OPTICAL CLEARING-ASSISTED OPTICAL SECTIONING MICROSCOPY FOR POINT-OF-CARE TISSUE IMAGING APPLICATIONS

AN ABSTRACT
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BY

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Abstract

Medical imaging techniques are crucial in providing medical professionals insight into human health, specifically for pathologists making diagnostic decisions from human biopsy samples. The standard technique for digital pathology imaging first requires generation of tissue sections on glass slides using extensive and slow tissue preparation techniques. However, an emerging method for imaging tissues near the patient and space and in time is a form of ex vivo microscopy called structured illumination microscopy (SIM). SIM is an optical sectioning technique that preferentially extracts the imaging focal plane from within a thick sample. Samples stained with fluorescent dyes and illuminated with the proper wavelength of light generate fluorescence in all directions, enabling images to be obtained in a back-reflection geometry rather than trans-illumination as required by traditional histopathology. However, the efficacy of SIM for thick tissue imaging is affected by the amount of light scattering in the tissue. As light passes through tissue, photons encounter different refractive indices as a result of inhomogeneous tissue, causing them to bend, changing their direction and impeding the modulation depth of the structured illumination, which in turn reduces the signal-to-noise ratio (SNR) of the resulting images. As photons moves deeper into a sample, they encounter more and more changes in refractive indices and eventually the illumination patterns become completely demodulated, and the optical section benefit of the SIM is lost. This study aims to reduce light scattering in SIM using rapid optical clearing. Optical clearing is a method of tissue preparation that makes tissues more homogenous, reducing the amount of differing refractive index, therefore, reducing light scattering. Using simple, non-toxic clearing agents in aqueous solution and a user-designed optical
clearing chamber, image clarity and the depth at which structures can be viewed have been studied in response to rapid clearing protocols in tissue phantoms and human prostate biopsy samples.
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Chapter 1:

Introduction/Background
I. Introduction

This study aims to improve point-of-care tissue histological imaging through the generation of high-quality images in rapid timeframes. Current methods of point-of-care histology include frozen section analysis, which requires tissue freezing and a multi-step processing protocol in order to generate multiple 4-5 micrometer thick sections of tissue samples on glass slides for pathological analysis.

The Translational Biophotonics Laboratory (TBL) focuses on the development and clinical application of optical technologies to improve the research, detection, management, and treatment related to cancer. To address the need for better methods for point-of-care pathology that require fewer processing steps and are non-destructive to the tissue, the laboratory utilizes structured illumination microscopy (SIM) and selective-plane illumination microscopy (SPIM) optical sectioning to obtain digital pathology images from minimally processed (i.e. fresh) biopsies and surgical resection specimens. Additionally, the laboratory is developing innovative computational models to enable automated cancer detection and analysis.

SIM is an optical sectioning microscopy technique that focuses on small, individual viewing planes, called focal planes, within a desired sample while SPIM generates full three-dimensional images of samples that are typically thicker than samples that can be adequately imaged using SIM [1].

Use of these techniques begins with a sample stained with a fluorescent dye that is struck by light waves of a specific wavelength so that the stained structures are highlighted in contrast to non-stained structures. The result is a molecular image. High
contrast images indicate an in-focus image whereas a low contrast image indicates that most of the field of view is out of focus.

Previous SIM works have focused on moving towards a more quantitative version of the SIM algorithm by quantifying the modulation contrast parameter, which controls the fraction of the measured light intensity that is recovered by the SIM demodulation algorithm (Figure 1.1, Figure 1.2). Additionally, after removing the out-of-focus light from the sectioned image, there is increased presence of noise from both the sectioned image and each of the out-of-focus planes (i.e., the shot noise of the background light is retained is retained in the final SIM image). [2].

$$i_{std}(x, y) = \sqrt{(i_1 - i_2)^2 + (i_1 - i_3)^2 + (i_2 - i_3)^2}$$

*Figure 1.1:* Root mean square calculation used by SIM to generate an optically section image at the focal plane from the 3 associated patterned images [2].

$$S(t_0, \omega_0) = 1 + m \cos(\omega t_0 + \phi_0)$$

*Figure 1.2:* Illumination mask pattern equation [13].

One potential drawback with optical sectioning using SIM is that deeper focal planes are difficult to clearly image when the modulation contrast of the pattern is demodulated, which limits SIM’s ability to image deep within a scattering sample. The pattern demodulation can be caused by a greater influence of out of focus light which degrades contrast, or distortion of the pattern by scattering and aberrations within the tissue, or most likely both. A technique that works to reduce the influence of tissue scattering with both SIM and SPIM is optical clearing, but the necessary extent of clearing differs between the microscopy techniques and the goal of the imaging task.
Optical clearing decreases the extent of light scattering in a sample by making a tissue more transparent. Light scattering occurs when wavelengths of light encounter structures of varying refractive indices in samples and change direction. This scattering of light blurs the patterns in SIM, reducing modulation contrast which results in blurred, out of focus images. However, optically clearing samples before using SIM decreases light scattering and improves the ability to image deeper into a sample [3].

Molecular images can be analyzed to detect changes and abnormalities in cellular function. This information can be extremely helpful in providing insight into the functioning of chemical and biological processes within a human being [4]. Optical clearing combined with microscopy techniques can be applied to image pathological samples, such as cancerous tissue. The increased viewing depth into a sample from optical clearing gives SIM increased potential to effectively image clinical samples.

Current methods for optical clearing include specific clearing agents that follow robust protocols, such as XCLARITY and saccharide clearing. This work focuses on finding the best clearing agent for SIM imaging of fresh tissue biopsies in rapid point-of-care pathology timeframes, taking into account clearing time, retention of molecular stain, decreased light scattering, and overall image quality.

II. Background

II.1 Current Rapid Histopathology Techniques

II.1.1 Frozen Section Analysis (FSA)

Frozen section procedure is an intraoperative consultation technique that provides insight into the presence of cancer cells in a patient. Samples ranging from small biopsies to larger organs that are removed from patients are sliced into very small sections (5-10
microns) using a cryostat. The frozen sections are then stained, usually with hematoxylin and eosin, and imaged for analysis. This procedure is much faster than traditional histology technique and can be performed in 10-20 minutes [5]. This method usually provides enough information regarding patient management immediately post-operation but is not used to make specific diagnoses. This is due to frozen section’s limited sampling and the quality of the sections produced are much lower and therefore less reliable than permanent final pathology sections. Additionally, the procedure is quite difficult to perform [6].

II.1.2 Touch Imprint Cytology (TIC)

Touch imprint cytology, sometimes referred to as “touch-prep”, is another form of intraoperative diagnostic technique for surgical resection margin analysis. The procedure works by touching a sample to a glass slide, which leaves an imprint of the sample on the slide that is then fixed with alcohol and treated with hematoxylin and eosin stain for imaging. Advantages of the procedure include the fact that it is a fairly simple technique and provides immediate results with minimal artifacts. It is also an inexpensive technique. However, the procedure limits the depth of imaging as it only allows for the observance of surface level cells. Additionally, thicker, well-differentiated tumors with dense fibrous stroma cannot be adequately observed through this technique [7].

As a result of the drawbacks of FSA and TIC, there is a need for higher quality rapid intraoperative histology techniques.
II.2 Emerging Slide-Free Imaging Techniques for Digital Pathology

II.2.1 Fluorescence Microscopy

Current imaging techniques for observing thick samples often utilize fluorescence microscopy due to the fact that using fluorescence contrast light does not need to be transmitted through the tissue to obtain an image. Standard (wide-field) fluorescence microscopy involves illumination and collection of emitted light through the same microscope objective in a back-reflection geometry called epi-illumination. Although wide-field fluorescence microscopy is suitable for thin samples such as cells in culture or thin tissue sections, it suffers from the same problems that limit traditional bright-field microscopy in thick samples. Alternatively, fluorescence optical sectioning microscopy enables preferential isolation of the focal plane from the out-of-focus planes in thick samples, which improves image clarity in scattering samples. The main types of optical sectioning fluorescence microscopy include confocal laser scanning microscopy, two photon microscopy, SPIM, and SIM. The basis of fluorescence microscopy lies in the excitation and relaxation of electrons in molecules. When a molecule is struck with a photon, electrons become raised from the ground state, $E_0$, to a higher energy state, called the excited state, $E_2$. The electrons then begin to relax to the lowest vibrational energy state of the excited state, $E_1$. At this point, the electrons have lost a portion of their excited, vibrational energy. Some of this energy is lost to the surrounding environment in the form of heat, while the rest is emitted in the form of light. The wavelength of light emitted must always be of a longer wavelength than the initial photon wavelength because some of the energy has been lost during the excitation-deactivation cycle and a longer wavelength indicates a lower energy level. The exact wavelength can be
determined by analyzing the difference in energy between the electrons at the E₁ and E₀ energy states. This process can be visualized using a Jablonski diagram (Figure 1.3) [8].

![Jablonski diagram](image)

*Figure 1.3: Jablonski diagram demonstrating the process of excitation, vibrational relaxation, and light emission. These processes are labeled 1, 2, and 3, respectively [8].

