interfaces and incomplete total internal reflection could be reduced with additional coupling optics. Three DUV LEDs and lightguide illuminators spaced 120 degrees apart to avoid shadowing were assembled on a 3D printed holder. Using water immersion, the illumination was quasi-collimated with a beam width of ~4 mm, and the total illumination area was ~12 mm². Using air immersion, the refraction at the hemispherical output lens focused the illumination before the coverslip which then diverged to an illumination area of 50 mm² at the coverslip.

4.4.2 Imaging system

The MUSE imaging system was incorporated into an existing commercial NLM system (Thorlabs Inc.) using the eyepiece port. The fluorophores PI and EY were selected as nuclear and counterstain because of their relatively long wavelength emission, which minimizes the contribution of the short wavelength autofluorescence. Fluorescent light from the tissue was separated by a dichroic beam splitter (cutoff of 590 nm) into two spectral channels and additional band-pass filters from 520 to 560 nm and from 620 to 680 nm were used for EY and PI detection, respectively. Images were acquired with two low-cost monochrome CMOS cameras (Point Grey BFLY-U3-23S6M: 41 fps at 1920x1200 resolution and 12 bits per pixel) in the MUSE system or by two photomultiplier tubes (PMTs) (H7422; Hamamatsu, Inc.) in the NLM system. Two 10x, 0.3 NA objectives (air immersion: Olympus MPLFLN10X, water immersion: Zeiss Achromplan 10x/0.3 W) were used to compare air and water immersion MUSE at equal NA. A 20x, 1.0 NA water immersion objective (Olympus XLUMPFL20XW) was also used for NLM as well as to investigate high NA MUSE imaging (Fig. 4.6). The depths of field of the objectives were calculated to be 7.5 µm (10x air), 10 µm (10x water), and 1.5 µm (20x water), respectively. The
fields of view of the MUSE system were 0.68 x 0.47 mm (10x Air), 0.62 x 0.43 mm (10x Water), 0.35 x 0.24 mm (20x Water), with an effective 1680x1150 pixel image after digital alignment of the two spectral channels. MUSE image acquisition was performed with 125 to 300 ms exposure time (up to 8 frames per second equivalent) for water immersion MUSE and 500 ms to 1 s exposure time for air immersion MUSE using unity detector gain (1 count per photoelectron). The long exposure time for air immersion MUSE was due to the wider illumination area produced when the water immersion illuminator was used in air. An air-only illuminator could be designed for much higher efficiency. The imaging speeds could be increased for preview or live imaging by increasing the detector gain at the expense of a minor decrease in dynamic range, which far exceeds the signal to background ratios observed in MUSE images. A ~150-fs tunable Ti:sapphire femtosecond laser (Mira Optima 900-F; Coherent) with a 76-MHz repetition rate operating at ~780 nm wavelength was used for NLM imaging. A detachable primary dichroic mirror was used to direct specimen fluorescence to the MUSE cameras and the NLM PMTs. For NLM, the femtosecond laser was scanned using a nonresonant galvanometer scanner (slow axis) and an 8 kHz (16 kHz bidirectional) resonant galvanometer scanner (fast axis). The NLM operated at 16 fps with <30 mW average power incident on the specimen.

### 4.4.3 Specimen preparation

Freshly excised skin tissue, thick frozen sections (~50 µm) of skin tissue stored in alcohol, digital images of frozen section slides, and freshly excised breast tissue were acquired under protocols approved by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects (COUHES) and the Beth Israel Deaconess Medical Center (BIDMC) Committee on Clinical Investigations (CCI). Only discarded and deidentified human
tissue specimens that were no longer required for clinical diagnosis were used, and the protocol was therefore exempt from informed consent. The discarded, freshly excised skin and breast tissue specimens were maintained in chilled Roswell Park Memorial Institute (RPMI) medium. All freshly excised tissues were imaged within 12 hours of excision. The fresh skin and breast specimens were transected to expose representative regions (3 to 5 mm width) of stroma or pathology immediately prior to staining and imaging to minimize surface contamination. The freshly transected skin and breast tissue specimens were stained for 2 minutes in a solution of 40 μg/ml PI and 200 μg/ml EY dissolved in 70% ethanol or in water as noted in each experiment. We observed that using 70% ethanol improves uptake of PI into fresh tissue, especially in dense ducts and lobule of breast as compared to using water (Fig. 4.8). Therefore PI/EY in 70% ethanol staining protocol was used for the qualitative comparison of images from fresh, unfixed tissue (Fig. 4.2, 4.4). The stained fresh, unfixed specimens were rinsed in buffered saline and placed on a 200 µm thick quartz glass coverslip attached to a specimen holder. The specimen holder was filled with saline-soaked biopsy foam pads so that the specimen surface maintained contact with the glass coverslip without dehydration. This method enables repeated imaging of the same specimen for several hours without noticeable alteration of cellular structures or fluorescent signals. Thick tissue sections taken from frozen Mohs blocks were maintained in alcohol and stained for 2 minutes in a solution of 40 μg/ml PI and 200 μg/ml EY dissolved in water. The stained thick tissue sections were then rinsed in buffered saline and covered by a 200 µm thick quartz glass coverslip. Specimens were rehydrated in water during staining/rinsing because alcohol rapidly evaporated during imaging.
4.4.4 Imaging procedure

Large tissue areas (~50 mm²) were imaged consecutively with MUSE and NLM using a high-precision linear motor stage (MLS-203, Thorlabs, Inc.). NLM images at multiple depths (z-stacks from surface to ~50 µm depth) were also acquired to quantitatively assess the optical sectioning thickness of MUSE. After MUSE and NLM imaging, the skin and breast tissues were fixed in 10% formalin for more than two days and processed for conventional FFPE H&E histology. Since the digitally scanned images of actual diagnostic frozen sections used to guide surgery were available for Mohs specimens, these images were used for comparison of the skin cancer specimens. A team of experienced pathologists and a Mohs surgeon evaluated the mosaicked images of each modality at full resolution. No image processing was performed for the images used in the quantitative analysis (Fig. 4.7), except for subtraction of the dark current of the CMOS cameras (MUSE) or PMTs (NLM). For all other imaging results (Fig. 4.2 - 4.6), the minimum value of each MUSE frame was also subtracted to suppress the background (Fig. 4.9). Histogram brightness normalization was used to equalize the brightness of each fluorescent channel such that 0.01% of pixels were overexposed and lens vignette correction was used to facilitate seamless mosaicking. Virtual H&E rendering was performed using a virtual transmission microscopy (VTM) algorithm⁴⁸. VTM is a physically realistic light absorption model that enables high-quality rendering to resemble absorptive stains such as H&E by using fluorescent data and simulating the transmission of white light through virtual slides. Wide area mosaics were generated by stitching rendered virtual H&E frames using Image Composite Editor (Microsoft Research), enabling comparisons to conventional H&E histology slides.
Fig. 4.8. AO/PI cell staining. Fresh, unfixed breast tissue stained with (a) AO/PI in water solution, (b) AO channel, and (c) PI channel imaged using NLM at 20 µm below the tissue surface. Many cells that are visible under AO staining are not stained with PI. Fresh, unfixed breast tissue stained with (d) AO/PI in 70% ethanol solution, (e) AO channel, (f) PI channel imaged using NLM at 20 µm below the surface. The ethanol solvent improves the uptake of PI and nearly all cells are now visible under both AO and PI. Fresh, unfixed skin tissue stained with (g) AO/PI in water solution, (h) AO channel, and (i) PI channel imaged using NLM at 20 µm below the tissue surface. Similar to the breast specimen, there are cells that are visible
under AO staining which are incompletely stained by PI, although correspondence is higher. Fresh, unfixed skin tissue stained with (j) AO/PI in 70% ethanol solution, (k) AO channel, (l) PI channel imaged using NLM at 20 µm from the surface. The uptake of PI in skin is improved by the ethanol solvent. Scale bar, 100 µm.

