Mapping the Histology of the Human Tympanic Membrane by Spatial Domain Optical Coherence Tomography

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Abstract

The tympanic membrane is one of the major structures of the ear that aids in the hearing process, giving humans one of the five major senses. It is hypothesized that sound induced displacements of the membrane, which allow humans to hear, are directly related to the membrane’s medial layer which is comprised of a network of collagen fibers. Limitations in available medical imaging techniques have thus far inhibited the further study of these fibers. In this paper we detail an imaging system that we developed with the capability to quantitatively and noninvasively image the internal structures of biological tissues in vitro through spatial domain optical coherence tomography (OCT). By utilizing spatial OCT, we can correlate the characteristics of internal collagen fibers to sound induced displacements in the tympanic membrane. This will eventually lead to improved modeling of the middle-ear and a better understanding of hearing mechanics.
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**Objective**

The tympanic membrane is an essential part of the human anatomy. It is one of the major structures that aids in the hearing process, giving humans one of the five major senses. Despite the importance of the tympanic membrane, its mechanics and anatomy are not yet fully understood because of limitations in the capability of available medical imaging techniques to image microstructures in biological tissues. Currently there is a need for a technique to quantitatively and non-invasively image and create volumetric models of the fibers present in the tympanic membrane. These models need to have a strong focus on the orientation and density of the fibers related to their position in the membrane. Optical Coherence Tomography (OCT) is a non-invasive method by which these 3-dimensional images can be created.

The volumetric representations of the tympanic membrane generated by OCT will have a wide range of potential applications. One application is the comparison of fiber density and orientation to displacement measurements of tympanic membranes under pressure waves generated by the Center for Holographic Studies and micro-mechaTronics (CHSLT) labs at Worcester Polytechnic Institute (WPI). Through this comparison, it can be determined whether or not the vibrations of the tympanic membrane caused by pressure waves follow a pattern based on the fiber distributions in the tympanic membrane. Additionally, the creation of a technique that can generate 3-dimensional images of the tympanic membrane will greatly advance computer modeling technology as it is applied to the hearing process. Current models of middle ear mechanics assume an isotropic tympanic membrane. Through our studies and observations, we will advance the understanding of the tympanic membrane being a non-uniform structure with varying mechanical properties as a function of position.
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1. Introduction

The hearing process is very complex and requires the synchronized involvement of dozens of anatomical structures. Pressure waves are collected by the structures in the ear and converted into electrical signals which are processed by the brain to give humans the sense of hearing. One essential structure in the hearing process is the tympanic membrane (TM), more commonly known as the ear drum. The TM is comprised of three layers. The epidermal layer and mucosal layer are the innermost and outermost layers respectively. These layers act as shields for the middle layer, known as the lamina propria, which converts pressure waves collected by the ear into mechanical vibrations. This conversion is of great importance to the hearing process making the lamina propria one of the most significant physical structures in the ear.

It is known that the lamina propria is composed of a complex network of collagen fibers primarily composed of collagen II, the type of collagen present in cartilage. These collagen fibers, which are oriented radially and circumferentially like a spider web, are responsible for the mechanical properties of the tympanic membrane. When excited by pressure waves from sound, the TM undergoes displacements which relate to the frequency of the sound waves being transmitted. Many tympanic membrane models which aim to demonstrate these deformations assume that the tympanic membrane has isotropic mechanical properties. Better understanding the histology of the tympanic membrane, as well as the structure, density, and orientation of the collagen fibers in the lamina propria, will lead to better understanding of TM displacement patterns caused by pressure waves. As a result, more accurate computer models of TM displacement patterns caused by pressure waves can be created.

Various imaging techniques have been used to examine the histology of the tympanic membrane. These techniques include direct microscopic observation, scanning electron microscopy, and confocal microscopy. While these techniques can be advantageous in measuring surface characteristics of the TM, they lack the ability to quantitatively measure the internal structures of
biological tissues. In this project, we considered optical coherence tomography (OCT) as a methodology to overcome these limitations. OCT has the ability to measure the internal structures of semi-transparent biological tissues, making it a viable method for imaging the fibers present in the TM. OCT also requires no sample preparation allowing for in vivo and in vitro imaging of biological tissues allowing for eventual clinical applications.

Our project aims to produce quantitative 3-dimensional images of the tympanic membrane through the use of optical coherence tomography. One of our major goals is to analyze these images to gain a better understanding of pressure induced deformations in the TM with varying frequencies to improve future models. Our first step in this process was to understand the mathematics and theory behind optical coherence tomography so that we could successfully implement an accurate imaging system. We then designed an OCT setup based on white light interferometry which used a Michelson interferometric setup. An image acquisition procedure was created using the programs MatLab, LabVIEW, LaserVIEW, and MathCad with the ability to produce quantitative three dimensional images with varying fields of view. We were able to analyze the images that we produced with our OCT system to determine the density of collagen fibers of the lamina propria in different locations in the TM as well as fiber diameter. Our findings will help to clarify that tympanic membrane displacements caused by sound waves are directly related to the structure, orientation, and density of the collagen fibers in the tympanic membrane, and that future TM displacement models should correspond to the collagen fiber orientation.
2. Background

2.1. The Anatomy and Physiology of the Human Ear

The process by which sound waves are gathered in the human ear and transferred to the brain is complicated. There are many components to the human ear, with the perfect function of all of them being essential for hearing. Similar to any machine, the failure of one small part in the ear can lead to the failure of the entire process. Therefore, understanding of the entire anatomy and physiology of the ear is essential if medical procedures to address issues in the hearing impaired are to take place. For our examination of the fibrous layer of the tympanic membrane (TM), we must first understand the importance of the surrounding parts of the ear if we are to perceive the significance of the TM structure to sound induced membrane displacements. The following sections examine the outer ear, the middle ear, and the inner ear with focus on their anatomy and their relation to the hearing process. The labeled anatomy of the human ear can be seen in Figure 1.

![Anatomy of the Human Ear](Image)

**Figure 1: Anatomy of the Human Ear (Lickstein, 2009)**
2.1.1. The Outer Ear

The outer ear, as seen in Figure 2, consists of the pinna, the external auditory canal, and the outermost tissue layer of the tympanic membrane. The pinna, which is the visible portion of the ear, is composed of cartilage and attaches to the skull on the temporal bone above the external auditory canal, which is commonly referred to as the ear canal (Alberti, 2001). The primary function of the pinna is collecting sound and directing it towards the external auditory canal, or ear canal. The ear canal itself is approximately four centimeters in length, with hairs and ear wax prevalent closer to the pinna to prevent foreign contaminants, such as dirt and insects, from entering the ear canal. Covering this cavity is a layer of skin comprised of simple epithelial skin cells which becomes thinner as distance to the TM decreases. The auditory canal is responsible for directing and amplifying sounds towards the tympanic membrane, which separates the outer ear from the middle ear. The third area of interest in the outer ear is the epidermal layer of the tympanic membrane. The epidermal layer, in terms of the outer ear, provides a protective layer for the rest of the tympanic membrane and the middle ear, and is a continuation of the skin of the ear canal. This layer will be discussed in more detail in section 2.2.1.

Figure 2: The outer ear (Cape Fear Otolaryngology, 2012)
2.1.2. The Middle Ear

The middle ear, also known as the tympanic cavity, is an air-filled compartment surrounded by the temporal bone (Heine, 2004). It is lined with a mucosal skin layer that extends over the tympanic membrane, creating the innermost layer of the tympanic membrane. This mucosal layer of the TM will be discussed in greater detail in section 2.2.1. The tympanic cavity is filled with air to equalize pressure on both sides of the tympanic membrane, a necessary condition for the membrane to vibrate naturally. Equilibrium is kept in each ear by a Eustachian tube, which connects to the nasopharynx. These Eustachian tubes provide passage to the outside air.

The auditory ossicles, which can be shown in Figure 3, are three small bones housed in the tympanic cavity. They transfer and amplify sound waves from the tympanic membrane to the vestibular window, which is the beginning of the inner ear structures. The auditory ossicles, which include the malleus, incus, and stapes, are the smallest bones in the body and are essential in conducting sound from the tympanic membrane to the inner ear (Alberti, 2001). The malleus attaches directly to the tympanic membrane, and then to the incus to transmit mechanical vibrations at a frequency dependent on the sound being transmitted. The malleus is also attached to the temporal bone for structural support. The incus then fastens and transmits vibrations to the stapes, which is firmly linked to the vestibular window by an annular ligament. This is where vibrations are transmitted to the inner ear. The ossicles work together to ensure sound transmission to the inner ear.

![Figure 3: The auditory ossicles (Lickstein, 2009)](image-url)
2.1.3. The Inner Ear

The inner portion of the ear is responsible for transforming mechanical vibrations produced by a sound into neural impulses which can be interpreted by the brain. The structure of the inner ear that is most essential to this process is the cochlea (Purves, Augustine, Fitzpatrick et al, 2001). The cochlea’s function is to convert sound waves to neural signals, but it also is responsible for breaking down acoustical waveforms into more simplistic components. The anatomy of the inner ear is shown in Figure 4 below.

![Figure 4: The anatomy of the inner ear (Givelberg, 2003)](image)

The human cochlea is approximately 10mm wide and forms a shape which appears similar to the shell of a snail. The cochlea is a tube however, and would be roughly 35mm long if outstretched (Purves, Augustine, Fitzpatrick et al, 2001). The oval window of the inner ear is attached to the stapes of the middle ear, and transmits vibrations to the cochlea. The round window and the oval window are each covered by membranes and are located at the base of the cochlea. These membranes are the basilar membrane and the tectorial membrane respectively and their functions are to vibrate and send...
pressure waves into the cochlea where approximately 30,000 hair follicles are located. These hair follicles convert vibrations to nervous impulses, which are gathered by approximately 19,000 endings. These endings transfer the nerve impulses to the brain (Alberti, 2001).