The fluorescence cycle can repeat many times, although a theoretical maximum number of cycles does exist. The main way in which the fluorescence cycle becomes disrupted is from photobleaching. Photobleaching is when a fluorophore becomes irreversibly destroyed and occurs when the intensity of light that excites the molecule, at step 1 from Figure 1, is too great causing an irreversible chemical structure alteration [8].

This project utilizes fluorescence in the form of fluorophores, which are fluorescent molecules that bind to macromolecules present within the tissue sample. Different molecular stains that contain fluorophores bind to different macromolecules and undergo the fluorescence cycle at different wavelengths. The major stains that will be
utilized in this project are DRAQ5 and acridine orange. DRAQ5 stains the double-stranded DNA in nuclei present in a given sample and most efficiently fluoresces when struck with photons that have a wavelength of 647 nanometers. Acridine orange is a nucleic acid stain that binds to DNA, RNA, and some stromal components, which includes adipocyte cell walls, cytoplasm (typically RNA), myocytes, and acidic vacuoles. Acridine orange is efficiently excited by photons that have a wavelength of 470 nanometers. Fluorescence can be analyzed one wavelength at a time (such as in a scanning spectrometer), or by collecting bands of multiple wavelengths at a time using optical filters (typically in microscopic imaging). This is typically achieved using sets of optical filters in the microscope that filter the excitation light incident on the sample (excitation filter), reflect the illumination light to the sample and prevent collection of reflected illumination light by the objective lens (dichroic beamsplitter), and filter the emitted light from the sample before detection (emission filter). Filter sets can be optimized for a single set of wavelengths or may be optimized to enable simultaneous imaging of multiple bandwidths, enabling multiple dyes to be imaged in the same system. Using multiple wavelengths, referred to as multicolor imaging, can provide powerful contrast and generate high-clarity images [8]. The contrast between fluoresced structures and non-fluoresced structures in tissues provides the basis for image production using fluorescence microscopy.

II.2.2 Confocal Microscopy

Confocal microscopy is an optical sectioning fluorescence imaging technique that offers several advantages over conventional wide-field microscopy techniques. It permits a shallow depth of field, minimal out-of-focus light collection, and the ability to image
optical sections of samples, including thick tissues. In conventional widefield optical microscopes, fluorescence emitted by the specimen is generated throughout the specimen thickness, which when collected by the objective lens in addition to light generated from the focal plane of interest, creates an out-of-focus signal that impedes the clarity of resulting images. This results from the way in which light interacts with samples. The entire sample within the cone of illumination of the microscope objective is exposed to excitation light, and all of the fluorescent light generated is collected by the objective lens and transmitted to the detector (either the eye via the eyepiece, or a digital camera via a tube lens). Confocal microscopes are able to exclude fluorescence originating from outside the focal plane as a result of its optical design (Figure 1.4). Confocal microscopes use a focused beam of laser light that is scanned across the sample. A small adjustable pinhole is placed in front of the light detector in a plane that is conjugate to the focal point of the laser beam. In this way, light that is generated from outside of the focused spot is rejected via spatial filtering, and out-of-focus light is thereby eliminated before detection. Images can then be generated by scanning the laser beam systematically over the field of view of the objective lens and recording the emitted intensity at each location, allowing images to be generated. (Figure 1.5) This approach allows for 3-dimensional image capture of living tissues and allows for the image capture of samples subjected to multiple fluorescent stains [9].
II.2.3 Selective Plane Illumination Microscopy (SPIM)

SPIM is a form of fluorescence microscopy that works by utilizing two optical paths and an orthogonal imaging system to illuminate a sample with a thin light sheet.
SPIM’s advantages include its capability to image larger samples, limit out-of-focus background, and to limit photodamage to samples.

An issue encountered with SPIM is the resulting image clarity is often hindered due to light scattering, particularly in larger samples [12]. A common way this issue is addressed is through optical clearing. In order to be imaged using SPIM, a sample must be optically cleared to the point at which it is almost completely transparent, which can be accomplished by using solvent-based methods, or the clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue hydrogel (CLARITY) (Figure 1.7).
Figure 1.7: Before and after of a mouse embryo that has been optically cleared utilizing X-CLARITY protocol [11].

A current accepted method for optimizing image contrast for SPIM is through the use of electronic confocal slit detection (eCSD). eCSD uses the rolling shutter mode of the camera sensor to create a confocal slit, allowing there to be a limited amount of out-of-focus light that is detected in an image [10].

II.2.4 Structured Illumination Microscopy (SIM)

Structured illumination functions by projecting a single-spatial frequency grid pattern onto the focus plane. This grid provides variation of illumination power within a specified optically sectioned focus plane, through use of a spatial light modulator. This variation creates a local contrast variation in the image that is effectively transferred to the image in the in-focus portions of the image, but not the out-of-focus portions. Thus, in effect, SIM allows the in-focus portion of the signal to be modulated preferentially, which in turn allows for the retention of this signal and the rejection of the non-modulated signal via an appropriate demodulation algorithm. In order to ensure that all areas of the in-focus image are recovered with equal intensity, usually 3 patterns phase-shifted by 1/3 of the grid period are projected onto the sample sequentially, and images
are taken of each phase-shifted pattern illumination. These phase-shifted images are the combined within a single demodulation step, which reproduces a seamless optical section image without the presence of the grid (suppressed carrier). The thickness of the optical section is governed by the spatial frequency of the illumination pattern, with higher frequency patterns giving thinner optical sections. The optical section thickness is limited by the achievable modulation frequency given by the system, which is limited either by the SLM pixel pitch or the attenuation of the illumination pattern by the imaging system modulation transfer functions, or a combination of the two [1].

The SIM system utilizes a sCMOS camera to take images of samples. For every location of the sample imaged, there are up to 5 images that can be generated. First, 3 patterned images are captured which vary slightly due to the projection of the grid pattern onto the sample. The fourth image is the sectioned image obtained by combining the 3

![Figure 1.8](image-url)
patterned images in the demodulation algorithm. The sectioned image is a combination of the three pattern images, imparted onto one another to create a high contrast image. The fifth image that can be generated is the wide-field image, which can be recreated by taking the average of the 3 patterned illumination images. This provides a convenient way to compare wide-field vs. optical sectioning improvements on the exact same area of a sample, which is difficult or impossible to do with other optical sectioning modalities. One of the biggest disadvantages of using structured illumination is the effect of tissue scattering which degrades the modulation pattern contrast. This study aims to alleviate this issue by applying optical clearing protocol to SIM.

II.3 Tissue Optics – Light scattering

Light scattering originates from the inhomogeneity of tissue. As light passes through tissue, it encounters various structures within the tissue that have varying refractive indices. Highly compact structures such as elastic fibers and collagen have a high refractive index and media such as cytoplasm and interstitial fluid have low refractive indices [3]. The movement of light through media boundaries that have different refractive indices cause wavelengths of light to bend and change direction (Figure 1.10). As light moves deeper into tissues and encounters more structures and media, bulk scattering occurs and the clarity of an image becomes more and more diminished. A result of this phenomenon is the limitation of image depth to approximately 100 micrometers for most optical imaging techniques and approximately 500 micrometers to 2 millimeters for multiphoton microscopy [16].
Figure 1.9: Depiction of the bending of photons as they pass through refractive boundaries from a less dense medium such as air into a higher density medium. In the case of this study, the light moves from air across various refractive indices present within different tissues [17].

II.4 Optical Clearing Technique

Optical clearing is a method of tissue preparation that makes tissues more transparent, prior to imaging. It is an incredibly useful technique because it makes the tissue more homogeneous which, in turn, helps to eliminate light scattering. This results in an increased depth of imaging while maintaining contrast and improving overall image quality. Optical clearing agents work by acting as a partial substitute for interstitial fluid, One of the biggest causes of light scattering comes from the movement of photons through high refractive index components, such as collagen and elastic fibers, to lower refractive index components, such as interstitial fluid and cytoplasm. Collagen, the most abundant tissue component, has a refractive index of 1.43-1.53 and water, which makes
up the bulk of interstitial fluid, has a refractive index of 1.33. Optical clearing agents have a high refractive index and closely resemble the refractive indices of higher refractive index fibers. This creates refractive boundaries that have much smaller changes in refractive index then would be the case without optical clearing treatment [3]. On a macro scale, the tissue becomes more transparent, which can be seen by the naked eye [18].

II.5 Optical Clearing Agents

There are many types of optical clearing agents and the application of some can require complex protocols. CLARITY works by forming a hydrogel in a given sample. The hydrogel mesh is formed at the same time as lipids are extracted from the sample through electrophoresis. The electrophoresis is carried out by applying an electric current through an ionic detergent solution that contains the sample and is accomplished using XCLARITY protocol. The resulting tissue is nearly transparent and slightly larger than originally, as a result of the hydrogel polymer expansion. The macromolecules and associated microarchitecture within the sample remain intact and the sample can be fluorescently labeled. However, XCLARITY protocol often takes multiple hours to several days to create near-transparent tissues and the apparatus and reagents for performing the protocol are expensive [11].