**Fig. 4.9.** Comparison of MUSE virtual H&E images with and without background subtraction. (Fresh, unfixed normal skin) MUSE images of normal human skin specimen acquired with air immersion MUSE; (a) raw image (RG), (b) uniform background-subtracted image (RG), (c) raw image rendered as virtual H&E, and (d) background subtraction & virtual H&E rendered image. Images acquired with water immersion MUSE; (e) raw image (RG), (f) background-subtracted image (RG), (g) raw image & virtual H&E rendering, and (h) background subtraction & virtual H&E rendered image. Background subtraction has a pronounced effect on air immersion MUSE images due to the large background signal. Scale bar, 100 µm.
Chapter 5

Randomized controlled clinical trial for intraoperative surgical guidance with NLM evaluation

5.1 Intraoperative surgical guidance, clinical trial protocol

Breast conserving therapy (BCT) is the standard of care for early breast cancer.\textsuperscript{105} a Current standard practice for assessing surgical margins is postoperative H&E histology of the surgical specimen. Up to 40\% of patients require a second surgery as the result of positive or close surgical margins, but intraoperative margin assessment with frozen section (FS) for BCT is not routinely used in the majority of centers because of the limited sensitivity, the destruction of tissue, and increased surgery time.\textsuperscript{29,62,68,69,106,107} Repeat surgeries can delay adjuvant therapy, increase patient morbidity, and increase health care costs. There is a clinical need for a high sensitivity method to intraoperatively assess surgical lumpectomy specimens in real-time.

Preliminary studies have demonstrated the feasibility of histological evaluation for freshly excised human tissue using NLM.\textsuperscript{4,55} However, translation of the technology to daily clinical practice requires solid clinical trial results, involving multi-center large-scale clinical trials. FDA approval will also be required for the commercial availability of the system. In order to provide a sound foundation for such a large-scale clinical study or commercial collaboration, a controlled randomized clinical trial for intraoperative surgical guidance with NLM evaluation is necessary.
In this chapter, we describe an ongoing study, investigating intraoperative assessment of breast cancer surgical margins using NLM. This study was supported by NIH R01-CA178636 “Intraoperative Real Time Breast Cancer Margin Assessment with Nonlinear Microscopy”. The clinical trial is designed in collaboration with Dr. James Connolly, M.D., breast pathologists, breast surgeons, breast radiologists, and a biostatistician at BIDMC, registered in ClinicalTrials.gov (NCT02926729 “Real-Time Assessment of Breast Cancer Lumpectomy Specimen Margins With Nonlinear Microscopy”). The primary endpoint of the clinical trial is to demonstrate that intraoperative feedback with NLM evaluation of surgical margin will lead to lower repeat surgery rate by decreasing positive/close surgical margin rate. The secondary endpoint is the agreement of margin assessment between NLM and postoperative FFPE H&E histology.

5.2 Clinical trial design

The flowchart of the study protocol is shown in Fig. 5.1. Patients who meet the eligibility criteria (Fig. 5.2) are recruited during pre-surgery visits by study surgeons. Enrolled patients are randomized into the study arm or the control arm. The randomization has been done in each stratum (IDC, ILC, and DCIS) so that stats are not dominated by frequent subtypes. The randomization was done by the study biostatistician, and the result was kept in sealed envelopes. Unblinding by opening envelopes is done immediately before the surgery by a study coordinator (including myself). Study surgeons, pathologists, and radiologists are still blinded when the study procedure starts. The first a few steps of the workflow are the same between study and control arms, closely following the standard clinical care protocol. The lumpectomy tissue is surgically resected by the study surgeons during operation, sent to a radiology room, and being assessed with an X-ray machine to check tumor mass, calcifications, and surgical/biopsy clips, following
standard clinical procedure. The radiologic reading results are immediately communicated to the surgeons. The study surgeons are unblinded when the X-ray result is communicated so that they can decide either to wait for the NLM procedure to provide feedback or to start the closing procedure immediately.

The NLM system is deployed in the radiology room so that the NLM study procedure can be started immediately after the radiological evaluation. The details of the NLM system, tissue preparation, and evaluation protocol are described in chapter 2. The tissue is first grossly examined and sliced into small pieces, ~ a few millimeters thickness by the study pathologist or residents. This is a standard clinical procedure, normally done in the pathology department. The gross examination includes 6 color inking for orientation (superior, inferior, anterior, posterior, lateral, and medial), measurement of size and weight, and slicing of tissue. The tissue slices to be submitted for post-operative histological processing are selected, and NLM examination is performed only on those selected slices rather than all tissue slices in order to minimize NLM evaluation time while maintaining consistency with the clinical standard. After the selection of tissue slices, pathologists are unblinded about which arm the patient is in. The selected tissue slices are fluorescently stained in acridine orange (40 μg/ml) and sulforhodamine 101 (40 μg/ml) in a 1:1 ethanol:water solution for two minutes using, rinsed with saline for ~30 seconds, and placed onto the glass window of the specimen holder. Formalin is poured into the specimen holder to avoid tissue drying and to expedite the tissue fixation process to keep the surface flatness of tissue. The specimen holder equips an air-tight lid, blocking stray light and dissipation of toxic formalin vapor. The specimen holder is set on the NLM system. First, the line scan camera takes a white light image to grossly guide the NLM microscopic field of view. During NLM evaluation, pathologists are primarily focusing on the examination of tissue near surgical margins, indicated by the surgical
inks. The surgical inks used in our study are conventional surgical inks used in the pathology department, but we applied fluorescent micro beads to improve the identification of ink under NLM. In a lumpectomy, often certain aspects of surgical cavities are not actionable because no tissue to be taken or too much risk to take additional tissue. That information is communicated from surgeons through radiologists to pathologists. Evaluation is focused on the aspects actionable with NLM feedback. Pathologists have the control of NLM such as gain change, objective magnification change, xyz rapid translation. The acquired NLM frames are recorded for post-operative comparison with H&E histology. Once all the margins of interest are evaluated, pathologists communicate with the surgeon in the OR about their findings if the patient is assigned to the study arm. Taking the NLM feedback information into consideration, the surgeon may perform additional shaving. The NLM feedback of the additionally shaved tissue is not performed as it will lead to substantially lengthened surgical time, posing risk to study participants. For patients assigned to the control arm, no feedback is made from pathologists until they examine FFPE H&E histology slides of the tissue a few days later.

After the NLM evaluation, tissue (both primary excision and additional shavings) is submitted to the clinical standard histology process and H&E histology pathology evaluation. The recommendation of repeat surgeries is made based on the final margin evaluation of the H&E histology slides. The study results included margin statuses (NLM, H&E for primary excision, H&E for final margin), duration of tissue processing and NLM evaluation, number of evaluated slices, tissue size and weight, and recommendation of a second surgery are recorded and summarized to assess the endpoints.
**Fig. 5.1.** Randomized controlled trial flowchart. Patients assigned to the control arm (N=49) will receive a standard of care. Patients assigned to the study arm (N=49) will have their excised tissue examined by NLM. The margin assessment result is communicated to the surgeon and may lead to additional shaving to minimize the risk of positive margin. The primary endpoint is to show that the study group has a lower second surgery rate than the control group.
Eligibility Criteria

- Patient of a study surgeon's scheduled to undergo lumpectomy for breast cancer at BIDMC.
- Core needle biopsy positive for invasive breast cancer or DCIS.
- Female.
- Minimum age of 21 years.
- Eligible for breast conserving surgery, lumpectomy and radiation.
- ER receptor positive on core needle biopsy, or if receptor negative, have evaluable ER receptor with positive internal control on core biopsy.
- PR receptor positive on core needle biopsy if biopsy indicates invasive cancer, or if receptor negative on biopsy indicating invasive cancer, have evaluable PR receptor with positive internal control on core biopsy.
- HER2 IHC and/or FISH ordered on core biopsy, if biopsy indicates invasive cancer.
- Oncotype DX or other DNA testing performed on core biopsy or not requested.
- Ability to understand and the willingness to sign a written informed consent document.

Exclusion Criteria

- Contraindicated for radiation therapy.
- Pregnancy. (Pregnant women will be excluded from this study because radiation therapy is contraindicated during pregnancy.)
- Current invasive cancer or DCIS at the site of a previous surgery.
- Any systemic neoadjuvant (or preoperative) therapy between the core biopsy and lumpectomy.
- Involvement in another therapeutic trial for breast cancer at Dana Farber or elsewhere.
- Risk of poor cosmetic outcome after initial lumpectomy and possible additional excision, as assessed by a study surgeon.
- Recommendation for mastectomy based on radiology.
- Patient's that have complex DCIS as indicated on radiology, which would require excising a large tissue volume.
- No or equivocal ER, PR or HER2 testing performed prior to surgery if biopsy indicates invasive cancer.
- No or equivocal ER testing performed prior to surgery if biopsy indicates ductal carcinoma in situ.