The entire hearing process concludes with the conversion of vibrations to nerve impulses inside of the cochlea. This could not happen, however, without the working condition of the rest of the structures of the human ear. One of the anatomical structures most important to this process is the tympanic membrane (TM). If the TM fails there would be no vibrations transmitted from the environment to the middle and inner ear structures, and no nerve impulses would be transmitted to the brain. Therefore, to better understand the importance of the TM to the entire hearing process, we will focus our study on the tympanic membrane structure and its relation to TM displacements when engaged by pressure waves.

2.2. The Tympanic Membrane

The human tympanic membrane (TM) is soft tissue membrane which separates the ear canal from the middle ear tympanic cavity. The tympanic membrane, which is approximately 10mm in diameter and 80 micrometers in thickness on average in human adults, is instrumental in converting sound pressure waves into mechanical vibrations (Cheng et al, 2007). The TM has been the subject of many studies, focusing mainly on its structure, composition, and mechanical properties. In the following sections, a brief overview of the anatomy of the tympanic membrane will be presented, as well as its physiological characteristics. In addition, the various fibers of the tympanic membrane are examined and the need to more clearly map out their orientation is justified. A human tympanic membrane is shown in Figure 5.
2.2.1. Anatomy and Physiology of the Tympanic Membrane

The tympanic membrane, commonly referred to as the eardrum, is the portion of the ear essential for transmitting sound from the outer ear canal to the ossicles and middle ear tympanic cavity. Figure 6 is a diagram of the anatomy of a human tympanic membrane.

Figure 5: The human tympanic membrane (Medicine Decoded, 2009)

Figure 6: Anatomy of the Human Tympanic Membrane (Jarvis, 1996)
The tympanic membrane is primarily conical in shape with the apex forming an umbo (Lim, 1970). The TM is divided into two main sections. The pars flaccida, also referred to as Shrapnell’s membrane, is the upper level of the tympanic membrane which can be identified by a small inverted triangle which is above the short process of the malleus, and the pars tensa is the lower portion of the tympanic membrane which is located around the umbo (Lim, 1970). Surrounding the pars tensa, there is a ligament referred to as the anulus. This ligament does not extend into the pars flaccida. The pars flaccida, which contains very few collagen fibers, is thicker than the pars tensa. A typical human pars flaccida is between 0.03 and 0.23 mm in thickness, while the pars tensa is typically between 30 microns and 90 microns in thickness. In contrast to the pars flaccida, the pars tensa contains a fiber arrangement that is very well-developed. The fibers in the pars tensa have an orientation similar to that of a spider-web, with radial fibers that are well developed and circular fibers which are moderately well developed. As the pars tensa approaches the pars flaccida, the collagen fibers present begin to thin out. Figure 7 depicts the general orientation of the collagen fibers in the pars tensa of the tympanic membrane.

![Figure 7: Fiber orientation of the pars tensa in the human tympanic membrane (Lim, 1995)](image)

When the depth of the tympanic membrane is examined in the pars flaccida and the pars tensa, three distinct layers can be identified. The first layer that the tympanic membrane contains is the
epidermal layer. This layer is made up epithelial skin cells filled with keratin, which is the protein that makes up fingernails and hairs, and is devoid of hair follicles and glands (Lim, 1995). The epidermal layer of the tympanic membrane migrates outward in the centripetal direction from the umbo. This process, which is similar to how the fingernails and toenails grow, is a self-cleaning mechanism for the tympanic membrane. Figure 8 is an image of a cross sectional drawing of the pars tensa of a tympanic membrane. The three distinct layers of the tympanic membrane can be seen in this figure.

The middle layer of the tympanic membrane when a cross section is examined is the lamina propria. The lamina propria is composed primarily of loose connective tissue which contains collagen and elastin fibers, with an orientation as of that in Figure 7. The lamina propria, or the main collagen
layer, has a stiff structure to it which gives the membrane many of its unique properties (Stenfeldt, 2006). The fibers of the lamina propria are made up of smaller collagen fibrils which are typically between 10nm and 300nm in diameter. These fibrils are weaved together in a triple helix to form the collagen fibers (Puria, 2008). The fibers of the tympanic membrane are primarily made up of type II collagen and can vary from 1-20µm in diameter. Collagen II is the type of collagen that is present in cartilage, which gives the tympanic membrane its stiff properties. Type II collagen also allows the tympanic membrane to transmit vibrations from sound waves to the inner ear. The lamina propria also contains sub-epidermal loose connective tissue with an external vascular plexus and a nerve network, as well as a thin sub-mucosal loose connective tissue layer which also contains a vascular plexus and nerve network. This is because of the connection of the lamina propria to the epidermal layer and the third layer of the tympanic membrane, the mucosal layer.

The mucosal layer of the tympanic membrane is very similar in function to the epidermis. While the mucosal layer does not experience migration like the epidermal layer, the mucosal layer’s primary function is to protect the lamina propria. The mucosal layer is composed of squamous cells (Lim, 1970). These cells are responsible for nutrient exchange as well as protection, and can be found in the epidermis of the tympanic membrane as well. The mucosal layer is resistant to water, and allows the lamina propria to be free from bacteria and other harmful substances which could be present in the tympanic cavity. The mucosal layer of the tympanic membrane is also a continuation of the mucosal layer which lines the tympanic cavity of the inner ear (Heine, 2004).

2.2.2. Investigating the Fibers of the Tympanic Membrane

The fiber arrangement of the tympanic membrane has been investigated previously through various methods such as scanning electron microscopy, multiphoton microscopy, and other microscopy derivatives (Kawabata and Ishii, 1971), (Hiraide et al, 1980), (Puria et al, 2008). Many studies have
focused on the relation between the fiber arrangement and the vibrations produced throughout the membrane. To the best of our knowledge, the fibrous layer of the tympanic membrane has not been imaged and analyzed through the use of optical coherence tomography. Optical coherence tomography has been used to measure the thickness of tympanic membranes previously, however (Djalilian et al, 2008). The ability of OCT to yield 3-dimensional high resolution images makes it an ideal candidate to map out the histology of the fibrous layers of the lamina propria. By producing high resolution maps of the tympanic membrane fibrous layer, better understanding of the physiological function of the TM can result, which can aid in surgical procedures and biomedical research. We will now justify the use of optical coherence tomography by investigating the advantages and disadvantages of other imaging techniques which are applied to the tympanic membrane.

2.3. Current Imaging Techniques Applied to the Tympanic Membrane

Imaging techniques have been applied to the tympanic membrane for decades. The aim of imaging the TM is to quantitatively define its physical characteristics to gain insight into its anatomy and physiology. Several methods utilized in the past include direct microscopic observation, scanning electron microscopy (SEM), and confocal microscopy. Each of these techniques has advantages and disadvantages in imaging biological tissues which will be discussed in detail in this section.

2.3.1. Direct Microscopic Observation

Direct microscopic observation is an imaging technique used to magnify objects in order to view details that would not be visible with the naked eye. It is utilized in many fields where qualitative observations of samples are required. Basic microscopic setups involve several lenses which collect light reflected from samples and focus it so that an observer can view it or a camera can make recordings. Many different types of microscopic observations are utilized to observe tissue samples with the main
differences between them being how the sample is illuminated. The type of sample illumination
determines the contrast of the viewed images.

Bright field illumination is a type of microscopy in which the sample is illuminated from below
with white light and observed from above (Carpette “Light Microscopy”, 2005). This is one of the
simplest methods of microscopy as almost no sample preparation is required and illumination can be
achieved using a light bulb. While this is advantageous for basic observations of most samples, bright
field illumination has several drawbacks when imaging biological samples. The spatial resolution of
observed samples is much less than with other more sophisticated imaging techniques because of the
requirement for fine focus on specific areas of the sample. This means that the imaging of a full sample
is not possible at one time if the area of the sample you are trying to observe is larger than the field of
view of the microscopic setup being used. The contrast attainable in biological samples is also not ideal
with bright field illumination as most tissues are semi-transparent, allowing background light to pass
through samples and flood the observation window with more light than is necessary for viewing
structures in tissues. This problem can be limited by applying stains to biological samples. Staining,
however, is generally not possible on living samples as the stains can in some cases kill tissues at the
cellular level. This means that in vivo staining of biological tissues must be done with great care or ruled
out entirely as a possible imaging technique for living tissues.

Dark field illumination is a technique which aims to improve the contrast of transparent and
semi-transparent samples as compared to bright field illumination (Carpette “Light Microscopy”, 2005).
The method of illumination in dark field is very similar to bright field with the light source being below
the sample. The main difference between the two methods is the addition of an annulus that allows
only light coming from the sample to be picked up by the objective lens of the microscope. Additionally,
a lens can be added between the light source and the sample which improves illumination of the sample
by increasing light focus. A typical dark field microscopy setup is shown in Figure 9.
As stated previously, the addition of an annulus to allow only light coming from the sample improves image contrast, allowing for improved imaging of transparent and semi-transparent samples, including most biological samples (Carpette, “Dark Field Viewing”, 2005). Bright field illumination relies on shadows being cast by structures on a sample making it difficult to pick up small surface details. Dark field illumination can pick up small features because the image contrast relies solely on light being reflected and refracted off a sample.
The main drawbacks of dark field illumination are the low illumination levels in final images because of the reduction in the amount of light gathered by the objective lens, and the fact that some features could be invisible to the observer because of the nature of the technique itself. In order to obtain a more complete representation of a sample, samples should be viewed using both bright and dark field illumination.

The two techniques described above are basic versions of microscopy and are good representations of the advantages and limitations of direct microscopic examination. In summary, direct microscopic observation is excellent for making qualitative observations of samples. It is extremely useful in experiments being conducted in the long term as data can be recorded with pictures at time intervals, as was done to view the TM healing time in rats when a perforation was made in the membrane (Rahman, 2007). Figure 11 shows a typical image of a TM using microscopic observation.
The resolution of images captured with microscopes can be very high, on the order of sub-micron (Foster, 2001). The contrast, however, suffers from backscattering of light or over illumination depending on the type of microscopy being utilized. There is one other major drawback with these techniques in terms of our goals of creating 3-dimensional representations of tissues. Direct microscopic observation is incapable of quantitatively measuring internal structures of samples. This means that constructing 3-dimensional models of the tympanic membrane is impossible using direct observation and is therefore not a viable method to be used for the purposes of this project.