2,2’-thiodiethanol (TDE) has been shown to increase the transparency and light penetration depth of tissues but is not widely used. This is because higher concentrations of TDE often extinguish the action of fluorescent proteins or pull the fluorescent proteins out of the samples during clearing, inhibiting fluorescence of fluorophores during imaging. However, lower concentrations of TDE have demonstrated strong clearing
properties while maintain fluorescence in 400-micron fixed mouse brain slices after 30 minutes of clearing [19]. While low-concentration TDE is able to produce near-transparent tissue in a shorter period then its XCLARITY counterpart, the 30 minutes time period required is still longer then desired for point of care applications. Additionally, 400-microns is smaller than the depth of most prostate biopsies.

Furthermore, disaccharides such as sucrose and maltose and monosaccharides such as fructose have demonstrated success as optical clearing agents of skin tissue of 1 to 3 millimeters in thickness. This thickness corresponds more analogously to the size of the average biopsy. Additionally, saccharide clearing agents have demonstrated rapid clearing success in shorter time durations than TDE. Saccharides can create near-transparent tissue with improved signal intensity and imaging depth as quickly as 6 minutes of clearing.

![Figure 1.10: Molecular structures of A) fructose B) sucrose and C) maltose [20].](image)
II.6 Need for Rapid Optical Clearing to Assist Optical Sectioning

It is clear that light scattering is an issue for even the more advantageous molecular imaging techniques. Our overall objective was to determine whether optical clearing protocols could be useful to improve the quality of structured illumination microscopy by reducing tissue scattering, thereby increasing pattern modulation contrast and recovered signal intensity. To test this, first, we developed a specialized sample cassette in order to reliably hold the sample while immersed in optical clearing agents for varying lengths of time. Then, we studied the effect of various non-toxic clearing agents to determine the potential improvement in image clarity and imaging depth in rapid timeframes (i.e. less than 10 minutes.) using non-human tissues. Finally, we tested the optimum protocols on human prostate tissues to determine whether there would be any benefit for rapid optical clearing in point-of-care biopsy imaging.
Chapter 2:
Initial Evaluation of Saccharide-Base Aqueous Clearing Agents
and Methods for Optical Clearing
I. Introduction

This portion of the study will focus on evaluating the suitability of saccharide-based aqueous solutions for rapid optical clearing of tissues, specifically within a clinically-viable time period of under 10 minutes. We will outline the reagents and methods for sample preparation and imaging techniques to be used throughout this study. We focused on saccharide-based clearing agents due to their non-toxic nature, ability to clear using simpler immersion, and ability to clear samples rapidly. We will identify the specific functional requirements needed for the design of an optical clearing chamber to address the disadvantages of the current techniques.

II. Current Methods

II.1 Materials and Methods

The reagents required for this portion of the study were chicken breast, 0.002% acridine orange fluorescent dye, 100% weight-to-volume maltose-in-PBS solution and 100% PBS. Chicken breast was selected as an analogous test sample to human biopsy tissue due to its similar light color and consistency. Acridine orange was used a general stain and fluoresces when illuminated by 470 nanometer wavelength photons [21]. 100% PBS was used to rinse the sample into after acridine orange staining to remove any excess stain on the tissue. 100% maltose-in-PBS solution was selected as an optical clearing agent because it has been shown to produce optically clear tissues. It is also a low-cost reagent, as compared to other optical clearing agents such as TDE and XCLARITY. Having a dry sample after staining was essential to minimize the bubbles or
liquid droplets present between the tissue and the glass slide so that the images generated had as minimal obstruction for analysis as possible.

II.2 Sample Preparation

The tissue samples were acquired by cutting roughly 2-3 millimeter thick pieces from a piece of chicken breast using a laboratory knife. 2-3 millimeters was the goal thickness to accurately mimic the size of biopsy samples that are evaluated in the clinical setting. Samples were then placed into a small plastic dish containing the acridine orange stain for 1 minute and 45 seconds. The amount of acridine orange used was arbitrary but in an amount that was enough to completely surround the biopsy in order to ensure maximum permeability and surface coverage of the stain. The sample was then removed from the acridine orange dish, rinsed in 100% PBS located in another dish, and dab-dried on a Kimwipe. Next, the midpoint of the sample was marked using The Davidson Marking System orange histology ink. This ink acted as a reference point for the researcher to be able to locate the surface of the sample during future image capture and evaluation steps.

The sample was placed into a glass dish and placed onto the SIM stage. The sample was then viewed in the live widefield mode in LabVIEW where the user could assess the quantity of air bubbles and determine if the sample could be adequately imaged. If not, the sample needed to be taken off the stage and further dried. If the sample needed to be dried further, the sample was further dab-dried on a Kimwipe and the glass slide was cleaned with 70% ethanol prior to the sample being placed back onto the glass slide. Once we deemed the sample dry enough for imaging, we moved on to capturing images of tissue structures.
II.3 Image Capture

The first step of image capture was to find the histology ink mark in live widefield imaging mode in LabVIEW. We then found a frame around this mark with as few air bubbles as possible, while also keeping part of the reference ink spot in the corner of the image frame. The purpose of this was to have a way of identifying which frame in the future z-stack analysis corresponded to the surface of the tissue. Once the frame for imaging had been determined, we switched from live mode to the image capture mode in the LabVIEW program. Here, we were able to input the settings for the z-stack image capture (Figure 2.1). For this study, 20 images were taken at 10 micrometer increments for a total of 200 micrometers worth of z-stack depth. The spatial frequency of the pattern was 36 pixel pitch (given in terms of SLM pixel pitch i.e. 36 pixels per cycle at the SLM), and it determined the optical section thickness of the image. The integration time of 40ms corresponds to the exposure time of the camera for each of the patterned images used to calculate the SIM image. After the 0-minute z-stack was taken, 1-milliliter of maltose/PBS solution was pipetted onto the glass dish to begin the clearing process and successive z-stacks were taken at 5 and 10 minute increments, giving in total 3 z-stacks for each sample. Once all the desired z-stacks had been acquired, we then moved on to image processing and analysis.

![Graphical representation of what happens during the z-stack image capture. A) The z-stack is initialized at a selected point below the z-stack. B) The camera moves incrementally up, deeper into the tissue taking images along the way at user-specified increments. C) The z-stack is complete once the objective has moved the full distance upward, as inputted in LabVIEW by the user.](image)
III. Results and Discussion

Sample stability, presence of air bubbles, and the chosen z-stack depth proved to be issues with the outlined protocol. Once the sample was immersed in the fructose/PBS solution, the liquid would cause the sample to peel off the glass dish and float to the top of the liquid volume. This inhibited the sample from being imaged at all and data on the clearing effects were unable to be recorded. An alternative situation occurred if the sample was in fact able to maintain adhesion to the glass slide during the 10-minute clearing process. Dark regions along the edges of the image capture frame appeared after the clearing process was finalized. Based on the current procedure and the first issue of the sample losing slide adhesion, it was unclear if the region occurred as a result of optical clearing or if that region had lost its adhesion to the glass slide and had begun peeling off of it, but had not peeled off completely (Figure 2.2). This uncertainty was also evident in that the observed surface frame appeared at different depths for each of the different time increment z-stacks (Figure 2.3). The observed surface frame should have been at the exact same depth from the initialization of the z-stack for all the clearing times.
An additional issue was the presence of air bubbles in the imaging frames. While the initial setup and imaging of the 0-minute cleared sample was pretty good at removing air bubbles, it did not always completely remove them, resulting in the need to remove the sample and re-dry it multiple times to achieve minimal air bubbles. Furthermore, even after this step, air bubbles would often appear as a result of the application of the optical clearing agent (Figure 2.4) This further indicated that the sample was not fully secured to
the glass dish and that there were passageways for air bubbles to present themselves in between the sample and the glass dish. At that point, the sample being used would have already undergone some degree of clearing, rendering the sample unusable for future rapid clearing experiments.

![Figure 2.4](image)

*Figure 2.4: Each of the images shows the 110 micron deep frame from the z-stack initialization. A) 0 minutes of clearing. Some mild air bubbles present. B) 5 minutes of clearing more intrusive air bubbles have become present. C) 10 minutes of clearing. Highly intrusive air bubbles have entered the image frame.*

We looked at the sample images from Figure 2.4 with a selected region of interest for analysis (Figure 2.5). ROI selection also proves troublesome with air bubbles because possible areas for selection are small and must lack air bubbles for all 3 stacks. If air bubbles change throughout between the sequential stacks, this process becomes much harder and the results are less representative of the entire image frame. Even sometimes ROI selection must include some small air bubbles because that is the only choice possible.
Furthermore, the third main issue with the outlined procedure was the choice to size the z-stacks at 200 microns. As evident in Figure 2.6, once the samples were quantitatively analyzed in MATLAB, it was evident that there were important data points missing that could have been acquired using a larger sized (i.e. deeper) z-stack. One of the metrics we will use to observe the effects of optical clearing on the images is the full width at half maximum value of the image intensity versus axial defocus curve. In this case, it is the distance in microns between half of the maximum value of each of the clearing time graphs on each side of the peak. This metric will provide insight into the impact of optical clearing on the depth into tissues that structures can be seen clearly. However, we are able to see that the 10-minute graph does not contain the second half maximum value for 5 minutes of clearing because it occurred deeper than 200 microns from the starting point of the z-stack.