**Fig. 5.2.** Eligibility and exclusion criteria for the randomized clinical trials. Numbers of criteria are prepared to avoid rare occasions which pose a higher risk than standard patients. For example, the requirement of pre-surgical ER, PR, or HER2 test results is to avoid the interference of NLM staining to those assays. However, preliminary results indicate that there is no significant interference between NLM staining and ER, PR, or HER2. Furthermore, receptor testings are usually performed on core biopsy specimens, and additional receptor testings on lumpectomy specimens are extremely rare. Therefore, the eligibility criteria related to the receptor testing do not have a significant impact on patient recruitment.
5.3 Interventional breast study

The interventional clinical trial involves the management of the enrollment process, team scheduling, data collection, data analysis, follow-up meetings, and maintenance of IRB documentation. During the surgery, preparation of NLM imaging/tissue processing setup, technical support of NLM imaging, and recording of evaluation details, results, and timing is needed. The NLM system needs regular check-ups and maintenance, and modifications if required. The real-time NLM images are archived and reviewed postoperatively to examine details of imaging such as evaluation time per pathology, visualization of each pathology and contaminations, a close comparison of real-time NLM imaging, and postoperative H&E histology, in addition to the margin status which is evaluated during surgery. All the documentation including source documents of the study procedure and informed consent has to be kept in order. IRB members from Dana Farber Cancer Institution occasionally review the documentation.

Target enrollment is 98 patients (49 study, 49 control). Informed consent is obtained for all study participants. All research is being performed according to protocols approved by Dana-Farber/Harvard Cancer Center Institutional Review Board and Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. This study is registered at Dana-Farber/Harvard Cancer Center’s IRB as Protocol 16-145, and at Massachusetts Institute of Technology’s Committee On the Use of Humans as Experimental Subjects. We are currently actively enrolling patients, and the study is expected to be completed in 2022. The result of the study will be published shortly after the completion of the study.

This study has been done in collaboration with Michael G. Giacomelli, Lucas C. Cahill, Oscar M Carrasco-Zevallos, Timothy D. Weber, Sagar Doshi, and James G. Fujimoto at Massachusetts Institute of Technology, and Liza Quintana, Yaileen Guzman, Hilde Vardeh, Nicole
B. Johnson, and James L. Connolly (Pathology), Ranjna Sharma, Maryjane Houlihan, Monica C. Valero (Surgery), Tejas S. Mehta (Radiology) at Beth Israel Deaconess Medical Center and Harvard Medical School, and Rebecca Gelman at Dana Farber Cancer Institute. This work was supported by the National Institutes of Health program R01-CA178636.
Chapter 6

NLM imaging of simulated nerve-sparing radical prostatectomy specimens for protocol development for intraoperative margin assessment

6.1 Introduction

Prostate cancer is the most common cancer in the US male population, with an estimated 160,000 new cases in 2018.19 Treating localized prostate cancer with radical prostatectomy (RP), complete surgical excision of the prostate, provides excellent oncologic outcomes with long-term survival benefits.108,109 Nerve-sparing RP is favored if cancer does not involve the neurovascular bundles since patients have better recovery of sexual function and continence, two major factors determining postoperative quality of life.110–119 However, current preoperative evaluation methods do not accurately identify patients who could be treated by nerve-sparing RP. This leads to a higher rate of non-nerve sparing RPs than necessary, reducing postoperative quality of life.120–123 Intraoperative evaluation of prostate surgical margins with frozen section (FS) has been shown to enable more accurate surgical decision making. The NeuroSAFE study demonstrated that comprehensive intraoperative evaluation of the posterolateral and apical surgical margins using up to 25 FS sections per patient increased the rate of nerve-sparing RPs (81% to 97%) and decreased the positive margin rate.67 However comprehensive evaluation with FS required extensive
infrastructure (5 cryostats, 2 pathologists, and 4 technicians) to maintain an average evaluation time of 35 minutes, which is impractical and expensive for most hospitals.

Techniques that image fresh surgical specimens without freezing or microtoming can dramatically reduce the time and personnel required for intraoperative evaluation. Nonlinear microscopy (NLM) can produce high resolution images that resemble H&E histology in freshly excised tissue by using a scanned femtosecond laser to excite fluorescence only at the laser focus, generating images without freezing or microtoming the specimen. We have developed custom NLM technology for rapid, high-throughput evaluation of prostatectomy specimens and a method for rapidly staining specimens in <3 minutes using nuclear vs cytoplasmic/stromal specific contrast and displaying fluorescence signals in an H&E color scale to facilitate pathologist interpretation.\textsuperscript{63} We demonstrated that NLM achieves 97% sensitivity and 100% specificity detecting prostate cancer compared to the clinical standard of formalin-fixed paraffin-embedded (FFPE) H&E histology in a study of 122 specimens from 40 patients with blinded reading by three pathologists (Table 6.1).\textsuperscript{5,124} This study strongly suggests that NLM promises to enable intraoperative evaluation with a rapid and simplified workflow that is practical for widespread clinical adoption. However, a blinded reading study of surgical margins was not practical because positive margin rates are low and large numbers of specimens with surgical margins were not available.

In this chapter, we describe the method to simulate surgical margins of nerve-sparing RP specimens and the preliminary results including margin detections, processing/evaluation time, and findings. The nerve-sparing RP protocol enabled the examination of positive margins in a simulated but realistic workflow. This study has been done in collaboration with Lucas C. Cahill, Timothy D. Weber, Sagar Doshi, and James G. Fujimoto at Massachusetts Institute of Technology, and Yue Sun, Yubo Wu, and Seymour Rosen (Pathology), and Peter Chang and Andrew Wagner
(Surgery) at Beth Israel Deaconess Medical Center and Harvard Medical School. This work was supported by the National Institutes of Health program R01-CA249151.

Table 6.1. Sensitivity and specificity of NLM for detecting carcinoma vs FFPE H&E.

This table was adapted from "Nonlinear microscopy for detection of prostate cancer: analysis of sensitivity and specificity in radical prostatectomies," by L.C. Cahill et al., Modern pathology (2019) Copyright 2019 by United States & Canadian Academy of Pathology.

<table>
<thead>
<tr>
<th>Reader</th>
<th>Sensitivity [95% CI]</th>
<th>Specificity [95% CI]</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
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<tr>
<td>Reader 1</td>
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<td>1</td>
<td>1</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td>Reader 2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Reader 3</td>
<td>0.97</td>
<td>1</td>
<td>1</td>
<td>0.95</td>
<td>0.98</td>
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<tr>
<td>Pooled 1-3</td>
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<td>1.00 [0.97, 1.00]</td>
<td>1</td>
<td>0.96</td>
<td>0.98</td>
</tr>
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6.2 Methods

We have enrolled 28 patients who were undergoing RP for postoperative NLM imaging. The protocols were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigations and Institutional Review Board and the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. Written informed consent was obtained from all subjects. Patients over 18 years old were enrolled without having any exclusion criteria. In this study, RP surgical procedure was not altered and the patients were performed following standard RP protocol. The study surgeons determined nerve-sparing or non-nerve sparing based on preoperative imaging results (CT, MRI), core biopsy histology results, urinary and sexual functions, and patients preference using the standard care criteria. Bilateral nerve-sparing cases, unilateral nerve-sparing cases, and no nerve-sparing cases were all included in the study. The prostate specimens (with or without neurovascular bundles) were grossly examined and inked following standard clinical protocol by pathologists/pathology residents. Gross examination
includes the measurement of weight and size of the prostate tissue. Left and right sides are inked with different colors, with blue for left and green for right. Black ink was commonly used for the inking of the right side of prostate tissue at BIDMC, but we changed the color to green to minimize the interference to NLM imaging. This ink color change is not considered as a deviation of standard protocol. After inking, if the neurovascular bundles were excised with the prostate gland during the RP procedure, a study surgeon or a study pathologist resected the neurovascular bundles ex vivo. This postoperative excision simulates a nerve-sparing RP procedure and creates artificial surgical margins with an increased positivity rate to mimic actual positive margin detection by NLM. The artificial surgical margin (not used for clinical decision making) was inked by a pathologist in a different color (yellow or orange) to distinguish it from the original intraoperative (clinically relevant) surgical margin (Fig 6.1A, 6.1B). After the nerve resection and additional inking, the prostate specimens were grossed by breadloafing into ~5 mm slices following standard procedure, stained with fluorophores, and evaluated using NLM by a pathologist (Fig. 6.1C). After NLM imaging, the specimens were submitted for standard histology processing (formalin-fixed, paraffin-embedded, microtomed, and stained), and histologically assessed in a clinical standard manner. Tissue processing and NLM evaluation time were recorded to assess the impact of NLM protocol on the duration of surgery if used intraoperatively. NLM margin assessment results were compared with standard H&E histology evaluation to assess the discordance.

In this study protocol, the primary RP excision was performed per clinical standard of care. NLM imaging including application of fluorophores does not interfere postoperative histology processing. Both the prostate and excised nerves were histologically evaluated for margin evaluation. The artificial margins (yellow or orange ink) were distinguished by color with the
original surgical margins and not included in the final pathology report. Therefore patient care was not altered.