2.3.2. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is an imaging technique that uses a high energy electron beam to image the surface topography of samples (Yakimov, 1998). An emitter, typically a metal with a high melting point and low vapor pressure to allow for heating without significant loss of mass, is heated to emit electrons. These electrons are then focused into a fine beam using a series of focusing lenses and electromagnetic coils. The beam is aimed at a sample to allow for electron interactions between the beam and the sample’s surface causing the emission of high energy electrons and other light rays,
typically in the X-Ray spectrum. Sensors pick up these emissions which can be analyzed to generate images of the sample being observed.

SEM has the ability to generate images of samples with high spatial resolutions on the order of 1 nm (Yakimov, 1998). The technique can magnify objects from 10x to 500,000x. This is roughly a 250 times greater magnification than the most powerful light microscopes currently available. Images generated have 3-dimensional characteristics albeit not quantitative in nature. Figure 12 demonstrates the power of SEM to generate high resolution images of structures on the surface of the tympanic membrane.

![Figure 12: Tympanic Membrane Image Using SEM (Gschmeissner, 2012)](image)

SEM has several drawbacks when imaging biological samples including the tympanic membrane. First, the surface of any sample using typical SEM must be conductive to allow for interaction with the electron beam (Swapp, 2012). Most biological tissues do not meet this requirement and must be coated with a metal layer in order to be imaged using SEM. The tympanic membrane is composed of non-conductive material and would have to be prepared, possibly damaging the microstructures in the
medial layer. Second, samples must not have water present in them as SEM is conducted in a vacuum chamber in most applications. The low pressure would cause the evaporation of the water leading to potential interference and damage to the sample. Finally, SEM can only image surface characteristics of samples. It cannot be used to image internal structures of samples without removing covering tissue first, which would force our group to dissect a TM layer by layer to obtain measurements which is impossible to do without damaging the samples. These three factors rule out SEM for imaging the internal structures of biological tissues.

It should be noted that there have been recent advances in SEM called Environmental Scanning Electron Microscopy (ESEM) that allows for imaging of biological tissues (Swapp, 2012). This technique eliminates the need to coat the samples in conductive materials and the need to dehydrate samples before imaging. ESEM does not, however, allow imaging of internal structures making it unsuitable for the needs of mapping the fiber orientation in the tympanic membrane.

2.3.3. Confocal Microscopy

Confocal microscopy is a technique used widely in biological imaging. It uses a point light source and pinhole aperture to illuminate small portions of a sample while blocking light is backscattered from areas of the sample not being imaged (Shotton, 1989). Full field images are achieved by scanning the illumination of the sample in a raster pattern, or a rectangular scanning pattern that moves along parallel lines to cover an area. This scanning technique can be seen in Figure 13.
Confocal microscopy is similar in function to dark field microscopic observation in terms of image resolution and basic microscopic setup with the major difference being the inclusion of the pinhole aperture. The major advantage gained as a result of the scanning acquisition is the ability to create 3-dimensional representations of samples as well as high resolution surface profiling. Confocal microscopy can optically “slice” samples into planes along an axis. These planes can then be reconstructed to create models of these tissues. Staining can be used as well to create more contrast in samples and create more vivid images of internal structures of cells. A confocal microscopic image of smooth muscle cells in annulus is shown in Figure 14.
While confocal microscopy has several advantages and the ability to create 3-dimensional images of tissues, it also has several drawbacks in terms of our needs for this project. Because of the point light source and pinhole aperture, the amount of light reaching a collector is limited. In order to obtain enough light from the sample without flooding the receiver, longer scan times are required (Pawley, 2006). In terms of the TM, this can lead to drying out of specimens and image distortion. Scans with typical confocal setups over the field of views we would require (roughly 1 square mm in size minimum) could take minutes to scan one plane. With the addition of multiple planes the time is multiplied, and with over 1500 planes per scan in our setup the time for a scan would be rather large. This problem could be overcome with more advanced methods of this technique but would require an ample amount of upfront cost and cutting edge hardware not available to our team (Kupers et al, 2005). While a viable option for 3-dimensional tissue imaging, confocal microscopy is not available to our group for the reasons stated above.
2.4. Using Optical Coherence Tomography to Image the Tympanic Membrane

To achieve our goals for this project, we require an imaging technique that can generate 3-dimensional representations of biological tissues. Optical Coherence Tomography (OCT) has this ability while overcoming most of the limitations of other more widely available imaging techniques discussed above. In the following sections we will discuss the advantages gained by using OCT to image biological tissues, and more specifically the tympanic membrane.

Optical coherence tomography is an umbrella name that encompasses several methods of image acquisition and analysis. These techniques are all based on low coherence white light interferometry (Huang, 1991). A sample is placed in an interferometer and imaged in sequential steps, essentially cutting the sample into planes. While this stepping is taking place, the interferometer causes constructive and destructive interference in the light waves being reflected by the sample causing fringe patterns on each “slice” of the sample. These patterns are acquired and saved using a camera recording short movie clips of the stepping. By analyzing these fringe patterns in post-processing, the images can be recombined to create quantitative 3-dimensional representations of tissues with nanometer resolution. The reconstruction process will be discussed in greater detail in Section 3.4.

2.4.1. Types of Optical Coherence Tomography

As stated above, there are several types of OCT currently being implemented to image biological samples. Each scheme differs in the domain the data gathered from imaging the sample is analyzed in. The first we will discuss is frequency domain OCT. In this technique, the images gathered are analyzed in the domain relating to the frequency of the light being reflected from the sample (Schmitt, 1999). This allows for faster sampling rates while sacrificing some spatial resolution and adds limits to the scanning depth capable of the optical system being used. Frequency domain OCT also allows for fast post-processing and 3-dimensional image generation. This is because analysis of the frequency of the wave holds information on the position of a pixel in the x, y, and z directions simultaneously. Frequency
domain OCT is an excellent technique for imaging biological tissues. However, the process requires hardware that is unavailable to our team and therefore would not be a viable option for this project.

Another method of OCT in use is time domain OCT. This method relies on scanning a reference mirror in the interferometer to adjust the coherence length of the system (Fercher, 2005). This adjusts the plane at which the interference patterns present in the acquired images will be visible. The rate at which the mirror is scanned is controlled giving accurate measurements of the scan time. The intensity of the gathered light reflected back from the sample over the time interval can be analyzed to extract shape information for each individual pixel of the gathered images. Reconstruction is possible using software in post-processing to generate 3-dimensional images of biological samples. Time domain OCT requires a Linnik interferometer setup. Our team did not have access to this setup and therefore we could not implement time domain OCT. It should be noted time domain OCT would have been a valid method for achieving our goals.

Spatial domain OCT is the third major type of OCT. This method is similar to time domain OCT with the exception that the physical step sizes between the images acquired are recorded. An interferometric objective lens is scanned in this method instead of a reference mirror (Fercher, 2003). This lens keeps the distance between the reference and sample arms within the coherence length of the light source. As the objective is stepped down in the direction of the sample, images are acquired cutting the sample into parallel planes. The known step size gives depth information while the analysis of the fringe patterns gives shape information of the structures present in the biological tissues. Spatial OCT can be achieved with a Michelson interferometer, a setup available to our team.

The three methods of OCT described above are the basis for all other methods of OCT. Other methods combine the analysis schemes of these three methods. All methods of OCT have the ability to generate 3-dimensional representations of biological tissues while providing spatial resolution in the nanometer range. The depth OCT can penetrate into tissues varies with the type of OCT being utilized,
but is typically from 0 to several millimeters. Figure 15 shows a 3-D image of the macula in the retina of the eye using spectral OCT.

![Figure 15: 3-Dimensional Representation of the Macula using Spectral OCT (Iowa Institute for Biomedical Imaging, 2007)](image)

### 2.4.2. OCT Applications

Optical Coherence Tomography is beginning to see widespread use in clinical settings and as a research tool for characterizing biological tissues and structures. One of the main applications of OCT in medical imaging applications is the characterization of the retina. OCT has been used to study the layers and thickness of the retina to diagnose diseases and give quantitative models of the retina for laser surgery (Shi, 2008). OCT is useful in this application as the retina is a transparent structure with distinct, multiple layers as seen in Figure 16 below. In this image it is possible to see the separation of these layers, denoting damage to the retina. OCT has also been used to research the layers and structures present in the layers of skin.
OCT can also be used in industrial settings. Surface metrology can be done effectively by OCT as it has sub-micrometer spatial resolution allowing it to generate highly detailed images of surface structures and characteristics. OCT can also be used to validate MEMS devices and microscopic electrical components. OCT is a highly useful technique not only in the medical imaging field but also in certain industrial applications.

2.5. Conclusions and Moving Forward

After researching the anatomy of the human tympanic membrane, the hearing process, and imaging techniques that could provide high resolution images of biological tissues, our team began to develop a strategy for imaging the fibers present in the lamina propria of the tympanic membrane. Because of its high resolution, ability to create 3-dimensional images of biological tissues, and availability to our team, we decided that spatial domain OCT would be the best option to image the fibers of the tympanic membrane. In the following sections we will detail the mathematics behind
spatial OCT, our system setup, acquisition procedure, and image analysis. The combination of these steps allows us to generate quantitative images of biological samples including the tympanic membrane.

3. Methodology

For reasons stated previously, our team has decided to use spatial optical coherence tomography (OCT) to generate 3-dimensional quantitative models of the human tympanic membrane. We developed an optics system and custom programs in several software packages in order to acquire data from samples. We then analyzed the data to extract shape information from the sample tympanic membranes, and generated representations of the structures present therein. The following sections describe our methods in developing this system and its accompanying software.

3.1. Interferometry

Interferometry defines a range of optical techniques that extract useable information from interfering electromagnetic waves (light). In this section we will discuss how interferometry is related to metrology in biological tissues.