Figure 2.5: This figure contains the same image frames as figure XXX, except and identified region of interest has been selected to be used for quantitative analysis in MATLAB.
IV. Conclusion

The outlined procedure proved to be a useful starting point for analyzing the effect of optical clearing agents, however, we identified several areas for improvement. The acridine orange staining protocol proved robust as the samples still fluoresced after the 10-minute clearing cycle, something that was unable to be achieved using the Eosin bulk stain in previous experiments. The main points of emphasis that needed to be addressed to improve this protocol were to improve sample adhesion to the glass plate, limit the presence of air bubbles between the tissue and glass slide, and increase the size of the z-stack capture.

Figure 2.6: The graphs illustrated show the A) average intensity values and B) standard deviation in the pixel values of each frame at the specified depth of the z-stack. Each of the graphs has been normalized to their first data point to more accurately compare the full width at half maximums of each of the graphs for the different clearing times.
Chapter 3

Design and Testing of an Optical Clearing Chamber
I. Introduction

We will address the drawbacks of the previous experimental protocols in place for optically clearing tissue by designing an optical clearing chamber. The chamber was designed to maintain the presence of a liquid volume without leaking, keep samples adhered to a glass slide while immersed in the optical clearing solution, and be compatible with the SIM imaging system used in this work.

II. Materials and Methods

The goal of the sample chamber was to design it such that a standard 2-millimeter biopsy sample could be imaged on a glass slide while the sample was fully immersed in an optical clearing agent, without any movement of the sample. The design requirements for the construct were that it must have a fast and intuitive assembly, maintain a liquid volume of 1-milliliter for 10 minutes, allow the sample to remain stable throughout the imaging process, and be compatible with the Translation Biophotonics Laboratory’s SIM system.

The chamber construct was designed using SolidWorks 2018 3D modeling design software. The construct consists of 2 main pieces that are secured together using 4 stainless steel 25-millimeter M6 machine screws and corresponding stainless steel M6 hex nuts to create a seal. The base and top piece are made of polylactic acid (PLA) plastic and printed on Ultimaker 3 3D printers.

The base is an 85x40x5 millimeter rectangle with a single 20-millimeter diameter hole cut entirely through the middle and a 25x50x1 millimeter rectangular cutout centered on the overall rectangle. The hole exists so the microscope objective can view the sample from underneath the construct and the smaller rectangle’s purpose is to act as
the resting place for the glass slide. Additionally, there are 4 7.5-millimeter holes located slightly off center from the central axis of the longer edge. This area functions as the area where the machine screws will be placed to secure the top piece to the base (Figure 3.1).

The top piece is a 50x35x4 rectangle, also with a 20-millimeter central cutout hole with mesh-like bars extending throughout the central hole cutout, coplanar with the bottom face. These bars will act as material to gently press the sample onto the slide for stability, while maintaining open area for optical clearing agent can be applied to contact the sample. The top piece also has 4 7.5-millimeter hole cutouts that correspond to the 4-hole cutouts in the base for the machines screws (Figure 3.1).

![Figure 3.1: This figure contains the basic components of the optical clearing chamber construct. A) Rectangular piece for glass slide to be placed onto SIM stage. B) Top piece to stabilize sample and maintain liquid volume. C) Sealing bolts.](image)

Next, 3 gasket materials were applied to 3 separate top pieces to be tested to see which of the materials created the best and most consistent seal. The gasket material was desired to create a seal with the glass slide so that optical clearing agent did not leak out of its reservoir. One of the gaskets was a Shop Craft fiber gasket material purchased from
Amazon. It was custom cut so that it could be placed on all the solid surface on the bottom face of the top piece. The second material was a 1/16 fractional width square-profile oil-resistant Buna-N O-ring, placed along the outside edge of the central opening. The third material was a 1/16 fractional width circular-profile oil-resistant Buna-N O-ring. Both O-rings were purchased from McMaster-Carr (Figure 3.2). All 3 materials were secured to the top piece using a hot glue gun.

![Figure 3.2](image)

*Figure 3.2: From the left: round O-ring, square O-ring, custom shop gasket material. Each shown as applied to the top piece of the clearing chamber.*

Once the gasket material had been placed in the desired locations, the glass slide could then be placed in the glass slide divot of the base piece and the top piece could be secured to the base via the 4 machine screws and corresponding hex nuts. The extent of the force used to secure the top piece to the base piece was variable. For the custom Shop Craft fiber gasket, the machine screws and hex nuts could be secured as tight as possible. For the O-rings, the situation was different. The O-rings add an approximate 2-
millimeters of depth towards where the place of the glass slide. As a result, the machine screws and hex nuts could not be secured as tight as possible due to the O-rings depth putting excess for on the glass slide, which would cause the glass the shatter inside of the construct. In order to secure the top pieces with the O-ring gaskets, the top piece had to be secured tight enough, but not too tight so that the glass slide would break under the force (Figure 3.3).

Once the chambers were assembled, the efficacy of each of the gasket materials was ready to be tested.

The 3 assembled chambers were tested using 1-millimeter of water placed onto the glass slide that was exposed by the central opening in the top piece. Each chamber underwent 5 separate trials where the assembly was timed, water was placed onto the slide, success of the seal was measured, the chamber was taken apart and dried and then
reassembled for the next trial. Success of the seal was measured on a scale of 0-3 where 0 was scored for no leakage, 1 was scored for minor leakage that would likely not impede the use of the chamber for imaging, 2 was scored for ample leakage that would likely be detrimental to imaging, and 3 was scored for aggressive leaks that rendered the chamber ineffective. Ease of assembly was measured as the time required to assemble the chamber for each trial. Each of the trials were performed for 10 minutes, the time determined to be clinically viable for optically clearing samples.

**III. Results and Discussion**

The round profile O-ring scored the best in preventing leakage with an average score of 0 and the custom Shop Craft gasket scored the worst with an average score of 2.6. Regarding assembly time, the custom Shop craft gasket scored the best with an average assembly time of 58 seconds and the round profile O-ring scored the worst with an average assembly time of 1 minute and 24 seconds. These results are summarized in table 1.

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<th>0 1:30</th>
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<th>0 1:15</th>
<th>0 1:24</th>
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</thead>
<tbody>
<tr>
<td>Square-profile O-ring</td>
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<td>1 1:13</td>
<td>2 1:31</td>
<td>2 1:07</td>
<td>1 1:07</td>
<td>1.6 1:15</td>
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<tr>
<td>Custom Shop Craft Gasket</td>
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<td>3 :59</td>
<td>2 :49</td>
<td>3 :57</td>
<td>2.6 :58</td>
</tr>
</tbody>
</table>

*Table 1*: The above table shows the test results of the 3 sealants in question for the final clearing chamber. The value in the top of each of the cells corresponds to the sealant grade and the value below it is the time required for the assembly of the trial.
From these results, it was determined that the round profile O-ring was the best option for the chamber sealant material. Even though it had the slowest assembly time, the difference in time, compared to the custom gasket, was on average only 26 seconds which is not significantly longer enough to be deemed unviable. Being able to consistently assemble the construct in 1 minute and 24 seconds would be clinically viable at the point-of-care. The more important metric was the ability to maintain the liquid seal. The fact that the round O-ring had an average score of 0, indicating that it never led to any liquid loss during the trials, led to it being selected as the most effective sealant for the clearing chamber.

IV. Conclusion

The drawbacks of previously described methods for optical clearing were addressed by the creation of an optical clearing chamber. The use of high-density infill PLA and rubber round-profile O-rings were proven to create an optimally liquid-sealed chamber with a glass microscope slide. Liquid chambers also have broader research applications. Microfluidic chambers are designed to hold sub-millimeter volumes of fluid to analyze chemotaxis of neutrophils, breast cancer cells and to promote neurite growth [21]. This concept will be applied to holding a liquid volume of test saccharide in PBS solutions to optically clear human biopsy analogous tissue samples to reduce light scattering and improve overall SIM image quality.
Chapter 4

Optimal Optical Clearing Agent Selection and Optical Clearing Chamber Stability Testing
I. Introduction

With the drawbacks of previous optical clearing protocols identified and the design of an optical clearing chamber proven to maintain an adequate liquid volume for the duration necessary based on experimental parameters, the next step was to identify the most effective optimal clearing agent. We tested the saccharides maltose, sucrose, and fructose on non-human tissue at 100% weight-to-volume ratios with PBS at 0, 5, and 10-minute increments. The effects of optical clearing were assessed by analyzing changes in the average intensity, standard deviation, and contrast of the image pixels and changes in the distance of the full width at half maximum after optical clearing.