Fig. 6.1. NLM evaluation of a simulated nerve-sparing RP. A. Photo of inked specimen with black (right), blue (left), and yellow (NVB adjacent tissue). B. NVB, which was dissected by surgeon post-operatively. C. Photo (whitelight image) of breadloafed slices on the specimen holder. Surgical inks with different colors (blue (left), green (right), and orange (artificial surgical margin)) are identified.

6.3 Results

We have collected 28 prostates during Jan/2020 – Oct/2021. In this study period, enrollment was halted for ~7 months because of COVID19. Real-time NLM margin evaluation by pathologists was compared to margin evaluation on H&E histology slides of the corresponding tissue sections. Of the first 24 patients, 9 had positive margins and 15 had negative margins on standard H&E histology. Among the 9 positive cases, NLM detected positive margins in 8 patients but was discordant (false negative compared to H&E) in 1 patient. Among 15 negative cases, NLM detected negative margins in 11 patients but was discordant (false positive) in 4 patients. The
confusion matrix table is shown in Table 6.2. The types of nerve-sparing are summarized in Table 6.3. Majority of the 24 cases were unilateral nerve-sparing. We also included 8 bilateral nerve-sparing cases. Prostate tissue from bilateral nerve-sparing RP do not have neurovascular bundles, so we did not perform additional excision and artificial margins were not created for those cases. NLM evaluation was performed for the prostates from bilateral nerve-sparing RP as well. Table 6.4 shows the location of the 9 positive margins. Large fraction of positive margin was found on the artificial margins where additional ex-vivo, postoperative resection of neurovascular bundles were performed.

Table 6.2. margin evaluation of NLM v.s. FFPE H&E

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<tr>
<th></th>
<th>H&amp;E</th>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>NLM</td>
<td>Positive</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
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Table 6.3. Nerve-sparing (NS) types of radical prostatectomy

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<table>
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<tbody>
<tr>
<td>Non NS</td>
<td>2</td>
</tr>
<tr>
<td>Unilateral NS (R)</td>
<td>9</td>
</tr>
<tr>
<td>Unilateral NS (L)</td>
<td>5</td>
</tr>
<tr>
<td>Bilateral NS</td>
<td>8</td>
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Table 6.4. Location of positive margin, original margin vs. artificial margin

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<tr>
<th>H&amp;E margin positive on</th>
<th>Original margin</th>
<th>Artificial margin</th>
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<tr>
<td></td>
<td>2</td>
<td>7</td>
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In this cohort, margin evaluation was performed during the period of continuous optimization to the tissue handling workflow, and therefore initial accuracy is not representative of the technique’s potential. However, the initial results are promising. Given the overall positive margin rate for RP is 15% on average [6.5%-32%]^{125}, one false negative (postoperative positive margin) out of 9 positive cases should result in a very small probability of error in the full cohort. Had NLM been used to guide secondary resection, the 4 false positive cases would have resulted
in unnecessary nerve resection. Note that in all 4 cases, the actual clinical decision was to perform non nerve-sparing RP, meaning that the current standard RP would have also resulted in unnecessary nerve resection. Of the 11 true negative cases determined by NLM, 8 patients had unilateral nerve-sparing but could have been indicated for bilateral nerve-sparing with intraoperative NLM.

We carefully reviewed the discordant cases and found that the false negative NLM case had only a minimally focal positive margin on H&E slides, while NLM indicated a close margin in the same area. Figures 6.2.A-C show the images of NLM and H&E for the false negative NLM case. Cancer approaching to tissue edge is visualized in NLM (6.2.A), but surgical ink is not clearly visualized and surgical margin status was not evaluable and considered as negative. In H&E (6.2.B, C), the focal contact of cancer to the tissue edge is visualized. The surgical ink on the tissue edge is disrupted and identified only limited areas without continuity. The tissue edge was considered as surgical margin and therefore the margin status was determined as positive on the H&E. We also found that in all of the false positive NLM cases, H&E slides had very close margins from <100 μm to 300 μm. Figures 6.2.D-F show the images of NLM and H&E for the false positive NLM case. The NLM image (6.2.D) shows the nuclei-rich grandular-like structure on the inked edge, appearing to be cancer. The H&E images (6.2.E, F) show cancer approaching to surgical margin (surgical ink) in a similar manner to the NLM image, but the closest cancer gland has minimal but identifiable distance with the surgical ink edge. Therefore this case was determined as negative on the H&E. Figure 6.3 shows the example images of true positive/negative margin cases. Both NLM and H&E images show clear stroma layer between surgical inked margin and cancer (true negative), or clear contact between surgical margin and cancer (true positive).
Occasional discordance between NLM and H&E margin status could occur because of differences in the specimen placement on the NLM imaging stage versus the paraffin microtome section histological plane. H&E slide preparation also involves tissue handling and chemical processing, which alter tissue position, shape, and size. Fresh tissue handling is also more complicated than fixed tissue handling, potentially obscuring surgical margin. These can result in discordance of margin evaluation between NLM and H&E. The study pathologists have gained sufficient experience to assess NLM images during preliminary studies and this reporting period, and could consistently identify tumor in prostate tissue in all of the cases, but found some discordance in margin evaluation.

**Fig. 6.2.** Surgical margin of a simulated nerve-sparing RP, NLM vs. H&E, false positive/negative. Top row shows surgical margin images of a false negative case (A. NLM, B. H&E, and C. H&E magnified). The NLM (A) shows cancer approaching to the edge of the specimen, but surgical inks are not identified. The H&E images (B, C) show cancer on the edge of the specimen with surgical inks (Green, non-artificial margin, right). The surgical ink margin is incomplete on the H&E likely because of tissue handling disruption/artifact. The red arrows in figure A and C indicate cancer at the edge of the specimen. Bottom row shows surgical margin images of a false positive case (D. NLM, E. H&E, and F. H&E magnified). The
red arrow in figure D shows regions suspicious for cauterized cancer. On the other hand, the histology slide (E, F) shows gap between cancer and surgical ink (Green, non-artificial margin, right). However, the distance of the gap is as small as the single cell size (the red arrow in figure F).

**Fig. 6.3.** Surgical margin of a simulated nerve-sparing RP, NLM vs. H&E, true positive/negative. Top row shows surgical margin images of a true negative case (A. NLM and B. H&E). Stroma between cancer and inked margin (Orange, artificial margin) is clearly seen in both NLM and H&E images. Bottom row shows surgical margin images of a true positive case (C. NLM and D. H&E). Contact between cancer and inked margin (Orange, artificial margin) is clearly visualized in both NLM and H&E images. The gap between ink and cancer in figure C is the region of minor out-of-contact. Realtime NLM imaging with focus depth adjustment can visualize the margin clearly. Red arrows show cancer closest/on margin.

For the most recent 4 patients, we adjusted NLM reporting to include a category for indeterminate intraoperative margin cases. This designation could potentially be used to trigger
secondary nerve resection, and hence ‘err on the side of caution’. We will continue recruitment and develop objective criteria. Determining appropriate criteria for nerve-sparing versus non nerve-sparing based on NLM findings as well as defining criteria for comparing NLM and H&E requires careful clinical evaluation and consensus.

The NLM protocol (inking, grossing, and evaluation) required 36 minutes on average [22-48 mins] from tissue acquisition to the end of margin evaluation. NLM evaluation can be conducted in parallel with standard surgical procedures after the prostate is removed, including cauterization and lymph node dissection which require ~45 minutes. Therefore, the NLM protocol will not extend the duration of surgery. Transporting the specimen from the operating room to the NLM unit in a different building required an additional 6 minutes, but the NLM system will be in a room adjacent to the operating room if used for an interventional study, and transportation time will be reduced.