Interferometry is based on the interference of light waves. This phenomenon is illustrated in Figure 17. When two light waves are aligned they will either constructively interfere, making it seem to an observer that the light is more intense than normal by increasing the amplitude or they will destructively interfere which decreases the amplitude of the resulting wave causing light to appear less intense to an observer (Sutter and Davidson, 2006). If the two waves are of equal intensity but are misaligned they will not interfere with each other, making them invisible to an observer. As seen in Figure 17, intensity is related to amplitude. The resulting waves from the combination of two waves of the same wavelength depend solely on the alignment of the light waves in relation to the observer.
As mentioned, if two waves from a light source overlap they will interfere with each other either constructively or destructively. The amplitude, or intensity \( E \), of the resulting wave at a given point is determined by the amplitudes of the two waves, \( E_1 \) and \( E_2 \), and is governed by the equation

\[
E = E_1 + E_2. \tag{1}
\]

For two waves of the same wavelength and intensity, interference is determined by the phase difference, \( \phi \), of the two waves. Figure 18 shows the phase difference between two identical waves. The phase difference is defined as

\[
\Delta \phi = \phi_2 - \phi_1 \tag{2}
\]

where \( \phi_1 \) and \( \phi_2 \) are the phases of the two waves interfering in radians or degrees. One wavelength of a wave is equal to \( 2\pi \) radians.
Interference of light waves can only occur when the distance between the reference arm and the distance to the sample are within the coherence length of the light source being used. The coherence length is the distance in which waves will interfere with each other. We focus on low coherence interferometry in this project, meaning the light sources used have a coherence length on the order of microns. The light source that we used for this project had a coherence length of 20 micrometers. Figure 19 shows a Michelson interferometer. In order for interference to occur in this interferometer,

$$Coherence\ Length > |d_2 - d_1|$$

where $d_1$ and $d_2$ are the lengths of the reference and object beams respectively and the coherence length is a constant defined by the light source being used. Ideally, $d_2 - d_1$ will be equal to zero for a well calibrated interferometer measuring a relatively flat surface.
When measuring a surface, the intensity of the resulting wave generated by the combination of the object and reference beams will vary due to phase changes in the object beam. The phase change in the object beam due to the characteristics of the sample surface can be seen in Figure 20 and is defined by

\[ \phi_2 = \phi(y) = \frac{2\pi}{\lambda} d(y), \]  

with \( \phi(y) \) being the phase of the retuning wave, \( d(y) \) being the distance from the objective lens to the sample, and \( \lambda \) being the wavelength of the light source.
Combination of the reference beam and sample beam yields a modulation of intensity \( (I) \) of the resulting wave for a single point on the sample surface. This is defined as

\[
I = e^{-\frac{d(y)^2}{\sigma}} \cos(\Delta \phi),
\]

where \( d(y) \) is the distance from the objective lens to the sample, \( \Delta \phi \) equals the phase difference between the reference and object beams, and \( \sigma \) is a constant related to the coherence length of the light source. Intensity of the waves will be a maximum when \( \Delta \phi \) is equal to \( \pi \) or \( 2\pi \) which relates to \( \lambda/2 \) and \( \lambda \) respectively. By recording the maximum intensity of each pixel in the images we acquire of the tympanic membrane while the intensity is modulated due to interference, it is possible to extract 3-dimensional shape information from the 2-dimensional images we acquire.

### 3.1.1. White Light Interferometry

White light interferometry is a subset of interferometry that uses a white light source with a dominant wavelength to illuminate samples. Because of the white light source, this technique can have a depth resolution of less than .01 nm making it an excellent tool for measuring microstructures with precision (Blunt, 2006). To extract depth information, fringe patterns caused by interfering light waves
are observed on the surfaces of samples. White light is advantageous in this application due to the number of waves present in the light. This means that white light will have a maximum intensity only when the surface is in focus. Lasers and monochromatic sources can have a maximum intensity at several points in the depth of focus of the objective lens.

3.2. Spatial Optical Coherence Tomography

Spatial Optical Coherence Tomography is an optical technique that is based on the principles of interferometry discussed in the previous sections. OCT uses a two beam interferometer to cause interference fringes to become visible on planes that are in focus. The intensity of the fringe patterns will be at a maximum when a surface is in focus allowing for depth information to be extracted. Combining this depth information with the planar coordinates, given by imaging the fringe patterns with an acquisition camera, allows OCT to generate quantitative 3-dimensional images of tissues. This section will discuss the mathematics behind Spatial OCT and how it applies to imaging biological tissues.

3.2.1. Mathematics and the Governing Equation behind Spatial OCT

Spatial OCT aims to find the physical boundaries of structures in semi-transparent media. This is done by finding the intensity of fringe patterns on different planes in a sample by scanning through it. Scanning through a sample is usually done with a nano-positioning system on the reference mirror or positioned above the objective lens.

Spatial domain OCT follows the basic principles of white light interferometry. In our case, we used a 780nm infrared light source which we sent into a beam splitter. One resulting beam given by $E_s$ was sent toward the sample that we wanted to image. The other resulting beam $E_r$ was sent towards a reference mirror. Both the reference beam $E_r$ and the sample beam $E_s$ each were reflected from the reference mirror and the sample surface respectively becoming $E'_r$ and $E'_s$. The reflected sample beam
contained shape information of the surface being imaged. These two reflected beams were then combined which resulted in an increase or a decrease in intensity if equation (3) was satisfied. By scanning in one direction over all values for which

$$\text{Coherence Length} > |d_2 - d_1|,$$

intensity modulation of interfering light waves can be simulated by the equation

$$I_1(x, z) = A \exp \left(-\frac{(x-z_1)^2}{\sigma^2}\right) \cdot \cos \left(4\pi \frac{z}{\lambda}\right) + n(x, z),$$

where $A$ is the amplitude of the intensity modulation, $\sigma$ is the standard deviation of the intensity modulation which is related to the coherence length of the light source, $\lambda$ is the wavelength of the light source, $z$ is the total depth of the axial direction being scanned, $z_1$ is the location of the maxima of the intensity modulation, and $x$ corresponds to displacements perpendicular to depth due to Gaussian-distributed random noise (Larkin, 1996). Gaussian-distributed random noise $n(x, z)$ is caused by error from the environment and acquisition procedure, and yields unintentional but unavoidable nanometer size displacements in the $z$ and $x$ directions. When simulating an intensity modulation using equation (6), $z_1$, $\lambda$, $\sigma$, and $z_1$ all must be plotted based on the total number of frames acquired and used to generate OCT images. These values are then converted into micrometers for analysis.

A simple single mode intensity modulation can be generated with equation 6 using the following parameters - $z$ varying from 0 to 3000 total frames (0µm to 60µm) with a step size of 1 frame (20nm), $z_1$ located at the 1000th frame (20µm), a wavelength ($\lambda$) of 60 frames (720nm), a standard deviation ($\sigma$) of 300 frames (6 micrometers), an amplitude of 50, and zero Gaussian noise. The resulting intensity modulation can be seen in Figure 21.
When closely examining equation (6) and its respective plot, there are a few important characteristics of this which are important to note. The overall shape of the plot takes the form of a bell shaped curve. This is because equation (6) contains a Gaussian distribution. This is given by

$$A \exp \left( \frac{-(z - z_1)^2}{\sigma^2} \right).$$

(7)

In engineering experimentation, the Gaussian distribution is extremely useful in statistical analysis of data. Whereas for our case the value $z_1$ corresponds to the point of highest reflectivity of interference fringes, in statistics this value represents the mean reading of a set of data. In statistics, standard deviation ($\sigma$) measures the width of the distribution curve. The flatter the curve, the larger the expected error of the measurements will be. This is true for our system as well, because if the peak of the intensity modulation is too flat, there could be error in finding the exact maxima of the intensity.
modulation. This is one of the reasons why we are using a light source with a low coherence length.

Equation (6) also contains

$$\cos \left(4\pi \frac{z}{\lambda}\right),$$

which gives the governing equation the oscillations of a cosine function. The specified wavelength ($\lambda$) is responsible for the frequency of the wave underneath the generated bell curve from equation (7).

The same governing equation for spatial domain optical coherence tomography can be used to create intensity modulations with more than one mode. This is done by addition of two or more intensity modulations and is given by

$$I(x, z) = I_1(x, z) + I_2(x, z)$$

(9)

where $I_2(x, z)$ is given by

$$I_2(x, z) = A \exp \left(\frac{-(z-z_2)^2}{\sigma^2}\right) \ast \cos \left(4\pi \frac{z}{\lambda}\right) + n(x, z).$$

(10)

$I_2$ differs from $I_1$ because the location of the maxima ($z_2$) is different from the maxima location in $I_1$. Figure 22 is a figure generated from equation 7 which includes an OCT signal with two modes. The amplitude of the second intensity modulation is 25 with a peak at 40 micrometers.
In order to accurately determine the exact maximum value corresponding to each peak in an intensity modulation, we had to investigate methods to apply an envelope to our OCT signal. The envelope represents the shape of an optical signal and will essentially “cover” it, connecting the peaks of each wavelength in the signal. To generate envelopes for our data, we applied a Hilbert transform to our data.