II. Materials and Methods

The methods for staining the sample and image capture were the same as outlined in Chapter 2. However, instead of being placed onto a glass dish and then onto the SIM stage, the sample was placed onto a glass slide and the glass slide was placed into the newly designed optical clearing chamber. Additionally, instead of only using 100% weight-to-volume maltose-in-PBS solution as a clearing agent, 100% sucrose and fructose in PBS solutions were added as test reagents. These saccharides have all demonstrated optical clearing properties in samples [23]. All 3 were tested for this experiment to determine which one achieved the best short-term optical clearing results, as desired for point-of-care SIM imaging.

In addition to observing the effects of the 3 saccharide solutions, the ability of the optical clearing chamber to adequately stabilize samples and limit the presence of air bubbles during optical clearing was tested.
II.I Image Processing

Image processing commenced with the viewing of the z-stacks for each of the 3 time increments for each sample using ImageJ software. The researcher viewed the images within the z-stacks aiming to identify a region of interest (ROI) that could be used for all 3 of the z-stacks. The goal was to find an area that did not contain any air bubbles or artifacts and contained clear, identifiable features throughout each of the stacks. Once this region had been identified, the researcher used the rectangle tool in ImageJ to specifically identify the ROI by its pixel coordinates. These coordinates are important for the next step, MATLAB analysis. The researcher used a user-generated MATLAB code that can read in all the images of the 3 z-stacks, identify the ROI within the stacks, and generate comparison graphs for each of the stacks. The parameters used for comparison were, average intensity, average standard deviation, modulation contrast, and full width at half maximum of the image pixels, as compared to depth into the tissue. The modulation contrast measures the ability of the SIM pattern to demonstrate contrast within the image as function of depth. The full width at half maximum parameter was used as a metric to identify changes in the depth at which adequate signal for pathological analysis could occur after optical clearing. The hypothesized curve for each of the graphs was a smooth bell curve shape. The goal was to observe any inconsistencies, consistencies, and shifts in the curve shapes for the specified parameters during z-stack analysis to determine the effects of optical clearing.

III. Results and Discussion

Upon observing the average intensity, standard deviation, modulation contrast, and full width at half maximum of the 3 clearing solutions at the 3 different time
increments, it is apparent that the optimal combination of factors to use is 100% fructose-in-PBS solution as the clearing agent for 10 minute of clearing time. Fructose led to the greatest increase in the full width at half maximum distance, almost double both sucrose and maltose’s results. Fructose also was the only clearing agent to yield an increase in the maximum average intensity of the resulting image pixels whereas sucrose and maltose led to decreases in average maximum intensity as the duration of clearing increased. Additionally, sucrose decreased the standard deviation of the image pixels whereas maltose and fructose had a positive impact on the standard deviation of image pixels. Fructose was the more favorable choice for this metric because fructose led to more than double the standard deviation (i.e. more contrast) at 10 minutes of clearing, compared to 0 minutes of clearing and maltose only resulted in a 10% increase after 10 minutes. Furthermore, the modulation contrast was improved by fructose, improved but less so by sucrose and harmed by maltose.

Qualitatively, all the solutions had an apparent, but mild impact on the observed transparency of the tissue after 10 minutes of clearing. None of the solutions yielded nearly transparent tissues the way XCLARITY does, but there appears to be some vision of text behind the optically cleared samples. Placing an optical clearing sample on top of text is a way to qualitatively observe the effects of optical clearing on tissues.
Figure 4.1: A) Average intensity and B) standard deviation and C) modulation contrast results for 100% w/v fructose/PBS solution.
Figure 4.2: A) Chicken breast sample after staining but prior to any optical clearing B) Sample after insertion into optical clearing chamber and 10 minutes of fructose clearing.

Figure 4.3: A) Average intensity and B) standard deviation and C) modulation contrast results for 100% w/v sucrose/PBS solution.
Figure 4.4: A) Chicken breast sample after staining but prior to any optical clearing B) Sample after insertion into optical clearing chamber and 10 minutes of sucrose clearing.

Figure 4.5: A) Average intensity and B) standard deviation and C) modulation contrast results for 100% w/v maltose/PBS solution.
Figure 4.6: A) Chicken breast sample after staining but prior to any optical clearing B) Sample after insertion into optical clearing chamber and 10 minutes of maltose clearing.

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<thead>
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<td>90</td>
<td>90</td>
<td>90</td>
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<td>Maltose</td>
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<td>120</td>
<td>120</td>
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Table 2: Depth of maximum average intensity, in microns.

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<th>10 Minutes</th>
<th>Overall % Change</th>
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<td>Fructose</td>
<td>5.572</td>
<td>6.144</td>
<td>6.717</td>
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<tr>
<td>Sucrose</td>
<td>4.730</td>
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<td>-15.03</td>
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<tr>
<td>Maltose</td>
<td>11.147</td>
<td>9.480</td>
<td>8.816</td>
<td>-20.91</td>
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Table 3: Max average intensity, normalized pixel values.
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<th>Overall % Change</th>
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<td>8.003</td>
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<td>+111.13</td>
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<tr>
<td>Sucrose</td>
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<td>3.838</td>
<td>3.672</td>
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<tr>
<td>Maltose</td>
<td>23.503</td>
<td>22.48</td>
<td>25.915</td>
<td>+10.26</td>
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</table>

*Table 4: Max standard deviation, normalized pixel values.*

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<tr>
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<td>+7.01</td>
<td>+5.16%</td>
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<tr>
<td>Sucrose</td>
<td>123.15</td>
<td>124.70</td>
<td>126.66</td>
<td>+3.51</td>
<td>+2.85%</td>
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<tr>
<td>Maltose</td>
<td>133.88</td>
<td>135.89</td>
<td>137.30</td>
<td>+3.42</td>
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</table>

*Table 5: Full width at half maximum, in microns.*

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<th>5 Minutes</th>
<th>10 Minutes</th>
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<tr>
<td>Fructose</td>
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<tr>
<td>Sucrose</td>
<td>0.0607</td>
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<td>0.0619</td>
<td>+1.98%</td>
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<td>Maltose</td>
<td>0.0743</td>
<td>0.0660</td>
<td>0.0632</td>
<td>-14.94%</td>
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</tbody>
</table>

*Table 6: Max Modulation contrast*

Additionally, the optical clearing chamber demonstrated the ability to stabilize the tissue onto the glass slide. None of the samples during this experiment floated to the top of the liquid due to their security to the glass slide by the 3D printed bars on the top piece of the construct. These bars were designed to allow for 2-3 millimeters of space between the bottom plane of the bars and the glass slide, an amount designed to mimic the thickness of the samples used for this experiment. They additionally allowed for enough
space for the optical clearing agent to be applied to maximize the area of the sample that was in contact with the optical clearing agent.

**Figure 4.7:** A) The PLA bars of the top piece that secure the sample to the glass slide are highlighted B) Ridges in the sample caused by the imposition of the PLA bars is evident.

**IV. Conclusion**

The goal of optical clearing is to improve image quality by reducing light scattering. Optical clearing agents have shown significant promise in being able to alter the physiology of tissues while also maintaining vital structures for observation. By making the tissue more homogenous, changes in refractive indices that light from fluorescence microscopy techniques encounter are reduced. XCLARITY and TDE have demonstrated strong clearing properties but this is often achieved over a period of hours or days and the reagents required are expensive. Other less expensive agents, specifically saccharides such as maltose, fructose, and sucrose, have been shown to achieve optical clearing. However, saccharide clearing solutions are much less expensive and their usage protocols are much simpler to achieve. Additionally, XCLARITY protocol cannot be
scaled down to the minute increments that are desired for possible point of care applications and TDE has demonstrated the ability to eliminate the fluorescence of applied stain after optical clearing treatment. From this experiment, it is apparent that saccharide clearing agent solutions with proper protocol can be effective in providing low cost and impactful optical clearing solutions. Specifically, 100% weight-to-volume fructose in PBS solution has produced significant results in improving recovered image signal intensity and demonstrated improved image contrast by reducing light scattering in SIM. Furthermore, the user-designed optical clearing agent chamber has demonstrated the ability to solve the main stability problem that is evident in the previous optical clearing protocol for saccharides.
Chapter 5

Clinical Test of New Optical Clearing Protocol and Chamber on Human Prostate Biopsies
I. Introduction

We proceeded with this study by applying our new optical clearing protocol and optical clearing chamber to human biopsy tissue. Similar to chapter 4, the effects of optical clearing were assessed by analyzing changes in the average intensity, standard deviation, modulation contrast, and full width at half maximum after optical clearing. We did, however, add an additional metric to observe, image contrast. The contrast metric measures the pixel contrast of the resulting recovered SIM image, as given by the SIM system, which differs from the modulation contrast’s assessment of the contrast of the pattern, specifically.

II. Materials and Methods

The methods used to stain and image the prostate biopsies initially followed the same protocol as outlined in chapter 3 except only fructose in PBS was used as a clearing agent and the samples that were imaged were human prostate biopsies, not chicken breast. Additionally, another metric was observed during image processing to assess the effects of optical clearing on the tissue. The metric was image contrast, which is calculated by taking the standard deviation of the pixels in each image and dividing them by the average intensities of the pixels of each image. This calculation was performed and graphed as a function of depth in MATLAB.