6.4 Conclusion

We demonstrate the NLM imaging of simulated surgical margins of nerve-sparing RP specimens. The preliminary results indicated that NLM can visualize prostate cancer and surgical margins as previous studies suggested. Tissue processing and NLM evaluation time is short enough that the duration of the RP surgery would not need to be extended if NLM would be used intraoperatively. Careful examination of discordant cases indicates that NLM and H&E can disagree when cancer is close to margin (<100-300 μm). Disruption of tissue edge can also become a problem because grossing fresh prostate tissue is more challenging than fixed prostate tissue. The simulated nerve-sparing RP specimen imaging study will continue to finalize the criteria and
evaluation/feedback protocol for the interventional clinical study. The interventional study is expected to be started in 2022.
Chapter 7

Rapid histological imaging of bone using nonlinear microscopy

7.1 Introduction

Histologic evaluation of bone typically requires fixation, decalcification, and paraffin processing to enable microtome sectioning. If decalcification is required, the procedure is especially time-intensive, and rapid analysis using frozen section is not feasible. The ability to rapidly process and visualize bone tissue pathology in such cases would enable intraoperative consultation for surgical margin evaluation, intraprocedural analysis of biopsies to assess adequacy, tissue triaging for specific assays, as well as near real-time access to diagnostic information. Optical sectioning fluorescence microscopy technologies, including two-photon excitation nonlinear microscopy (NLM), confocal fluorescence, structured illumination, and light sheet microscopy are being investigated for rapid histologic evaluation of freshly excised tissue. NLM is of particular interest because blinded reading studies of NLM compared to paraffin-embedded hematoxylin and eosin (H&E) histology demonstrated high sensitivity and specificity for detecting breast and prostate cancer. The NLM protocol enables rapid staining of tissue with nuclear and stromal/cytoplasmic fluorophores and images can be displayed in an H&E color scale to facilitate interpretation. Specimens can be rapidly imaged without microtome sectioning using a scanned, focused laser beam that excites fluorescence from a few micron axial ranges of tissue. Furthermore, by adjusting the focus depth, NLM can image up to ~100 μm below the tissue surface, analogous to serial sections. In this chapter, we describe
a method for rapid tissue preparation and visualization of bone using NLM. We describe imaging of normal bone and cartilaginous tissue, as well as orthopedic surgical and biopsy specimens with osteoarthritis, osteomyelitis, and malignancy.

The work in this chapter was first published in Bone. This work was done in collaboration with Lucas C. Cahill and James G. Fujimoto at Massachusetts Institute of Technology, and Seymour Rosen, Simon Lamothe, and Ashley Ward at Beth Israel Deaconess Medical Center and Harvard Medical School. This work was supported in part by the National Institutes of Health programs R01-CA178636 and R01-CA252216.

7.2 Methods

NLM imaging was performed on 71 specimens of discarded bone (not required for clinical diagnosis) from 40 patients who underwent joint replacement (hip or knee), amputation (hand, arm, or foot), bone marrow biopsy, or autopsy. The protocols were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigations and Institutional Review Board and the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. Informed consent was waived by both committees.

The bone specimens, with the exception of biopsies, were cut to expose the surface of interest using a scalpel, saw, or hammer (Fig. 7.1a) in the same manner as conventional histology processing. The surface was gently brushed and/or rinsed with water to remove debris from cutting. Biopsy specimens were taken using a 16-gauge needle during autopsy or excess tissue from abundant bone marrow biopsy specimens. Additional cutting, brushing, and rinsing were not performed on the biopsy specimens because of their small size. The specimens were stained in acridine orange (40 μg/ml) and sulforhodamine 101 (40 μg/ml) in a 1:1 ethanol:water solution for
5 minutes and rinsed for 30 seconds in saline to remove excess staining solution. Acridine orange provides nuclear contrast, while sulforhodamine 101 provides stromal and cytoplasmic contrast, analogous to hematoxylin and eosin. The protocol is similar to a previously published NLM staining protocol for breast and prostate tissue but requires longer staining time because both cortical and cancellous bone were found to be stained weakly compared with soft tissues. Immediately after staining, the specimens were placed on a glass specimen holder, covered with a lid to block room light, and imaged using an inverted NLM microscope (Fig. 7.1b).

The NLM instrument had a 10x, 0.45 numerical aperture (NA) air objective with a 2 mm field of view (FOV) (CFI Plan Apo Lambda, Nikon) and a 5x, 0.25 NA air objective with a 3.4 mm FOV Fluar, Carl Zeiss Microscopy) that could be rapidly interchanged under servo control. The wide field 5x objective was used for rapidly surveying regions of interest, and the 10x objective was used for visualizing details of pathology. Specimen holders for the wide FOV (5x and 10x) objectives had rigid glass windows (400 µm thickness) that accommodate up to 7 cm x 10 cm size specimens. Alternatively, the NLM instrument had an option to manually change to a higher magnification using a 20x, 0.75 NA air objective with a 1 mm FOV (CFI Plan Apo Lambda, Nikon). Specimen holders for the 20x high magnification objective required thin glass windows (170 µm, standard coverslip thickness) to avoid aberration and achieve the desired resolution. The thin glass is fragile and easily bendable, so it is difficult to prepare large windows. The specimen holders for the 20x objective were therefore designed to accommodate specimens with the size of a histology cassette or biopsy.

A femtosecond laser at 1 µm wavelength was used to excite two-photon fluorescence at the focus of a raster-scanned beam. Digital magnification up to 40x (or 80x for the 20x objective) was available by changing the scan area and speed. The fluorescent signals from acridine orange
and sulforhodamine 101 were collected and separated using a low-pass dichroic beam splitter (T588lpxr, Chroma Technology) and two emission filters (ET540/40m, Chroma Technology and FF01–650/60, Semrock), and then detected using two photomultiplier tubes (7422-40p, Hamamatsu). NLM images were displayed at 16 frames per second in a color scale that resembles H&E histology (Fig. 7.1c)\(^\text{48}\).

The specimens were imaged in real-time on NLM and evaluated in a manner similar to standard transmission light microscopy of histology slides. A pathologist examined the specimen by translating it horizontally (x-y plane) to regions of interest and changing magnification. A white-light image of the gross specimen surface was used to guide positioning (Fig. 7.1b). The microscope focus can be vertically adjusted (z-axis) to image tissue below the surface, analogous to serial sectioning. The rapid focus depth adjustment was also important for imaging the three-dimensional structure of bone as well as the irregular specimen surfaces caused by preparation, saw marks, and cracks.

During the real-time evaluation, the NLM images were recorded along with position data for subsequent analysis. The NLM images were then stitched together using commercial software (Microsoft Image Composite Editor) to provide a single 2D image of the entire real-time NLM imaging sequence for review/publication. After NLM imaging, the specimens were processed for standard histology and paraffin H&E slides were digitally scanned (20x magnification, Leica Aperio slide scanner). A pathologist performed an unblinded comparison of the digital NLM images and corresponding paraffin H&E slides to identify similarities and differences in normal tissue and pathology.
Fig. 7.1. Tissue processing and NLM imaging. (a). Specimens were cut to expose surfaces of interest, washed to remove surface debris, stained in a solution of acridine orange (AO) and sulforhodamine 101 (SR101) for 5 minutes, and rinsed for 30 seconds in saline to remove excess stain. (b). The specimens were placed on a glass window of a specimen holder in an inverted NLM instrument. The specimens were translated in the horizontal (x-y) plane to regions of interest and the microscope focus depth, vertical (z-axis) translation, could be adjusted to image irregular surfaces and subsurface features. A position indicator was displayed on a white-light image of the gross specimen to guide NLM image positioning. The specimen holder allows imaging of up to 7 cm x 10 cm specimens. (c). The NLM images were displayed in an H&E color scale at 16 frames per second. All figures in this chapter 7 were adapted from "Rapid histological imaging of bone without microtome sectioning using nonlinear microscopy," by T. Yoshitake et al., Bone 154 (2022) 116254. Copyright 2021 by Elsevier Inc..

7.3 Results

7.3.1 Imaging large specimens vs. biopsies

NLM enabled the visualization of histological features of bone such as bone architecture, degenerative changes, infection, and malignant alterations in specimens ranging from biopsies to multi-centimeter femoral heads. Figure 7.2 shows a transected, entire femoral head cross-section (4.5 cm width) imaged with NLM prior to decalcification and paraffin processing for standard
histology. The white-light image of the gross specimen (Fig. 7.2a) was used to guide NLM imaging. The articular cartilage at the periphery was imaged to assess osteoarthritis, and the internal trabecular bone network and bone marrow could also be imaged. The specimen surface was irregular and could not be placed flat against the glass NLM specimen holder. However, the NLM focus depth could be adjusted (vertical or z-axis specimen position) to compensate for surface contour variations up to ~500 µm and to image up to ~100 µm deep in tissue. The stitched sequence of real-time images in Figure 7.2b shows the areas imaged by NLM with focus depth adjustment. The regions of the femoral head shown (periphery cartilage and trabecular network) were evaluated in <5 minutes. Corresponding paraffin H&E histology required 4 separate slides (Fig. 7.2c). The dissection and decalcification/paraffin processing for histology slide preparation resulted in partial detachment of fragile tissue regions.