A Hilbert transform adds a phase of π/2, or 90°, to a Fourier function (Tanimoto, 2009). A Fourier function, which can be defined by

\[ f(z) = e^{-\frac{(z-z_0)^2}{\sigma^2}} \cos \left( 4\pi \frac{z}{\lambda} \right), \tag{12} \]

has a phase shift of π/2 applied to it while it is in the frequency domain. This yields \( f'(z) \) which is given by
\[ f'(z) = e^{-\frac{(x-x_0)^2}{\sigma^2}} \sin \left(4\pi \frac{z}{\lambda}\right). \] (13)

and is the Hilbert transform, or allied function, of \( f(z) \). It is important to note that the Hilbert transform and original Fourier function both exist in the frequency domain, which means that they each have real and imaginary components. Therefore, to generate the envelope from the Hilbert transform, the magnitude of the function \( F(z) \) which is given by

\[ F(z) = f(z) + f'(z) \] (14)

or

\[ F(z) = e^{-\frac{(x-x_0)^2}{\sigma^2}} \left( \cos \left(4\pi \frac{z}{\lambda}\right) + i \sin \left(4\pi \frac{z}{\lambda}\right) \right) \] (15)

must be taken in order to return the signal to the time domain. When computing the magnitude of the function \( F(z) \),

\[ |F(z)| = \sqrt{\left(e^{-\frac{(x-x_0)^2}{\sigma^2}}\right)^2 \left(\cos \left(4\pi \frac{z}{\lambda}\right)^2 + \sin \left(4\pi \frac{z}{\lambda}\right)^2\right)}, \] (16)

a basic trigonometric identity known as Euler’s Identity becomes present. Euler’s Identity is defined by

\[ \cos^2 \left(4\pi \frac{z}{\lambda}\right) + \sin^2 \left(4\pi \frac{z}{\lambda}\right) = 1 \] (17)

and therefore the presence of this identity reduces the function \( F(z) \) to

\[ |F(z)| = \left| e^{-\frac{(x-x_0)^2}{\sigma^2}} \right| \] (18)

which yields the envelope of our OCT signal when plotted. An envelope applied to the data seen in is shown in Figure 23.
The Hilbert Transform is also very effective in creating envelopes over intensity modulations with more than one mode. Figure 24 contains the same multi modal signal as seen in Figure 22, but with an envelope generated through Hilbert transform.
By using the Hilbert transform and generating ideal OCT signals with clear envelopes using the mathematics behind OCT, we can very effectively isolate the maxima of our intensity modulations. This process is described in more detail in section 3.4.4. With a strong grasp on the concepts of interferometry and the mathematics behind OCT, the next step in our process was to develop an OCT system and then create an image acquisition procedure which we could use to generate quantitative three dimensional images of the tympanic membrane. In the next sections we will examine these topics.

3.3. Development of a Spatial OCT System

After understanding the theory related to Spatial OCT, our team developed an optical system to image biological samples. This process included developing a microscopic setup that would be most compatible for imaging the tympanic membrane, an image acquisition procedure, and software to
automate the procedure. This section outlines the physical setup and software driving our imaging system.

3.3.1. Microscope Setup and Hardware

The physical setup of our microscopic system was developed to allow for 3-dimensional imaging of biological tissues. It is optimized for our needs of imaging the tympanic membrane and can be applied to image other tissues as well.

The light source was decided on after researching into the wavelength of light that would best penetrate the layers of semi-transparent biological tissues. Several recent papers into the topic let us determine that infrared sources would help us achieve this. Infrared light has been shown to penetrate deeper into semi-transparent tissues than other ranges of the spectrum, such as the tympanic membrane of gerbils (Cosatas, 2011). Infrared sources also cause less scattering allowing for higher resolution images of internal structures present in tissues. The light source also had to have a low coherence length in order to allow for Optical Coherence Tomography. The model of the light source used is a M780L2 mounted LED produced by Thorlabs Inc. It is a white light source with nominal wavelength of 780 nm and a coherence length of 20 microns. The light source used for this project is shown in Figure 25.
Based on the need for an infrared source in this project, a camera with a high sensitivity for infrared wavelengths was needed. The CHSLT labs at WPI had several options on hand that met this need. Our team decided that the best option available was a Pixelink PL-A741 CMOS machine vision camera. The camera has a 1.3 megapixel (1280x1024) receptor with 6.7 micron square pixels and a maximum frame-rate of 33 fps at full resolution. The camera uses a fire-wire interface to connect to the computer to allow for image acquisition. This camera has spectral sensitivity to light in the range of 350-1000nm (PL-A741). The camera used for image acquisition in this project is shown in Figure 26.
Scanning through the membrane was achieved using a piezo-electric nanopositioner, shown in Figure 27. To achieve full imaging of a healthy adult tympanic membrane the minimum travel of the piezo needed was approximately 65-70 microns (Lim, 1970). The piezo chosen was a PI P-726.1CD on loan from Physik Instrumente for several weeks during the course of our project. The piezo had 100 microns of travel allowing for full depth imaging of human tympanic membranes. The piezo is closed loop with linear repeatability of $\pm$ 3nm and a step resolution of .4 nm. The combination of the piezo’s travel distance, closed loop configuration, and linear resolution makes it an ideal positioner for imaging the full thickness of the tympanic membrane. The piezo expands 10 microns linearly for every 1 Volt AC applied to it, meaning for full travel 10 Volts of input would be required.
Figure 27: PI P-726.1CD Piezo Nano-positioner. This piezo has 100 microns of travel and is accurate to within .4nm.

The piezo requires the use of a driver to deliver a controlled voltage to allow for accurate scanning over any distance. The driver used was a PI Model E-501.00 with an E-504 Amplifier Module, and is shown in Figure 28. It comes equipped with a single channel control with closed loop feedback for accurate linear control. The closed loop feedback eliminates the positional hysteresis affect present in open loop piezo configurations. The maximum operating voltage of this driver is 90-120 Volts AC with a 10x amplifier. This means that for every volt put into the driver, 10 volts are outputted to the piezo. This was taken into consideration when designing a voltage ramp to drive the piezo which will be discussed in the software section later in this paper. The voltage supplied to the piezo driver is delivered through a National Instruments USB-6229 DAQ (P-726 PIFOC).
In order to perform spatial OCT, interference of the light source was required. The challenge with scanning arises with the need for the distance between the reference and object light beams to be within the coherence length of the light source being used. This was solved by using a Michelson interferometric objective lens. The model used was a Leica HCX PL Fluotar 5x/0.15 Michelson Interferometric lens. A Michelson interferometer uses a semi-reflective glass to cause interference between the reference and object beams. This interference takes place inside the objective lens, keeping the object and reference beam distance constant while scanning. The objective used also allows users to control the beam ratio between the beams. This allows for finer control of the contrast of interference fringes observed in images collected using our system. The objective has a working distance of 12 mm, and is shown in Figure 29.
The computer used to control the system is a Dell Optiplex GX620 running Windows 7 Enterprise 32-bit OS. It is used to control the voltage input to the piezo through the DAQ and image acquisition from the camera through a firewire interface.

The full microscope system can be seen in Figure 30. The camera is mounted on the top of the system, the light source on the arm to the right, and the piezo and objective mounted on the bottom of the microscope body. The sample is placed on an optical stage below the objective. The system as it was built had a maximum field of view of 1.37mm x 1.71mm. The spatial resolution of the system is 100nm as verified in Section 3.5.1., making this system an excellent tool for imaging biological tissues. The system also has the capability of creating 3-dimensional volumes of tissues, making it unique from most other systems capable of performing OCT.
3.3.2. Software

The software used to physically drive the piezo was developed by our team in lab using National Instruments LabView 2009. Appendix A shows the block diagram and front panel for the VI developed for this project. The block diagram consists of two voltage control loops that step the voltage delivered to the piezo up and down at a specified rate. The loop in between these two control loops is a time delay that can be set to halt the piezo at the maximum displacement set by the user. This allows for easier verification of the maximum displacement and is useful when acquiring images. The two voltage control loops are identical in function with the only difference being that the first loop increases voltage while the second decreases voltage.

The VI created has several user controls that can be accessed and altered from the front panel. These include the number of steps used to create the voltage ramp, the time delay between steps, and
the voltage increment for each step. The voltage increment is the input that ultimately determines the number of steps necessary to image a sample. The maximum voltage allowed is input in the block diagram and is defined by the characteristics of the piezo being used. For example, the piezo used in this project had a maximum travel of 100 microns and would reach that voltage at 10 volts of input. If we wanted to step every half micron, we would set the maximum voltage to be 10 volts in the block diagram. The piezo travels 10 microns per every volt of input. Therefore, based on the system characteristics, our step number would be set to 1/20th of a volt which coincides with one half of a micron. A MathCAD sheet was developed as well to calculate inputs for the VI. The use of both of these tools will be discussed in greater detail in section 3.5.1.

In addition to National Instrument’s LabVIEW, our system required the use of a few other computer programs as well. In order to acquire images, our group used a computer program that was designed in house at the Center for Holographic Studies and Laser Micro mechaTronics at WPI. This program, known as LaserVIEW, is used to view live images of a sample under our microscope, and has the capability to acquire images and video files from our camera. It can also be used to specify field of view of our system as well as frame rate and exposure time of our camera. Our use of LaserVIEW is discussed in more depth in section 3.4.1. All of the image processing and image generation of our system was done using Matlab 2009b. This includes single pixel analysis, 3D image generation, image filtering, and reading video files. Our video files were in .llvid format, and could be viewed using a program called Holostudio. In this program, all pixel (x,y) coordinates could be seen which was very useful when analyzing single pixels in Matlab. Additionally, Holostudio contained the total number of frames acquired in scanning a sample, which was a quantity that was necessary to generate single pixel analysis and three dimensional images. Finally, to reduce time required to generate three dimensional OCT images, our .lvvid files were reordered using C++. This process will be described in more depth in section 3.5.1.
3.4. Image Acquisition and Implementation

After we gained full understanding of the mathematics behind OCT, designed a microscope setup, and gathered all of our software, the next step was to implement our mathematics to create an image acquisition procedure. In the following section, we will present the procedure that we designed in order to generate three dimensional images of biological tissue. Each step of the process will be discussed in detail. In addition, we will explain difficulties that we faced and how these problems were overcome.

3.4.1. Scanning in the axial direction

As discussed earlier, our OCT system needed to be able to scan samples in the axial direction. This means that cross sectional images had to be acquired with sub micrometer separation in the depth of the tissue. In theory, this could be achieved by moving the entire microscope objective down in the z direction a set distance and taking a cross sectional image at that location. This method, however, was not feasible as it would be impossible to consistently repeat the distance that the microscope moves due to the weight of the entire system. Manual scanning would also add in too much human error. By using a piezo-electric nano positioner, it was much more feasible for us to accomplish this task. A piezo-electric nano-positioner (piezo for short) can be used to change the distance from the objective lens to the sample surface which increases the length of the sample beam while keeping it within the coherence length of our light source so interference can occur with the reference beam. In order for a piezo to increase the travel distance of our object beam, a voltage must be applied to it from a piezo driver which will increase the traveling distance based on how many volts are applied. For our model piezo, which could increase the travel distance of our object beam by up to 100µm, 0.1volts needed to be applied for the travel distance to increase by 1µm.
The traditional method in which piezo-electric nano-positioners have been used in OCT systems to scan a sample is by increasing the voltage manually using the piezo driver. For example, a voltage of 0.01V could be applied to the piezo manually to increase the travel distance of the object beam by 100nm, and then an image could be gathered. Then, another voltage of 0.01V would be applied to manually increase the travel distance by 100nm and another image could be taken. This process could be repeated for the total distance that needed to be scanned for the object being imaged, and then images would be analyzed to produce three dimensional images. There are a few issues with this method, however. When the piezo is stepped and stopped like in this process, the piezo tends to oscillate before coming to equilibrium. These oscillations can lead to inaccurate measurements, and intensity modulations which are very uneven. The oscillations caused by stepping and stopping can be seen in the displacement vs. time graph in Figure 31.