III. Results and Discussion

This experiment began with 6 prostate biopsies to have the newly created optical clearing protocol tested on. However, the transition from chicken breast slices to human
biopsy samples demonstrated a need for further iteration of the protocol. First, the size choice of the chicken breast samples proved to be larger than the human prostate biopsy samples. As a result, the first test ran with a human biopsy sample saw the sample peel off the slide and float in the clearing solution because the PLA bars on the top piece of the chamber construct were not deep enough toward the glass slide plane to adequately secure the sample to the slide. The top piece of the chamber construct was then iterated to have multiple depths between the PLA bars and the glass slide plane in order to be able to have multiple options for securing the for various sizes of samples.

Figure 5.1: Front plane of the 3 depth sizes of PLA bars of the optical clearing chamber top piece. The depth of the PLA bars was designed for prostate biopsies that are A) 2 millimeters, B) 1 millimeter, and C) 0.5 millimeters in thickness.
Additionally, the concentration of the acridine orange stain used was altered to have a concentration of 0.02% in PBS as opposed to 0.002% in PBS. The 0.002% acridine orange stain yielded very low fluorescence in the human biopsy samples, even though it permitted adequate fluorescence in chicken breast. As a result, the images generated from using the 0.002% acridine orange stains had very low pixel counts and were not adequate for analysis. Once the stability and fluorescence issues were addressed from the previous protocol, the effects of optical clearing on 3 human biopsy samples were ready to be adequately assessed.

The 100% weight-to-volume fructose solution had a positive impact on the average intensity of the pixel values after treatment, demonstrating a 30.65% average increase in recovered signal intensity at the focus peak after only 10 minutes of clearing. An observation that was made for all 3 of the samples was that there was a large spike in average intensity from 0 to 5 minutes of clearing and another increase, but much less significant, from 5 to 10 minutes of clearing, suggesting that maximum benefit of SIM image quality can be obtained in only 5 minutes immersion in fructose solution prior to imaging. However, there was no impact on the depth at which the maximum average intensity occurred for all the time increments for all 3 of the samples.

Similarly, optical clearing treatment of samples demonstrated a positive impact on the standard deviation of pixel values. There was a 38.51% average increase in pixel standard deviation across the 3 samples and the most significant change in values occurred between the 0 to 5-minute clearing increment for each of the 3 samples, similar to the results for average intensity. 2 of the samples demonstrated the expected smooth bell curve shape but one of the samples showed something different. There appeared to
be 2 peaks in the standard deviation of pixel values for the smaller 50J1TRZ2 sample, approximately 90 microns apart within the z-stack. Additionally, while none of the samples saw a more shallow appearance of the maximum standard deviation of pixel values, only one sample saw an increase in the depth at which the max value occurred, the sample being the larger 50J1TRZ2, which demonstrated a 30 micron depth increase. The modulation contrast saw an average increase of 6.82% in at the peak of the 3 graphs, with one of the samples demonstrating a 2.76% decrease.

The contrast graph results were much less consistent between samples then the average intensity and standard deviation results. Overall, there was an average increase of 30.37% in the maximum image contrast (image quality) across the samples. The 4PV1TRZ2 samples saw a decrease in contrast until approximately 150 microns deep into the tissue and, overall, did not demonstrate the predicted bell curve shape. The larger 50J1TRZ2 sample demonstrated a very large increase in contrast after 10 minutes of optical clearing, with the greatest incremental increase between 0 and 5 minutes of clearing. Additionally, the depth at which the maximum contrast occurred increase in depth by 30 microns. The smaller 50J1TRZ2 sample demonstrated a smooth bell curve shape and while it demonstrated an overall increase in maximum contrast, the depth at which the maximum contrast occurred decrease by 10 microns. Furthermore, the sample that demonstrated the decrease in modulation contrast also saw the decrease in overall contrast.

Similar to the contrast metric, the full width at half maximum metric demonstrated differing results across the 3 samples. The average change in the full width
at half maximum after 10 minutes of clearing was 13.33 microns but 2 of the 3 samples showed a negative or no effect.

Figure 5.2: Images from 4PV1TRZ2 prostate biopsy z-stack with the identified region of interest for analysis at A) 0 minutes, B) 5 minutes, and C) 10 minutes of fructose in PBS clearing.
Figure 5.3: A) Average intensity and B) standard deviation C) contrast, and D) modulation contrast of pixel values for the different optical clearing time increments of the ROI for 4PV1TRZ2. Normalized values.

Figure 5.4: Images of the 4PV1TRZ2 prostate biopsy placed on paper text after A) 0 minutes and B) 10 minutes of optical clearing.
Figure 5.5: Images from 50J1TR22-larger prostate biopsy z-stack with the identified region of interest for analysis at A) 0 minutes, B) 5 minutes, and C) 10 minutes of fructose in PBS clearing.
Figure 5.6: A) Average intensity and B) standard deviation, C) contrast, and D) modulation contrast of pixel values for the different optical clearing time increments of the ROI for 50J1TRZ2 - larger. Normalized values.

Figure 5.7: Images of the 50J1TRZ2 - larger prostate biopsy placed on paper text after A) 0 minutes and B) 10 minutes of optical clearing.
Figure 5.8: Images from S0J1TRZ2 – smaller prostate biopsy z-stack with the identified region of interest for analysis at A) 0 minutes, B) 5 minutes, and C) 10 minutes of fructose in PBS clearing.
Figure 5.9: A) Average intensity and B) standard deviation C) contrast, and D) modulation contrast of pixel values for the different optical clearing time increments of the ROI for 50J1TRZ2 - smaller. Normalized values.

Figure 5.10: Images of the 50J1TRZ2 – smaller prostate biopsy placed on paper text after A) 0 minutes and B) 10 minutes of optical clearing.
<table>
<thead>
<tr>
<th></th>
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<th>5 Minutes</th>
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<th>Overall Distance Change</th>
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<tr>
<td>50J1TRZ2_larger</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>50J1TRZ2_smaller</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table 7:* Depth of maximum average intensity, in microns.

<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PV1TRZ2</td>
<td>2.5231</td>
<td>2.9113</td>
<td>2.9914</td>
<td>+18.56%</td>
</tr>
<tr>
<td>50J1TRZ2_larger</td>
<td>2.8067</td>
<td>3.7547</td>
<td>3.8835</td>
<td>+38.37%</td>
</tr>
<tr>
<td>50J1TRZ2_smaller</td>
<td>5.6712</td>
<td>7.0384</td>
<td>7.6581</td>
<td>+35.03%</td>
</tr>
</tbody>
</table>

*Table 8:* Maximum average intensity, normalized pixel values.

<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall Distance Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PV1TRZ2</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>50J1TRZ2_larger</td>
<td>60</td>
<td>80</td>
<td>90</td>
<td>+30</td>
</tr>
<tr>
<td>50J1TRZ2_smaller</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table 9:* Depth of maximum standard deviation, in microns.
<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4PV1TRZ2</strong></td>
<td>3.0968</td>
<td>3.4343</td>
<td>3.5890</td>
<td>+15.89%</td>
</tr>
<tr>
<td><strong>50J1TRZ2_larger</strong></td>
<td>2.1779</td>
<td>3.4354</td>
<td>3.7680</td>
<td>+73.01%</td>
</tr>
<tr>
<td><strong>50J1TRZ2_smaller</strong></td>
<td>3.5212</td>
<td>4.3951</td>
<td>4.4586</td>
<td>+26.62%</td>
</tr>
</tbody>
</table>

*Table 10: Maximum standard deviation, normalized pixel values.*

<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4PV1TRZ2</strong></td>
<td>0.2947</td>
<td>0.2843</td>
<td>0.2793</td>
<td>-5.23%</td>
</tr>
<tr>
<td><strong>50J1TRZ2_larger</strong></td>
<td>0.3686</td>
<td>0.5520</td>
<td>0.6791</td>
<td>+84.24%</td>
</tr>
<tr>
<td><strong>50J1TRZ2_smaller</strong></td>
<td>0.4357</td>
<td>0.4729</td>
<td>0.4884</td>
<td>+12.10%</td>
</tr>
</tbody>
</table>

*Table 11: Maximum contrast, normalized pixel values*

<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall Distance Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4PV1TRZ2</strong></td>
<td>70</td>
<td>60</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td><strong>50J1TRZ2_larger</strong></td>
<td>160</td>
<td>210</td>
<td>210</td>
<td>+50</td>
</tr>
<tr>
<td><strong>50J1TRZ2_smaller</strong></td>
<td>210</td>
<td>200</td>
<td>200</td>
<td>-10</td>
</tr>
</tbody>
</table>

*Table 12: Depth of maximum contrast, normalized pixel values, in microns.*
Table 13: Full width at half maximum, in microns.