**Fig. 7.2.** NLM imaging of large specimens. An example showing NLM imaging of a transected femoral head (4.5 cm width). (a) A white-light image of the gross specimen was used to guide NLM positioning. (b) Stitched sequence of NLM images showing the pathologist examination which included the articular cartilage and the trabecular network. (c) Corresponding paraffin H&E histology. The specimen was dissected into quadrants for 4 processing (30 x 26 mm) cassettes. Detachment of tissue caused by tissue processing for slide preparation is seen (top left corner).

NLM can rapidly image small specimens such as biopsies. Figure 7.3 shows 16-gauge needle core biopsies of bone marrow obtained from discarded tissue. Although the biopsy
specimens were small, their cylindrical shape required adjusting the focus depth to image regions of interest. The ~2-mm-wide x 2-cm-long specimen was imaged in 2 minutes (Fig. 7.3a and 7.3b). Red bone marrow with granulopoiesis and erythropoiesis having approximate cellularity of 50% are shown in Figure 7.3c, as well as in the corresponding paraffin H&E histology slide (Fig. 7.3d). Endosteal lining along with trabecular bone is also seen in Figure 7.3e. The white area of trabecular bone shows imaging deep inside of the trabecular bone, where fluorescent signals are limited because of limited fluorophores and/or excitation laser light penetration into tissue. Bone marrow in some of the marrow spaces is missing because of aspiration artifacts due to the needle biopsy procedure. Nuclear details are well represented in NLM, but cellular borders are less sharp. Cell cytoplasm is darker and granulocyte granules are more distinctive than in paraffin H&E.

Higher-resolution imaging using a high NA objective and a thin imaging window are shown in Figures 7.3f and 7.4. The NLM images of core needle biopsy specimens visualize subcellular details with the high resolution 20x objective. The thin optical sectioning with the high NA 20x objective enables visualization of osteocytes embedded in trabecular bone with its lacunar and canalicular spaces (Fig. 7.3f). Figure 7.4 demonstrates NLM imaging of bone marrow biopsy specimens, visualizing different types/stages of hematopoietic cells. Figure 7.4a shows hematopoietic marrow. All three cell lineages, myeloid, erythroid, and megakaryocytic, are represented. Neutrophils and eosinophils are readily identified, including different maturational stages of neutrophils such as myelocytes, metamyelocytes, band forms, and mature neutrophils. Numerous erythroblasts at all stages of maturation are present as well. The cells with abundant cytoplasm and clock face chromatin are plasma cells. Stromal cells, endothelial cells, and a venous sinus are clearly visualized. Figure 7.4b shows a group of eosinophils and neutrophils at different stages of maturation and a lymphocyte. Eosinophils are distinguishable from neutrophils by their
larger and redder “eosinophilic” cytoplasmic granules. Orthochromatic erythroblasts are also easily distinguishable. Figure 7.4c shows a multinucleated (normal) megakaryocyte.

**Fig. 7.3.** Needle core biopsy imaging with NLM. A white-light gross image (a) and corresponding composite NLM image generated from the real-time image sequence (b) of a 16-gauge core needle biopsy from pelvic bone marrow. The biopsy surface was irregular and necessitated adjustment of the focus depth during imaging. Out-of-focus frames were excluded from the image composition to minimize image processing artifacts. Red bone marrow with granulopoiesis and erythropoiesis with approximate cellularity of 50% are shown in images with NLM (c) and paraffin H&E histology (d). NLM has less sharp cell boundaries, darker cytoplasm, and more distinctive granulocyte granules than paraffin H&E images. (e). NLM shows endosteal lining. NLM with a 20x objective enables visualization of sub-cellular details more clearly such as (f) osteocytes embedded in trabecular bone. (Scale bars = (a, b). 3 mm, (c-f). 50 μm) T: trabecular bone; Es: endosteal lining; MS: marrow space; Oc: osteocyte; Nb: normoblast.
Fig. 7.4. High resolution bone marrow imaging with NLM. 20x objective enables visualization of finer details of hematopoietic cells near the marrow such as (a) cells associated with all three cell lineages, myeloid, erythroid, and megakaryocyte, (b). eosinophils and neutrophils at different stages of maturation, and (c). megakaryocytes. (Scale bars = (a-c). 50 μm) Nt: Neutrophils; Eo: eosinophils; My: myelocytes; Mm: metamyelocytes; Bn: band forms neutrophils; Eb: erythroblasts; Pc: plasma cells; St: stromal cells; Ed: endothelial cells; Vs: venous sinus; Ly: lymphocyte; Mk: megakaryocytes.

7.3.2 Bone architecture

NLM can visualize the characteristic architecture of trabecular bone from the femoral head and knee joint, as shown in Figure 7.5. Figure 7.5a is a stitched sequence of NLM images showing the trabecular network and displaying trabecular lamellar bone with associated osteocytes. The layered lamellae structure and osteocytes are clearly recognized within the trabecular bone (Fig.
In articular cartilage, NLM can visualize the basophilic matrix, clusters of chondrocytes, and the tidemark (Fig. 7.5c). NLM imaging of bone marrow enables the identification of maturing trilineage hematopoiesis (Fig. 7.5d). Synovium is also readily visualized, including synovial epithelium and small vessels (Fig. 7.5e). A corresponding paraffin H&E histology slide image is shown in Figure 7.5f. Lacunae around osteocytes are white and much more distinct in paraffin H&E than in NLM. The adipose tissue in the marrow spaces is also better visualized in paraffin H&E.

Fig. 7.5. NLM of bone architecture. (a) NLM images of bone architecture that includes the lamellar structure of trabecular bone and osteocytes. The bone surface is irregular and requires images from different focus depth positions to be combined in order to generate a composite image. (b) High magnification NLM image showing trabeculae containing osteocyte nuclei. (c) NLM image of basophilic matrix and chondrocyte clusters of articular cartilage. (d) NLM image of bone marrow. Megakaryocytes,
granulopoiesis, and erythropoiesis are visualized. (e) NLM image of synovium. (f) Corresponding paraffin H&E histology of trabecular bone specimen (a). (Scale bars = (a). 300 μm, (b). 100 μm, (c). 100 μm, (d). 50 μm, (e). 200 μm) MS: marrow space; RC: resorption cavity; T: trabecular bone; CC: chondrocytes; TM: tidemark; AP: adipose; MK: megakaryocytes.

### 7.3.3 Osteoarthritis

Figures 7.6a and 7.6b show NLM images and corresponding paraffin H&E histology of articular cartilage from a knee. NLM images have more pronounced eosinophilic and basophilic staining when compared with paraffin H&E. Irregularity of the cartilage surface, which is a feature of osteoarthritis, is apparent in both the NLM and paraffin H&E. Chondrocytes proliferation and tidemark duplication are also more prominent in the NLM image (Fig. 7.6a, toward left) than in paraffin H&E histology.

![Fig. 7.6](image)

**Fig. 7.6.** NLM of articular cartilage. Both NLM (a) and paraffin H&E histology (b) show surface irregularity/clefts with the surface cartilage replaced by fibroconnective tissue (center). The staining is more pronounced in NLM than H&E histology, with basal cartilage calcification evident. (Scale bar = 500 μm)

### 7.3.4 Osteomyelitis
Figure 7.7 shows NLM images and corresponding paraffin H&E histology of osteomyelitis in a knee. NLM shows neutrophils with multilobed nuclei on the bone surface (Fig. 7.7a). Corresponding H&E images (Fig. 7.7b) show the margin of bone and infection. Figures 7.7c and 7.7d show NLM images with focus depth settings 50 µm apart, which visualize reactive changes with partial trabecular dissolution (black arrow). Osteoblasts lining the edge of trabecular bone, multinucleated osteoclasts, fibroblasts, and histiocytes are seen inside the cavity. (Fig. 7.7e, corresponding H&E histology)

**Fig. 7.7.** NLM of osteomyelitis. The NLM image (a) shows large numbers of neutrophils within the bony space (red square). Corresponding paraffin H&E histology (b) includes the bony margin. The NLM images (c, d) show reactive changes with partial trabecular dissolution (black arrow). The two images are 50 µm apart in depth. The bony edge is seen in both NLM and corresponding H&E histology (e) but the osteoblastic proliferation and occasional osteoclasts are more distinct in the NLM images. (Scale bars = 100 µm) T: Trabecular bone; Oc: Osteoclast; Ob: Osteoblast.
7.3.5 Malignancy in bone