![Graph](image.png)

**Figure 31:** Displacement vs. Time experienced when stepping and stopping using a piezo-electric nano-positioner.
To remove the issue caused by oscillations when stepping the voltage applied to the piezo, our group decided to take a different approach. We hypothesized that if a linear voltage was applied to the piezo rather than stepping and stopping, that we could produce much cleaner intensity modulations when analyzing our images. Additionally, this would make our acquisition procedure much quicker, because we could synchronize our camera with the linear voltage ramp so that images would be gathered after a certain voltage increment. In order to create this linear voltage ramp, we had to create a Virtual Instrument (VI) in LabVIEW which would send the corresponding signal to our DAQ, which would then travel to our piezo driver and to our piezo.

The goal of our LabVIEW linear voltage ramp was to send a certain amount of voltage to our piezo driver which would send the voltage to our piezo, increasing the traveling distance of our object beam. We had to design it so that we could customize the total amount of volts that the ramp would travel, and the time that it would take to completely ramp up. Additionally, the voltage ramp had to stall when the highest specified voltage was reached so that the camera could be stopped, and then ramp back down to zero to avoid damage to the piezo. The interface for our linear voltage ramp can be seen in Figure 32 as a downward voltage ramp occurs after voltage had been ramped to 6V. As seen in the interface, the voltage increment, total number of voltage steps, and time delay between voltage steps can be customized. The interface also includes charts which track the increase and decrease in voltage over time. In Figure 32, since the voltage increment is 0.001 and there are 6000 total steps, the voltage will be ramped up to 6V. As mentioned earlier, 0.1V applied to the piezo yields 1µm of travel distance added. Therefore, the current setup will add a travel distance of 60µm to our object beam. Additionally, with a time delay of 0.08 seconds, this means that it will take eight minutes for the voltage to ramp up, and eight minutes for the voltage to ramp back down.
Our voltage ramp required the creation of three separate loops in our LabVIEW block diagram. The function of the first loop was to ramp the voltage up. The only function of the second loop was to stall the voltage at the top of the voltage ramp when it reached maximum voltage. The last loop in our
VI was created to ramp the voltage back down. The three sections of our block diagram can be seen in Appendix A.

During the ramping of our piezo from 0µm to 60µm, it was essential for our camera to acquire images at a constant rate. The distance between images is directly related to the depth of structures in a tissue, making a repeatable distance between frames essential for 3-dimensional tissue modeling. As mentioned earlier, Laserview is the program that was used to trigger our camera to gather cross sectional pictures of the sample we are imaging. Laserview is a program designed by the Center for Holographic Studies and Laser micro-mechaTronics at WPI and is essentially a program which allows for live viewing of the magnified surface of a sample under a light source, with the capability to record images. The live image that is viewed on the computer screen is referred to as an interferogram.

There are a few features to Laserview which are important in our image gathering process, the first of which is determining the size of the field of view. Since our camera has a maximum capability of taking images with 1024 pixels in the X direction and 1280 pixels in the Y direction, we can choose any amount of pixels within these confinements. Additionally, because the pixel size is exactly $6.7 \times 10^{-6}$ µm, we can determine the exact field of view of our images. The field of view in the X direction is given by

$$X \text{ Field of View} = \frac{(\text{Pixel size}) \times (\# \text{ Pixels} \ X)}{\text{Magnification}}, \quad (19)$$

and the field of view in the Y direction is given by

$$Y \text{ Field of View} = \frac{(\text{Pixel size}) \times (\# \text{ Pixels} \ Y)}{\text{Magnification}}. \quad (20)$$

Some field of views which we have used include 746 pixels x 746 pixels which is equivalent to a 1mm x 1mm field of view and 560 pixels x 560 pixels, which is 750µm x 750µm. The interface for choosing the number of pixels in the X and Y directions in Laserview can be seen in Figure 33.
After determining the field of view for our interferogram, the next step in the Laserview program was to set the exposure time of our camera and the frame rate. These two parameters were the most important parameters in determining the resolution of our OCT images. Despite the fact that the frame rate of our camera was set in Laserview, it was not what the frame rate actually was once images began to be recorded. The actual frame rate of our system was strongly influenced by the exposure time of the camera. The exposure of a camera is directly related to the amount of light that reaches the camera sensor when the shutter is open. If the shutter of the camera is open longer, more light can be captured from a sample and higher resolution images can be taken. Because our camera needed to take images while the travel distance of our sample beam was increasing however, higher exposure times could cause smearing of images. This is similar to trying to take a picture of a moving car, and would cause high amounts of noise in our images as seen in Figure 34 below.
Shorter exposure times may avoid the smearing issue, but will not have as high of a resolution. It was important for us to find a medium in which the resolution was decently high and there was not much smearing with our images. The actual frame rate being recorded by our images is given by

\[
Frame Rate = \frac{1}{\frac{1}{\text{specified frames per second}} + \text{exposure time}}.
\]  

The Laserview interface to select the exposure time and frame rate of our system can be seen in Figures 35 and 36 respectively.
The final step in the scanning in the axial direction of a sample is determining the total number of images that is needed. This number is calculated by multiplying the total number of frames taken per micrometer by the total distance scanned. The total amount of frames per micrometer is based off of all of the specified values of the linear voltage ramp as well as the actual frame rate that is calculated from
the exposure time and set frame rate in Laserview. The equation relating all of these parameters to the number of total images per micrometer is given by

\[ \text{Frames per micrometer} = \text{Frame rate} \times \frac{\text{Time Delay}}{\text{Voltage Increment}} \times \frac{\text{Volts}}{\text{Micrometer}}. \]  

Therefore, the total amount of frames gathered for the entire distance scanned is given by

\[ \text{Total Frames Gathered} = \text{Frames per micrometer} \times \text{Total Distance Scanned}, \]  

and the distance between frames is calculated by

\[ \text{Distance Between Images} = \frac{1 \text{ micrometer}}{\text{Frames per micrometer}} \]  

The Laserview interface to select the number of frames taken can be seen in Figure 37.

![Laserview interface to select total number of frames taken.](image)

Figure 37: Laserview interface to select total number of frames taken.
All of the cross sectional images that we acquire are saved in a video file. The format that we have used for our videos is an .lvvid file. The next step of our process is to analyze the intensity modulations present in each pixel of our images in the .lvvid file using Matlab 2011 to determine surface characteristics of the sample being imaged.

3.4.2. Intensity Analysis of an Individual Pixel in Axial Direction

After experimentally gathering a video file which included the cross sectional images of our sample, the next step in our process was to individually examine each pixel in the axial direction. This was done by stacking all of our images on top of each other in the order in which they were taken so that all of the pixels lined up in the X and Y directions. Then, the coordinates of one pixel could be chosen on the very top image, and the intensity modulation could be looked at through the entire stack of images for that pixel. The idea behind this individual pixel intensity analysis can be seen in Figure 38.

Figure 38: Isolating the intensity of one pixel in the axial direction.
As discussed earlier, intensity modulations result from interference of the reference beam and the sample beam of our light source. These intensities increase as the distance of the object beam to the surface of the sample decreases, and are at a maximum when the object beam comes into contact with the surface of the sample. Additionally, modulation increases and reaches another maximum as internal structures in transparent biological samples are reached by the object beam. These increases of modulation can be seen as the traveling distance of our object beam penetrates the surface of the tympanic membrane in Figure 39. In this image, modulation increases again as the object beam travels through the lamina propria and again through the mucosal layer of the TM.

![Figure 39: Intensity modulations resulting from our object beam traveling through tissue (Lim, 1995).](image)

Using Matlab 2011, we were able to plot the intensity variation for an individual pixel in the Z direction. With our designed code, we could choose the pixel that we wanted to examine based off of...
the X and Y coordinates and see the intensity modulation that occurred at that location. The intensity modulation for the specified pixel would vary in amplitude, and the peak of the modulation would vary in location depending on where the point of highest reflectivity was. Figure 40 is an example of an intensity modulation for a pixel that we have gathered during one of our tests.

![Intensity modulation for an individual pixel.](image)

**Figure 40:** Intensity modulation for an individual pixel.

### 3.4.3. Difficulties with Envelope Generation by Hilbert Transform

After experimentally gathering a video file containing cross sectional images of our sample being imaged and analyzing one pixel to return an intensity modulation, our next step was to put an envelope over that modulation so that we could isolate the maxima which would correspond to the location of a
point on the surface of the sample. As discussed earlier, to generate this envelope we applied a Hilbert transform.

In section 3.2 an envelope was applied to the spatial OCT governing equation using the Hilbert transform. This envelope was very smooth, and the maximum point could clearly be identified. Experimentally, however, this was not the case. In a real life testing and image acquisition, there is always some amount of noise that is encountered that can affect image processing. It had turned out that the presence of noise in our acquisition procedure severely altered the envelope that we generated through our Hilbert transform. The experimentally acquired signal with a noisy envelope can be seen in Figure 41.