<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall Distance Change</th>
<th>Overall % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PV1TRZ2</td>
<td>102.6840</td>
<td>103.2876</td>
<td>103.6511</td>
<td>+0.9671</td>
<td>+0.9418%</td>
</tr>
<tr>
<td>50J1TRZ2_larger</td>
<td>115.4607</td>
<td>115.3634</td>
<td>116.0024</td>
<td>+0.5417</td>
<td>+0.4691%</td>
</tr>
<tr>
<td>50J1TRZ2_smaller</td>
<td>122.2124</td>
<td>117.9242</td>
<td>116.0231</td>
<td>-3.8107</td>
<td>-3.12%</td>
</tr>
</tbody>
</table>

Table 14: Modulation Contrast

<table>
<thead>
<tr>
<th></th>
<th>0 Minutes</th>
<th>5 Minutes</th>
<th>10 Minutes</th>
<th>Overall % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PV1TRZ2</td>
<td>0.0689</td>
<td>0.0681</td>
<td>0.0670</td>
<td>-2.76%</td>
</tr>
<tr>
<td>50J1TRZ2_larger</td>
<td>0.1079</td>
<td>0.1153</td>
<td>0.1258</td>
<td>+16.59%</td>
</tr>
<tr>
<td>50J1TRZ2_smaller</td>
<td>0.1612</td>
<td>0.1637</td>
<td>0.1719</td>
<td>+6.64%</td>
</tr>
</tbody>
</table>

IV. Conclusion

The effects of optical clearing were observed on human biopsy samples after a protocol was established using chicken breast as a test sample. Upon testing this protocol on human tissue, it was evident that some of the aspects of the protocol did not perfectly transition from chicken breast to human tissue and adjustments were made accordingly. Once the protocol was adjusted to treat human biopsy samples, the effects of optical clearing on the samples could be assessed. 100% weight-to-volume fructose in PBS solution demonstrated a highly favorable impact on the average intensity and standard deviation of image pixel values. However, the observed effect on the full width at half maximum, the depths at which the maximum average intensity and standard deviation occurred, and the contrast varied across the samples. In some cases, optical clearing decreased the values of these metrics, which quantifiable means the optical clearing agent...
decreased the clarity of some images. Overall, these results are promising and suggest that rapid 5-10 minute immersion in fructose solutions can significantly improve both SIM image intensity, quality, and clarity with depth, but require further assessment and testing on a larger number of samples is warranted to adequately determine the impacts of optical clearing agents on tissue image clarity.
Chapter 6

Future Work and Conclusion
I. Future Work

While the outlined experiments demonstrate success in establishing a robust, successful optical clearing protocol, there is room for improvement.

I.1 Optical Clearing Chamber

The optical clearing chamber’s securing mechanism proved its ability to create a firm seal for the optical clearing agent, but the bolts required to do so proved to be obtrusive. Due to the length of the bolts selected, the imaging area of a sample in the chamber is limited to an 8-millimeter by 8-millimeter square area. This is because the bolts are too long for the orientation of the microscope objective to the stage and come into contact with the microscope objective when moved outside of this area. This causes the chamber to dislodge from its fixation on the stage and impede imaging, in addition to limiting the imaging area. As a result, the sample needed to be placed as close to the center of the bottom piece opening as possible to ensure that the entire sample view could be accessed. An additional change to the chamber that could be made would be to make the bottom piece thinner. The piece is currently 5 millimeters thick and solely needs to house a 1 mm divot for the glass slide. The same function can be accomplished with a thinner base. Furthermore, the current thickness of the bottom piece requires that the objective break the plane of the bottom edge of the center opening circle. Even with optimal bolts in place, the imaging area would be limited to the area of the circular opening due to the height at which the objective must be placed in order to focus on the sample.
A material consideration that can be made pertains to the material of the top piece of the construct. The current PLA iteration does not conform to and seal with glass well, which justified the need for a gasket to be applied to it to create a liquid seal. Instead of PLA, the top piece could be made of polydimethylsiloxane (PDMS). It is the current favorite material in the field of microfluidics due to its clear and viscoelastic nature [24]. As a result, it conforms quite well to the surfaces it touches and has shown an ability to create glass seals [25]. It is also inexpensive as it is a cheaper derivative of basic silicone.

We could further adjust the material considerations by introducing magnets into the design. Specifically, we could substitute the bolt openings and the bolts themselves by imbedding magnets into the chambers at the cutout points that currently exists for the connecting bolts. This could be done by strategically placing magnets into their desired locations during the 3D printing process so that the rest of the print from that point would surround and imbed the magnet into the design. This would eliminate most of the assembly time and provide the chamber with full range of motion around the stage, with no fear of any bolts or other connecting pieces coming into contract with the objective lens.

The top piece of the construct could be modified to have a greater quantity and thinner PLA bars in the center opening. The current design stabilizes samples well, but due to the size of biopsies, often stabilizes them in only one of 2 places. By adding more and thinner bars, there can be more points of contact between the PLA bars and samples to provide a more even downward pressure on the sample for stability.
I.2 Optical Clearing Agents

The concentrations of the test solutions could be altered to reflect saturated solutions of each reagent, as opposed to standardizing a 100% weight-to-volume solution for all 3 of the clearing agents tested. The added solute in PBS likely contributes to increased viscosity of the current solution, which would slow down the optical clearing agent’s diffusion time. By using the minimum concentration of solute needed to create a saturated solution, the saccharides optical clearing agent’s effectiveness can be further optimized.

Additionally, a wider array of clearing agents can be tested going forward to determine if there are even more optimal clearing agents for rapid clearing applications than fructose. Sorbitol is a sugar-alcohol found in fruits and plants has shown promise as an optical clearing agent [26]. There are a multitude of other monosaccharides and disaccharides such as glucose and lactose that could be tested. Similarly, further testing can even involve polysaccharides as optical clearing agents. By expanding the array of clearing agents tested, the formation of a pattern between the success of monosaccharides vs disaccharides vs polysaccharides can provide insight into the specifics of what kinds of chemical structures work well for rapid optical clearing. This could pave the way for the discovery of other reagents to aid in rapid optical clearing.

Several protocols that exists use agents that accomplish both dehydration and optical clearing within the same solution, whereas the outlined protocol just accomplished the optical clearing step. Instead of using PBS as the solvent for the saccharide agent, the use of ethanol could be favorable to accomplish dehydration to
remove lower refractive index water molecules and then allow for subsequent clearing agent penetration into a sample.

II. Conclusion

This study outlines the establishment of a new optical clearing protocol. After identifying a need to improve the quality of intraoperative tissue imaging, an optical clearing chamber construct was created. After demonstrating the ability to stabilize tissue subjected to optical clearing, an optimal optical clearing agent was selected and tested in the newly designed chamber. Once design requirements were satisfied and an optical clearing agent was the selected, the new protocol was tested on human biopsy tissue to observe the effects of the new protocol, as translated from test samples to human samples. Overall, this new protocol is very promising and the user designed optical clearing chamber allows for robust, repeatable and stable testing, but the design could be optimized further to be more compatible with the SIM stage setup. Additionally, 100% weight-to-volume fructose in PBS shows promise as a rapid optical clearing agent but further tests to increase sample size and the analysis of other quantitative metrics that provide insight into the changes undergone by the tissue are needed.
I. Average Intensity, Standard Deviation, Full Width at Half Maximum and Contrast Calculations and Graph Generation

% 0 minute clearing data
% MATLAB can only run for loops for a range consisting of positive, nonzero % integers. LABVIEW records images with the first image being indexed as % "0". This step reads in the first image from LabView and will be combined % with images 2-50 from the z-stack.
I_0_1 = imread('AO_no_clear_0_0_0.tif');
a = [];
b = [];

% range of i is based on the number of images in the zstack plus the % previously written in 0th image.
for i = 1:49
    a{i} = imread(sprintf('AO_no_clear_0_0_%d.tif',i));
    b{i} = mean(mean(a{i}(408:1696, 400:1740)));
    c{i} = std(mean(a{i}(408:1696, 400:1740)));
end

% combines the first image (image number 0) with the loop images
fullimage_avg_1 = cell2mat([mean(mean(I_0_1(408:1696, 400:1740))),b]);
image_st_dev_1 = cell2mat([std(mean(I_0_1(408:1696, 400:1740))),c]);
contrast_1 = image_st_dev_1./fullimage_avg_1;

% This step is only sometimes necessary to account for "odd-frames-out"
fullimage_avg_1(24) = [];
image_st_dev_1(24) = [];
\% The following sets up the representation of the 3 main quantitative
\% parameters of this study:
\hspace{1cm} halfmax_1 = \max(\text{fullimage Avg}_1)/2;
\hspace{1cm} normal\_avg\_1 = \text{fullimage Avg}_1./\text{fullimage Avg}_1(1);
\hspace{1cm} normal\_stdev\_1 = \text{image st dev}_1./\text{image st dev}_1(1);

\% The following 2 read-in sections follow the same principles as outlined,
\% except they set up arrays for the 5 minute clearing images and the 10
\% minute clearing images