Many cancers can spread to bone and bone metastases are more common than primary bone cancer in adults. Figure 7.8 shows example NLM images and paraffin H&E histology of bone metastases and a primary bone tumor. Figure 7.8a shows an NLM image of squamous cell carcinoma metastatic to the bone. Cellular aggregates of atypical epithelial cells with keratin pearl formations, which are characteristic of squamous cell carcinoma, are visualized. Figure 7.8c shows NLM of a renal carcinoma metastasis. Nested cells with cleared out cytoplasm are characteristic of clear cell renal cell carcinoma. Figure 7.8e shows NLM of pancreatic cancer metastasis. The glandular formation can be identified. Figure 7.8g shows NLM of a primary bone sarcoma depicting a cellular spindle cell neoplasm, which is consistent with a high-grade sarcoma. Figures 7.8b, d, f, and h show corresponding paraffin H&E histology images. Typical appearance of malignancies, such as more prominent nuclei and darker cytoplasm, are similar in NLM to paraffin H&E. Differences between NLM and paraffin H&E include visualization of the stroma, which is more prominent in paraffin H&E than NLM images and visualization of erythrocytes, which are not clearly defined in NLM images.
**Fig. 7.8.** NLM of representative malignancies. (a) NLM of metastatic squamous cell carcinoma showing atypical cells with abundant cytoplasm and keratin pearl formation, and (b) corresponding paraffin H&E histology. (c) NLM of clear cell renal cell carcinoma metastatic to the femoral head, and (d) corresponding paraffin H&E histology. Clusters of cells with cleared out cytoplasm are seen in both H&E and NLM, but erythrocytes are defined only in H&E. (e) NLM of pancreatic cancer metastatic to the femoral head, and (f) corresponding paraffin H&E histology. A cellular proliferation with enlarged pleomorphic nuclei and glandular structures can be seen. (g) NLM of a primary bone sarcoma of the femoral head showing an atypical spindle cell proliferation, and (h) corresponding paraffin H&E histology. (Scale bars = 100 μm)

### 7.4 Discussion

NLM enabled visualization of bone within minutes, without requiring decalcification or microtome sectioning. NLM could visualize normal bone architecture, articular cartilage, osteoarthritis, osteomyelitis, and malignancy involving bone.

The structural context of trabecular bone containing osteocytes with intervening bone marrow was readily distinguishable by NLM. The NLM images of cartilage, including the basophilic matrix and chondrocytes, were remarkably equivalent to paraffin H&E. Neutrophils with multilobed nuclei on the bone surface were clearly visualized on NLM. In carcinomas and
sarcomas, the morphologic features of malignancy are maintained. The clear cell appearance of renal cell carcinoma was similar in NLM to paraffin H&E and the membranes of the poorly differentiated sarcoma were well delineated by NLM. High magnification NLM images of small needle biopsy specimens clearly visualized sub-cellular details of pathology, including characteristic nuclear patterns and cytoplasmic staining of hematopoietic cells and osteocytes embedded in trabecular bone with lacunar and canalicular spaces.

Despite the similarities between NLM images and paraffin H&E histology, several differences were noted. Cellular borders were less sharp in NLM, cells appeared rounder and larger and nucleoli were more prominent than in paraffin H&E histology. Erythrocytes are not clearly visualized in NLM but are prominent in paraffin H&E histology. Some histologic features that rely on well-establish color patterns in paraffin H&E histology (such as the color of cytoplasm or the “blandness” of nuclei) were less evident on NLM. These differences are due to a combination of factors: dehydration, decalcification, and paraffin processing used in H&E histology result in cellular shrinkage and cytoplasmic contraction. Differences in staining specificity of acridine orange and sulforhodamine 101 in fresh tissue vs. hematoxylin and eosin staining of microtomed tissue specimens in histology slides were also observed. For example, cytoplasmic and nuclear elements can be more darkly stained and marrow granulocytes staining is distinctive and bright using acridine orange and sulforhodamine 101 in NLM.

NLM enabled real-time imaging of large specimens by rapidly translating the specimen horizontally (x-y plane) and adjusting the focus depth (z-axis) using 5x and 10x objectives and specimen holders with a rigid large glass window. Large specimens could be imaged without dissection into the smaller sizes typically required for microtoming, enabling imaging of intact bone with attached soft tissue as well as whole tumor margins. Real-time adjustment of the focus
depth enabled imaging of subsurface tissue and complex architectural structures such as trabecular bone, glandular structures, branching components, and cavities.

All NLM images shown in this chapter, except Figs. 7.3f and 7.4, were acquired using a 10x, 0.45 NA microscope objective and a 400 µm thick glass for the imaging window. The 10x wide-field imaging objective has an 8.7 µm axial resolution, which is thicker than histology slide sections (5 µm). Therefore the 10x NLM images appear to have lower resolution compared with the high magnification (20x) view of the corresponding H&E histology slides. However, NLM can achieve higher resolution imaging by using a high NA 20x objective and thin glass window as shown in Figs. 7.3f and 7.4. Standard coverslips are fragile and bendable to hold multi-centimeter square large specimens, limiting the size of the imaging window for high-resolution NLM imaging. Biopsy or histology cassette size specimens are easily accommodated by specimen holders with thin glass. Large soft tissue specimens can be easily cut into small pieces without appreciably increasing imaging time if high resolution is required. However, large tissue specimens involving bone are more time-consuming to cut into smaller sizes.

NLM generates images at a given focal depth position. A flat and clean tissue surface, which can be placed flat against the specimen holder glass, maximizes NLM image quality. However, bone must be coarsely cut with tools like saws and hammers, which create surface fractures and debris. Brushing or washing the surface prior to NLM reduced surface debris and irregularity, while real-time focus depth adjustment can keep regions of interest in focus. However, imaging bone with NLM is still more challenging than soft tissue.

The ability of NLM to acquire images from different focal depths can pose a challenge for achieving exact correspondence between NLM and histology slides. Mismatches in the imaging/sectioning plane between NLM and histology can cause discordance if pathologies are
focal. However, NLM can acquire multiple images at continuously adjustable focal depths and therefore achieve more comprehensive sampling than possible with isolated histological sections. For example, NLM can visualize the curved surface of bone, three-dimensional structure of trabecular networks, and bone marrow embedded deep inside of marrow spaces. Serial sectioning can provide denser sampling for standard histology but is not commonly performed because it is time-consuming and costly. The diagnostic performance of NLM will need to be validated in blinded reading studies compared with standard histology. However, if NLM diagnostic accuracy is high, it has the potential to achieve higher accuracy than histology because sampling errors can be reduced.

The NLM images are generated using fluorescent signals from acridine orange/sulforhodamine 101 and are displayed in the color scale analogous to H&E histology to facilitate pathologist interpretation and minimize the training required. As an example, a previous blinded NLM reading study of prostate cancer by three pathologists achieved pooled sensitivity and specificity of 97.3% (93.7–99.1%; 95% confidence interval) and 100.0% (97.0–100.0%) with only three hours of training. Bone tissue imaging involves more frequent focus depth adjustment because of surface irregularities, therefore more training will likely be necessary. However, we expect that the total training time required will be on the order of hours rather than days.

Further technological improvements will be necessary to develop ergonomic and robust NLM evaluation protocols for bone. Cutting tools like diamond bone saws or ultrasonic bone scalpels will be helpful to minimize debris, saw marks, and cracks. Automated z-axis focus adjustment can compensate for the surface irregularity from grossing as well as the innate three-dimensional structure of bone without delays associated with manual focus adjustments. Using a low magnification (low NA) objective, NLM imaging is less sensitive to surface irregularity...
because the optical section is thick (e.g., 27 µm, with 5x objective\textsuperscript{63}). However, the low magnification reduces cellular/subcellular resolution and rapidly interchangeable higher magnification objectives are needed to achieve finer resolutions to visualize details of pathology.

The NLM tissue processing and imaging protocol is non-destructive and does not consume or alter tissue by decalcification and physical sectioning. After grossing to expose areas of interest, the fluorescent staining is rapid, requiring only ~5 minutes. After NLM imaging, the tissue can be used for standard assays such as H&E histology, immunohistochemistry, and fluorescence in situ hybridization\textsuperscript{142}.

The NLM system and tissue processing protocol were originally developed for rapid H&E-like histological evaluation of fresh tissue for use in intraoperative evaluation applications. Acridine orange and sulforhodamine 101 were chosen because they can label fresh surgical tissue specimens rapidly, with specificities very similar to H&E histology. However, NLM is a versatile technology and multiple contrast agents with different specificities have been demonstrated in other biological/biomedical studies\textsuperscript{146-149}. Future studies will investigate other contrast agents, which may further extend NLM imaging capability by improving visualization and diagnostic accuracy for specific pathologies.