![Noisy envelope generated over data from an actual test.](image)

Figure 41: Noisy envelope generated over data from an actual test.
The main issue associated with a noisy envelope such as the one in Figure 41 is that it can harshly alter the location of the maxima of the intensity modulation. If we attempt to find the maximum point of our envelope while there is noise present, the maximum can be off by a factor of micrometers from where it should truly be located, which will lead to very inaccurate three dimensional images. Because each peak corresponds to a structure on the surface of a tissue or within, accurately locating the maxima is extremely important. In Figure 42, the maximum of a noisy envelope can be seen which differs greatly from where the maxima should be located. The location on the X axis where the maxima should be located can be calculated by averaging the distance between two points on the opposite side of the intensity modulation. This is given by

\[
\frac{17.75\mu m + 25\mu m}{2} = 21.38\mu m
\] (25)

and the closest point to this result is graphed in Figure 42.
To account for the noise generated in our envelopes our group investigated filtering methods which could reduce the high frequency components in our envelopes. The type of filter we ultimately decided to adopt was a digital low-pass Butterworth filter. The advantage to using a low-pass filter is that it accepts low frequency signals but reduces the amplitude of high frequency signals which are above a certain value (Morisak, 2012). This specified value is referred to as a cutoff frequency. All frequencies that are accepted and are not blocked by the low pass filter are referred to as the bandwidth of the filter, and those which are blocked and are reduced by the filter are referred to as part of the stop band.

Our filter is a 6th order low-pass filter. The order of the filter determines how steeply the signal amplitude is reduced and for each order it is reduced by approximately half. A Butterworth low-pass filter can be summarized by

\[ |H_c(j\Omega)|^2 = \frac{1}{1 + (\frac{j\Omega}{\Omega_c})^{2N}} \]  

(26)

in which \(H_c\) is referred to as the frequency response function (Kwon, 1998). In this form, \(\Omega\) is defined as the frequency of the signal being processed, \(\Omega_c\) is the cutoff frequency specified by the filter, and \(N\) is the filter order. A low pass filter also acts on a time delay. This means that it will take into consideration the location and frequency of preceding data points and will generate the new set of data points depending on those preceding points. This time delay is directly related to the filter order. Higher filter orders correspond to more data points being included in the time delay allowing for a closer approximation. Figure 43 is the same intensity modulation as Figure 41, except with the low pass filter applied. The maximum of the envelope can be clearly identified with no issue at all, and the envelope is extremely smooth.
3.4.4. Determining Shape Information using the Generated Envelopes

As noted in earlier sections, it is extremely important for us to gather accurate maxima on our intensity modulation for each pixel because the location of the maxima corresponds to either a structure on the surface or interior of a sample being imaged. Using Matlab 2011, it was necessary for our code to analyze the intensity modulation for one pixel in order to find the corresponding frame out of the total amount of cross sectional images we gathered in which a maxima occurred. Our code also had to store that location so that a three dimensional representation could be created at a later time. In order to sufficiently isolate the location in which a maximum occurred for an individual pixel, we implemented a built-in Matlab function to our code. The max function in Matlab returns the largest elements either in an array, a vector, a matrix, or a multidimensional array. Since our intensity
modulations are processed as arrays, the max function in matlab will find the point with the highest amplitude and will return that value as well as the location on the X axis where that occurs. We are interested in the location on the X axis, as the X axis corresponds to the depth of surfaces in a tissue that we are imaging. We can also store this value into our workspace so that that process can be repeated for all pixels. By repeating this process for all pixels in our image field of view, we can generate three dimensional images of the sample.

3.5. Three-Dimensional Image Generation

As discussed earlier, optical coherence tomography has the ability to yield three dimensional images of biological tissue. This means that both surface characteristics and internal structures can be imaged using our system. When examining intensity modulations for various pixels in the axial direction, we encountered numerous cases in which multi-modal intensity modulations occurred. This means that the envelope of the intensity modulations for these pixels had 2 or more peaks. Some of the multi-modal intensity modulations for an axial scan of a tympanic membrane can be seen in Figures 44, 45, and 46. Because of constraints regarding computational power and time allowed for this project, we ultimately decided to only concentrate on creating three dimensional images using the first maxima of our multi-modal intensity modulations. The reasons behind this decision are explained in more detail in the following section (3.5.1).
Figure 44: Multi-Modal Intensity Modulation for Tympanic Membrane Scan - Pixel 1.

Figure 45: Multi-Modal Intensity Modulation for Tympanic Membrane Scan - Pixel 2.
The multi modal intensity modulations in Figures 44, 45, and 46 each only have two peaks. Because our scanning in the axial direction began a few micrometers from the surface of the tissue, the maxima of the first peak corresponded to a surface characteristic of the tympanic membrane. The second peak therefore corresponds to an internal structure. It is very likely that the maximum of the second peak represents a collagen fiber of the tympanic membrane. Because the membrane is transparent, our 780nm wavelength light source was able to penetrate the surface of the TM. Both the surface of the TM and the internal collagen fiber reflected light, which caused interference with our reference beam yielding a double mode intensity modulation. Both of these peaks could be extracted and three dimensional images with the surface and collagen fiber could be generated from this data.

3.5.1. Using maxima of all pixels to generate three dimensional images

After devising very efficient methods to calculate a smooth envelope over the intensity modulation for an individual pixel and locating the maxima, the next step was to complete this process
for every pixel in our entire field of view. As we discussed earlier, our system had the ability to generate field of views up to a maximum of 1024 pixels in the X direction and 1280 pixels in the Y direction, which is a total of 1,310,720 pixels.

$$\text{#Pixels } X \times \text{#Pixels } Y = \text{Total amount of pixels}$$  \hspace{1cm} (27)

$$1024 \times 1280 = 1,310,720$$

A giant problem that we faced with this project was the creation of a Matlab code that could efficiently find the maxima of all of these pixels, and then save the location of them in memory and generate a three dimensional image from those values in a reasonable amount of time.

In our first attempts to generate three dimensional images, the amount of pixels that we needed to analyze was a serious issue for us. To generate a three dimensional image with a field of view of 1mm × 1mm it took 13 hours for our computer system to locate the maxima for every single pixel in our field of view. This was an unreasonable amount of time for our images to be processed. If our system was going to have any future viability at all it had to be optimized so that image processing time was less than our goal of two hours. In order to do this, we had to re-think the way in which we were analyzing pixels to locate the maxima. Rather than examining one pixel at a time to locate the maxima of the intensity modulation, we re-ordered our .llvid video files using a custom made C++ program to examine groups of pixels based on which row they appeared in on an image. By doing this, the intensity modulations for a whole row of pixels would be acquired at the same time, and then the maxima were identified before moving onto the next row. This was much faster than producing intensity modulations and isolating maxima for pixels one at a time. By reordering our .llvid files in this fashion we were able to reduce our image processing time from 13 hours down to an hour and half. All of our Matlab and C++ code used to acquire 3-D OCT images can be found in appendix B.
3.6. Validation and Characterization of the System Setup

In order for our spatial OCT imaging system to be validated, we had to devise a method in which we could determine the accuracy of our measurements. Our OCT system would not be very useful if we could not very accurately determine the spatial resolution as well as the minimum step size that could be imaged. In this section, we describe the methods which we used to validate and characterize our system setup.

3.6.1. System Calibration

To determine the spatial resolution and minimum step size that could be imaged by our spatial OCT system we had to acquire measurements on a device that had an exact step size that was recognized globally. To do this, we acquired measurements using NIST traceable gauge blocks. NIST traceable gauge blocks are recognized by the ISO (International Organization for Standardization), meaning they are very suitable for use to characterize our system. Our team had access to a Dektak Calibration Standards Set from the Sloan Company which included calibration blocks of the step sizes 50KÅ, 5KÅ, 1KÅ, 500Å, and 200Å. By imaging the steps on these blocks using the acquisition procedure described in sections 3.4 and 3.5, we could determine the accuracy of our system, the spatial resolution, and the minimum step size that could be imaged. A 50KÅ NIST traceable gauge block that we image is shown in Figure 47.
To determine the minimum step size that could be imaged by our system and the resolution, we decided to first image the NIST traceable gauge block with the largest step size, and then to image decreasingly smaller step sizes until we could no longer see the resulting step size. The first step size that we imaged was the NIST 50KÅ step size shown in Figure 47. The resulting three-dimensional image is shown in Figure 48.

Because one angstrom is equivalent to \(1.0 \times 10^{-10}\) m, the 50KÅ NIST traceable gauge block is equal to 5 micrometers in size. As shown in figure 48, the resulting change in height in the z direction is
18.19µm – 13.35µm, which is equal to 4.84µm. This value is off of the specified value of 50KÅ by 3.3%, which is a very reasonable result. The resolution of the image in Figure 48 is 5nm.

The next NIST traceable gauge that we imaged was a 5KÅ step size. This is equivalent to a step size of 500nm. Although this step size was considerably smaller than the 50KÅ step size that was imaged previously, we were successful in generating a three-dimensional spatial OCT representation of the step size. The resulting spatial OCT image of the 5KÅ step is shown in Figure 49. As shown, the change in height in the Z direction is 8.027µm – 7.524µm which is equal to 503nm. The image resolution is 500pm.

![Figure 49: Spatial OCT image of a NIST traceable gauge with a step size of 500nm.](image)

After successfully imaging a step size of 5µm and 500nm, we next attempted to generate an OCT image of a step size of 1kÅ. This is equivalent to a step size of 100nm. By successfully imaging a step size this small, it would ensure that the 3D images of the tympanic membrane anatomy that we wanted to acquire would be very detailed and in a high resolution because the fibers in the lamina propria of the
TM were on the order of 1-20µm as reported in the literature (Puria, 2008). The resulting spatial OCT image of the 1kÅ step size NIST traceable gauge is shown in Figure 50. We found a height difference of 98nm with a resolution of 500pm.

![Figure 50: Spatial OCT image of a NIST traceable gauge with a step size of 100nm. The field of view is 1mm \times 1mm](image)

We were unsuccessful in acquiring OCT images of NIST traceable gauge blocks with step sizes of 500Å or 200Å. It is likely that this is because the distance between images that we were acquiring using our camera was on the order of 20nm, which was not small enough to distinguish the step sizes. By analyzing the results of the measurements performed on the NIST traceable gauge blocks, we were able to determine that the minimum step size that we could distinguish was 100nm with a spatial resolution of 500pm. This is extremely sufficient to produce images of the collagen fibers in the lamina propria, as literature has identified them as being between 1µm and 20µm in diameter.