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% 5 minute clearing data

I_0_2 = imread('AO_5\_min\_0\_0\_0.tif');
d = \[];
e = \[];

for i = 1:49
d{\{i\}} = imread(sprintf('AO_5\_min\_0\_0\_%d.tif',i));
e{\{i\}} = mean(mean(d{\{i\}}(408:1696, 400:1740)));
f{\{i\}} = std(mean(d{\{i\}}(408:1696, 400:1740)));
end

\hspace{1cm} fullimage\_avg\_2 = \text{cell2mat}([mean(mean(I_0_2(408:1696, 400:1740))),e]);
\hspace{1cm} image\_st\_dev\_2 = \text{cell2mat}([std(mean(I_0_2(408:1696, 400:1740))),f]);
\hspace{1cm} contrast\_2 = image\_st\_dev\_2./fullimage\_avg\_2;

\hspace{1cm} halfmax\_2 = \max(\text{fullimage Avg}_2)/2;
\hspace{1cm} normal\_avg\_2 = \text{fullimage Avg}_2./\text{fullimage Avg}_2(1);
\hspace{1cm} normal\_stdev\_2 = \text{image st dev}_2./\text{image st dev}_2(1);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% 10 minute clearing data

I_0_3 = imread('AO_10\_min\_0\_0\_0.tif');
g = \[];
h = \[];

for i = 1:49
g{i} = imread(sprintf('AO_10_min_0_0_%d.tif',i));
h{i} = mean(mean(g{i}(408:1696, 400:1740)));
j{i} = std(mean(g{i}(408:1696, 400:1740)));
end

fullimage_avg_3 = cell2mat([mean(mean(I_0_3(408:1696, 400:1740))),h]);
image_st_dev_3 = cell2mat([std(mean(I_0_3(408:1696, 400:1740))),j]);
contrast_3 = image_st_dev_3./fullimage_avg_3;
halfmax_3 = max(fullimage_avg_3)/2;

normal_avg_3 = fullimage_avg_3./fullimage_avg_3(1);
normal_stdev_3 = image_st_dev_3./image_st_dev_3(1);

% z represents the depth range of the z-stack the other
% z_'x' arrays are
% only sometimes necessary and are used to account for the
"odd-frame-out"
% images that have been removed from analysis
z = [10:10:500];
z_1 = [10:10:230, 250:10:500];
z_2 = [10:10:330, 350:10:500];
z_3 = [10:10:210, 230:10:500];

% The following creates a graph, plotting the average
% intensities of the 0, 5, 10 minute clearing images as a
% function of depth
figure
plot(z,normal_avg_1)
hold on
plot(z,normal_avg_2)
hold on
plot(z,normal_avg_3)
legend('No Clear', '5 Min', '10 Min');
xlabel('Depth')
ylabel('Average Intensity')
title('Average Intensity vs. Depth')

% The following creates a graph, plotting the standard
% deviation of the 0, 5, 10 minute clearing images as a
% function of depth
figure
plot(z,normal_stdev_1)
hold on
plot(z,normal_stdev_2)
hold on
plot(z,normal_stdev_3)
legend('No Clear', '5 Min', '10 Min');
xlabel('Depth')
ylabel('Standard Deviation')
title('Standard Deviation vs. Depth')

% The following creates a graph, plotting the contrast of
% the 0, 5, 10 minute clearing images as a function of
% depth
figure
plot(z,contrast_1)
hold on
plot(z,contrast_2)
hold on
plot(z,contrast_3)
legend('No Clear', '5 Min', '10 Min');
xlabel('Depth')
ylabel('Contrast')
title('Contrast vs. Depth')

% The following interpolates the half maximum value of
% average intensity
% on both sides of the peak of the graph and outputs the
% distance in
% microns between the 2 values.
z1 = interp1(fullimage_avg_1(1:7), z_1(1:7), halfmax_1);
z2 = interp1(fullimage_avg_1(8:49), z_1(8:49), halfmax_1);
fwhmx_no_clear = z2 - z1

z3 = interp1(fullimage_avg_2(1:7), z_2(1:7), halfmax_2);
z4 = interp1(fullimage_avg_2(8:49), z_2(8:49), halfmax_2);
fwhmx_5_min = z4 - z3

z5 = interp1(fullimage_avg_3(1:7), z(1:7), halfmax_3);
z6 = interp1(fullimage_avg_3(8:50), z(8:50), halfmax_3);
fwhmx_10_min = z6 - z5
II. Modulation Contrast Calculations and Graph Generation

% 0 Minutes

% Reads into MatLab the SIM image and the 3 associated patterned images
% for all 50 frames of the z-stack.
for i = 1:50
    i = i-1;
    sim = double(imread(sprintf('AO_no_clear_0_0_%d.tif',i)));
    pat_1_1 = double(imread(sprintf('AO_no_clear_0_0_%d_1.tif',i)));
    pat_1_2 = double(imread(sprintf('AO_no_clear_0_0_%d_2.tif',i)));
    pat_1_3 = double(imread(sprintf('AO_no_clear_0_0_%d_3.tif',i)));

    % Calculation of uniform image
    uni = (pat_1_1 + pat_1_2 + pat_1_3)./3;
    % The patterned image divided by the uniform image is the modulation
    % contrast image
    pat_over_uni_full_non_sized = pat_1_1./uni;
    pat_over_uni_full = reshape(pat_over_uni_full_non_sized,1,[]);
    mod_contrast(i+1) = std(pat_over_uni_full)./(mean(pat_over_uni_full));
end

% 5 Minutes

for i = 1:50
    i = i-1;
    sim_2 = double(imread(sprintf('AO_5_min_0_0_%d.tif',i)));
    pat_1_1_2 = double(imread(sprintf('AO_5_min_0_0_%d_1.tif',i)));
    pat_1_2_2 = double(imread(sprintf('AO_5_min_0_0_%d_2.tif',i)));
    pat_1_3_2 = double(imread(sprintf('AO_5_min_0_0_%d_3.tif',i)));

    uni_2 = (pat_1_1_2 + pat_1_2_2 + pat_1_3_2)./3;
    pat_over_uni_full_non_sized_2 = pat_1_1_2./uni_2;
pat_over_uni_full_2 = reshape(pat_over_uni_full_non_sized_2,1,[]); mod_contrast_2(i+1) = std(pat_over_uni_full_2)./(mean(pat_over_uni_full_2)); end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

% 10 Minutes

for i = 1:50
  i = i-1;
sim_3 = double(imread(sprintf('AO_10_min_0_0_%d.tif',i)));
pat_1_1_3 = double(imread(sprintf('AO_10_min_0_0_%d_1.tif',i)));
pat_1_2_3 = double(imread(sprintf('AO_10_min_0_0_%d_2.tif',i)));
pat_1_3_3 = double(imread(sprintf('AO_10_min_0_0_%d_3.tif',i)));

  uni_3 = (pat_1_1_3 + pat_1_2_3 + pat_1_3_3)./3;
pat_over_uni_full_non_sized_3 = pat_1_1_3./uni_3;
pat_over_uni_full_3 = reshape(pat_over_uni_full_non_sized_3,1,[]);
  mod_contrast_3(i+1) = std(pat_over_uni_full_3)./(mean(pat_over_uni_full_3));
end

% Graph generation of each of the 3 time point's modulation contrast
% parameters as a function of depth

z=[10:10:500];

figure
  plot(z,mod_contrast)
  hold on
  plot(z,mod_contrast_2)
  hold on
  plot(z,mod_contrast_3)
title('Modulation Contrast'); xlabel('Depth'); ylabel('Modulation Contrast'); legend('No Clear', '5 Min', '10 Min');
III. Optical Clearing Protocol

1. Obtain sample.
2. Fill glass dish with enough stain (in this case, 0.02% acridine orange) to completely cover the bottom of the dish.
3. Stain sample for 30 seconds.
4. Dab sample in another dish filled with 70% ethanol to remove any residual stain.
5. Dab dry sample.
6. Place sample onto center of glass slide.
7. Place glass slide in the glass slide divot of the optical clearing chamber.
8. Secure top piece to bottom piece of chamber using bolts and nuts.
9. Place sample onto SIM stage.
10. Determine imaging frame in LabVIEW and take a z-stack without any optical clearing treatment. Once the frame is determined, the stage is not to be moved as this frame must remain the exact same for subsequent imaging steps (Use 470 laser to image with acridine orange stain).
11. Pipette optical clearing agent into center opening of optical clearing chamber.
12. Repeat z-stack imaging after 5 and 10 minutes of optical clearing treatment.
List of References


Biography

Derek Spillane is a 4+1 master’s student in the biomedical engineering department at Tulane University and is originally from Long Island, New York. He joined Dr. Brown’s Translation Biophotonics team in January 2018. In addition to his coursework and lab work, Derek has been awarded 2nd prize in the 2019-2020 Novel Technology Challenge as part of team AccuTractor for inventing an innovative dissection tool to aid in the improvement of anatomy lab procedures.

Upon graduation, Derek will be working with a Tulane professor and one of his thesis committee members, Dr. Ronald Anderson and an orthopedic surgeon, Dr. Uwe Pontius, on the development of a novel knee implant to prevent complications that often arise post knee surgery.