NLM imaging might benefit applications where rapid histological evaluation is needed, such as intraoperative surgical margin evaluation of bone resection, rapid and nondestructive determination of biopsy adequacy, and triaging fresh tissue for specific assays. For example, intraoperative surgical margin evaluation for osteomyelitis surgery may decrease positive margin rates by guided additional resection. The positive margin rate of amputation for osteomyelitis is currently high (40.7%) and associated with a higher likelihood of poor outcome (81.8% for positive margin vs 25% for negative margin)\textsuperscript{131}. Postoperative antibiotic treatment can be longer
and more intense for patients with positive surgical margins. If NLM can provide accurate osteomyelitis margin assessment intraoperatively, NLM-guided resection may reduce positive margin rates, decrease the burden of postoperative care (antibiotic treatment and hospitalization), and improve patient outcomes.

Achieving clear margins is also critical in oncological surgery. For example, surgical margins of head and neck cancers are often intraoperatively evaluated with frozen sections. However, head and neck cancer specimens often involve bone, making sampling difficult and limiting the accuracy of tissue orientation for frozen sections.\textsuperscript{150} NLM can evaluate intact surgical margins from tissue specimens including bone and may improve sampling and tissue orientation accuracy compared to current frozen section protocols.

Non-destructive adequacy testing for bone needle biopsy is another potential application of NLM. A retrospective study that investigated CT-guided bony needle biopsy found a high frequency of cases with non-diagnostic cores (21.6%).\textsuperscript{141} Amongst these cases, \textasciitilde{}40\% were followed up with surgical biopsy, but the majority of biopsies were benign (>85\%). Other, low-risk patients (~50\%) had a clinical watchful follow-up, and most patients did not develop cancer (~98\%). Repeat core needle biopsy is shown to be effective to improve diagnostic yield (38\%, converted from non-diagnostic to diagnostic). NLM can non-destructively evaluate needle biopsy specimens and has the potential to perform intraprocedural adequacy testing to reduce sampling error and increase diagnostic yield. This will benefit patients by decreasing surgical biopsies or reducing anxiety caused by a long clinical follow-up with an unknown diagnosis.

NLM also may be useful for triaging large specimens. For example, histological examination of arthroplasty specimens is costly and sometimes only gross examination is performed because of the large specimen size and low incidence of unexpected findings.\textsuperscript{151}
However, the discrepancy rate between preoperative diagnosis and postoperative histology is shown to be small, but not negligible.\textsuperscript{152} The example in Fig. 7.2 shows NLM imaging of an entire femoral head cross-section can be performed in ~10 minutes (5 minutes staining + 5 minutes imaging, excluding standard grossing/sawing time). Therefore, NLM might be able to decrease the cost of histological examination while maintaining the diagnostic accuracy of standard histology.

NLM might also be a useful clinical research tool that is complementary to a radiographic analysis of bony microarchitecture or other biological studies. NLM could be used to guide sampling/microdissection of viable cells from targeted regions of interest. Bone growth and regeneration have been investigated with the ingestion of fluorophores such as calcein or tetracycline\textsuperscript{153,154}, which may have synergetic benefits when combined with NLM imaging technology.

NLM is extensively used in biological/biomedical research on cell cultures or small animals. However, the NLM instrument in this study was custom designed for rapid imaging of pathology specimens.\textsuperscript{63} Research NLM instruments use high-cost Ti:sapphire femtosecond laser light sources, however, our pathology imaging protocol is designed to be compatible with Yb-fiber femtosecond lasers at ~1 µm wavelength which have a much lower cost.\textsuperscript{142}

This study has several limitations. The range of pathologies and sample size are limited. For example, two of the most common cancers that metastasize to bone, breast and prostate cancers, are not included because of limited tissue availability, although NLM imaging of breast and prostate cancer was previously investigated and had high diagnostic accuracy.\textsuperscript{4,5} NLM images were read unblinded to paraffin H&E in order to assess similarities and differences. Therefore, the ability to diagnose bone pathology on NLM was not demonstrated. Sensitivity and specificity were not measured, and statistical analysis was not performed. More comprehensive studies are needed
to evaluate NLM performance for specific applications, and specimen preparation and imaging times must be within clinically acceptable limits.

In summary, NLM can provide rapid histological visualization of pathology in bone tissue, and may enable rapid diagnosis and intraoperative/procedural consultation. Rapid histological evaluation of bone may have applications in orthopedic surgery, bone biopsy, and cancer management. Future larger-scale studies on specific pathologies which compare blinded evaluation of NLM with paraffin histology to assess sensitivity, specificity, and inter-reader agreement are warranted.
Chapter 8

Summary of Thesis

In this thesis, we developed rapid NLM evaluation system and protocol, compared the performance of NLM with that of other imaging modalities, and validated it for several clinical scenarios.

In Chapter 2, we developed NLM technology and methods for the assessment of surgical specimens at rates sufficient to comprehensively assess surgical margins during breast conserving therapy and other intraoperative imaging scenarios. We introduced a multiscale imaging approach combining widefield white light imaging for gross specimen evaluation and identification of inked margins, low magnification NLM for rapid tissue surveying, and high magnification NLM for detailed evaluation at cellular resolution. We demonstrated rapid NLM assessment of centimeter-scale surgical specimens in a workflow analogous to FS processing of tissue, but faster, no freezing artifacts, and compatibility with subsequent histological processing.

In Chapter 3, we reported a direct comparison between CFM and NLM with dual agent staining and rendering of virtual H&E images for rapid histopathological evaluation of unfixed human breast tissue. We demonstrated that CFM can image the surface of unfixed breast tissue with comparable quality to NLM. The images of the surfaces of the specimens demonstrate that 20X CFM and 20X NLM have comparable imaging performance for individual nuclei in both normal breast tissue and IDC. Both 10X CFM and 10X NLM show degraded image quality as compared with 20X due to reduced lateral resolution and axial sectioning, but still enable visualization of individual nuclei in normal breast tissue. The 10X CFM images showed a
significant increase in background signal and loss of individual nuclei identification when imaging IDC because of the reduced optical sectioning and increased contribution of out-of-focal plane signal, although this effect was partially attributable to the lower objective NA.

In Chapter 4, we developed a video-rate MUSE system and performed a preliminary evaluation of surgical specimens for two potential applications where surgical margins are assessed intraoperatively: Mohs surgery for basal cell carcinoma of skin and lumpectomy for breast cancer. MUSE images of basal cell carcinoma showed high correspondence with frozen section H&E histology, suggesting that MUSE may be applicable to Mohs surgery. However, correspondence in breast tissue between MUSE and paraffin-embedded H&E histology was limited due to the thicker optical sectioning in MUSE, suggesting that further development is needed for breast surgical applications. The limited transverse image resolution of MUSE and the estimation of MUSE optical sectioning using co-registered NLM were also demonstrated.

In Chapter 5, we designed and has performed a clinical study for a prospective 98 patient interventional randomized controlled trial at BIDMC on patients who are receiving a lumpectomy for breast cancer. Intraoperative NLM real-time surgical feedback is provided to guide re-excision. The hypothesis is that NLM evaluation of surgical margins will reduce the rate of indication for repeat surgeries. The primary endpoint of the clinical trial is to demonstrate that intraoperative feedback with NLM evaluation of surgical margin will lead to lower repeat surgery rate by decreasing positive/close surgical margin rate. The secondary endpoint is the agreement of margin assessment between NLM and postoperative FFPE H&E histology. We are currently recruiting patients to participate in this study at BIDMC.

In Chapter 6, we demonstrated NLM imaging of simulated nerve-sparing radical prostatectomy specimens. Simulated surgical margins enabled observation of realistic surgical
margins of nerve-sparing specimens and optimization of NLM workflow for the future intraoperative radical prostatectomy NLM imaging project. The preliminary results indicated that NLM can visualize prostate cancer and surgical margins as previous studies suggested. Tissue processing and NLM evaluation time is short enough that the duration of the RP surgery would not need to be extended if NLM would be used intraoperatively. Discordant cases indicate that NLM and H&E can disagree when cancer is close to margin (<100-300 μm) or tissue edge is disrupted via fresh tissue grossing.

In Chapter 7, we demonstrated that NLM can provide rapid histological visualization of pathology in bone tissue. NLM enabled visualization of normal and pathological bone features with an appearance resembling the paraffin H&E. Differences such as changes in cell border sharpness, cellular and nucleolar size, and color patterns were noted, suggesting that training is required for accurate evaluation of bone pathology with NLM. Irregular surface contours and debris generated by gross tissue preparation of bone can make some regions difficult to evaluate with NLM, but the ability to perform rapid three-dimensional translation and sub-surface imaging reduced these problems. NLM is a promising technique for rapid evaluation of bone pathology, and rapid histological evaluation of bone may have applications in orthopedic surgery, bone biopsy, and cancer management.
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