As a fun experiment to further test the validity of our OCT system, we decided to image Abraham Lincoln on the back of the penny. The location of Abraham Lincoln on the back of the penny
and our resulting spatial OCT image is shown in Figure 51. The total depth of Abraham Lincoln from the highest point on his knee to the flat penny surface was identified as being approximately 60µm.

Figure 51: Spatial OCT image of Abe Lincoln on the back of the penny. Depth from Abe’s knee to the flat penny surface was identified as approximately 60µm.

4. Tympanic Membrane Measurements

After characterizing our system, determining the smallest step size that could be imaged, and finding the resolution, it was finally time to our team to take measurements on tympanic membrane samples. In this section, we present the experimental plan that we used when gathering spatial OCT measurements on a human tympanic membrane. The imaging of a chinchilla tympanic membrane to validate the imaging of a human tympanic membrane is presented. Additionally, we discuss the human tympanic membrane sample we were donated, preparation of the sample, imaging locations, resulting
OCT images, fiber density, and fiber size. Finally, our results are interpreted and the implications of our measurements are discussed.

4.1. Experimental Plan

Our overall goal in imaging tympanic membrane samples was to quantitatively image the fibers of the lamina propria. We planned to accurately determine the diameter of collagen fibers in the TM as well as the fiber density in different locations. To begin with, our first tympanic membrane measurements were to be taken on a chinchilla. By testing our OCT system on a chinchilla TM, we could demonstrate the ability to determine information on the collagen fibers in the lamina propria. This would validate our system, allowing us to take measurements on a human TM. All TM samples were donated and prepared for imaging by the Massachusetts Eye and Ear Infirmary.

To determine the fiber diameter and fiber density, we planned to take advantage of the field-of-view customizability of our spatial OCT setup. By customizing the field of view, we could image a location in the lamina propria on the order of 135µm × 135µm or less. By examining a field-of-view of this size, the diameter of collagen fibers could very clearly be identified and fiber density could be determined. By examining 135µm² sized areas throughout the TM, it could also easily be determined whether fiber density changed as a function of location.

In the following sections, our tympanic membrane images for the chinchilla are presented as well as information on the collagen fiber size and density. Additionally, we discuss the preparation of a human TM for imaging with our spatial OCT system and the locations in which it was imaged.

4.1.1. Chinchilla Tympanic Membrane Measurements

The first tests of our OCT system on an anatomical structure were on the fibrous layer of a chinchilla tympanic membrane. The TM was imaged in a dry state in vitro. Experimentally, we had never imaged a semi-transparent tissue sample before, so we were concerned that the TM would not reflect
our sample beam so that it could interfere with the reference beam to yield interference fringes. When we placed the TM under our microscope however, our interferogram expressed that interference fringes were indeed present. An interferogram is essentially a live video feed of the surface of a sample being imaged. The three dimensional tomographic OCT images that we were to produce would have the same field-of-view as the interferograms that we generated. An interferogram of the surface of the chinchilla TM can is shown in Figure 52.

![Interferogram of the chinchilla TM surface. The field of view is 0.87mm × 0.65mm](image)

All interferograms are created by the program LaserVIEW. Since interferograms are live feeds of the surface of a sample, they display the constructive and deconstructive interference of light, or fringe patterns, which appear as we scan through a sample. As mentioned earlier, by locating the maxima of these intensity modulations for each pixel in our stack of images, we could then plot those locations to generate three dimensional images. By using this process, we were able to generate a spatial OCT image.
corresponding to the location on the chinchilla TM surface as seen in Figure 52. The resulting OCT image of the chinchilla TM is shown in Figure 53. The exact location of this image on the surface of the TM is not documented.

Figure 53: Spatial OCT image of the chinchilla TM. The field of view is 0.87µm × 0.65µm.

The spatial OCT image of Figure 53 shows a very clear definition of the collagen fibers as well as a variation in orientation. Because the collagen fibers of the TM are below the surface, they do not show up as clearly in the interferogram in Figure 52. The 780nm light source that we used to image the TM however, was able to penetrate the surface of the TM and reflect back from the collagen fibers present therein to yield a representation of the fibers. With this generated spatial OCT image of the chinchilla TM, we could make our first measurements on fiber diameter and density. Figure 54 shows two 135µm
× 135µm locations on the spatial OCT generated image of the chinchilla TM in which fiber density and size can be clearly determined.

Figure 54: Fiber size and density for two 135µm × 135µm locations on the spatial OCT image of the chinchilla TM. Section (a) shows 16 collagen fibers with a diameter ranging from 3µm and 10µm in size. Section (b) shows 18 collagen fibers on the order of 2µm to 10µm.

From section (a) of Figure 54, 16 collagen fibers can be identified in the 135µm × 135µm location. Fiber diameter is measured as between 3µm and 10µm in size. Section (b) of Figure 54 contains 18 collagen fibers with fiber diameters ranging from 2µm to 10µm in size. Because section (b) contains collagen fibers at an angle, more appear in the 135µm × 135µm field-of-view. It was very clear from our preliminary results however, that spatial OCT was a very viable method to determine collagen fiber density and diameter in the TM. Figure 55 (a) shows a second spatial OCT image of the chinchilla TM
with the corresponding interferogram (b). The fiber size and density of two locations on this spatial OCT image are shown in Figure 56. The exact location of this spatial OCT image on the surface of the TM is not documented.

Figure 55: Second spatial OCT image of the chinchilla TM. The field of view is 0.87µm x 0.65µm.
From the analysis of the second chinchilla TM spatial OCT image, fiber diameter was determined to be on the order of 5µm for both section (a) and section (b) of Figure 56. Section (a) yielded 16 collagen fibers in the 135µm × 135µm field-of-view, while section (b) contained 15.5 collagen fibers. It appears in Figure 56 that the fiber density decreased from section (a) to section (b). It is also noticeable from Figure 56 that the orientation of the collagen fibers changes throughout the spatial OCT image. This means that the umbo would be located outside of the field-of-view to the bottom left of Figure 56.

With excellent results from the tests on the chinchilla TM, we had the confidence in our system to begin measurements on a human TM. The next sections describe the background on the human TM.
sample which we were donated as well as how it was prepared for imaging. Additionally, we describe the locations on the TM in which spatial OCT images were to be gathered. All of our spatial OCT images on the chinchilla TM can be found in Appendix C.

4.1.2. Tympanic Membrane Sample and Preparation

The findings that we had gathered from imaging the chinchilla tympanic membrane were sufficient enough to justify recording measurements on a human tympanic membrane. As mentioned, the Massachusetts Eye and Ear Infirmary was generous enough to donate the sample for our use in this project. The human TM sample measured was that of the left ear of a 52 year old male. The donor was known to have mild tympanosclerosis which is a condition leading to scarring and mild calcification in the tympanic membrane and middle ear. The TM sample was transported in a saline solution and was prepared for imaging by the Massachusetts Eye and Ear Infirmary. The human tympanic membrane measured is shown in Figure 57.

Figure 57: Human tympanic membrane donated by the Massachusetts Eye and Ear Infirmary.
To prepare the human TM sample for imaging, the epidermal and mucosal layers were removed, exposing the lamina propria containing the network of collagen fibers. The human TM was dissected whilst it was submerged in the saline solution. After dissection, the lamina propria was placed on a glass microscope imaging slide while maintaining the membrane in the saline solution. Once the lamina propria was uniformly laying on the microscope slide, it was removed from the saline and placed underneath the microscope objective and imaging could take place. A live image of the human TM dissection by the Massachusetts Eye and Ear Infirmary is shown in Figure 58.

Figure 58: Dissection of the human TM by the Massachusetts Eye and Ear Infirmary to prepare for imaging.
When we placed the human TM under our microscope and began to bring it into focus to find interference fringes, we ran into an issue. For some reason we could not clearly see the surface of the TM because everything appeared cloudy in the interferogram. After repeatedly adjusting the focus of the microscope to find interference fringes, we noticed some that were barely visible on the surface of the tissue. As we contemplated why this was happening, it occurred to us that the TM was just taken out of a saline solution. The surface of the membrane was still covered in the solution, with droplets of the saline sitting on the surface of the TM. The interferogram was showing the saline solution on the surface of the membrane, which gave the interferogram a very cloudy appearance. The very faint fringes that we were able to see were from our light source penetrating the solution and reflecting back from the surface of the membrane. We then realized that our best results would come from imaging a sample that is dry as opposed to damp or wet. We removed the microscope slide with the TM on it from the microscope and dried it off, which allowed us to be able to see the characteristics of the TM very nicely in our interferograms. The following section discusses the locations in the lamina propria of the human TM which we were to image.

4.1.3. Imaging Locations

One of the major drawbacks of the results that we obtained with the chinchilla tympanic membrane was the fact that we did not know the exact locations in which our spatial OCT images were taken. This made it hard to come to conclusions on the fiber density and size throughout the TM. Therefore, one of our major goals when imaging the human TM was to very accurately know the location in which our images were taken. To do this, we planned to begin imaging next to the umbo and to then gather images radially in one direction until we reached the edge of the TM. Additionally, only the lamina propria of the pars tensa would be imaged because the TM was dissected prior to imaging. The approximate locations in which the human TM was imaged are shown in Figure 59. Eight spatial OCT images with field of views of 750µm x 750µm were gathered radially across the tympanic membrane.
4.2. Human Tympanic Membrane Measurements

After confirmation that spatial domain optical coherence tomography could be used to quantitatively image the fibers present in the lamina propria of the tympanic membrane of a chinchilla, we began to gather images on the human tympanic membrane sample donated by the Massachusetts Eye and Ear Infirmary. The goal of imaging of the human tympanic membrane with spatial OCT was to determine whether or not distance from the umbo had an effect on the collagen fiber density present in the lamina propria of the pars tensa. In the following sections, we present our findings as well as discuss their implications to hearing mechanics and medical imaging.
4.2.1. Images of the Lamina Propria in the Human Tympanic Membrane

As previously mentioned in Section 4.1.3, eight spatial OCT images were gathered in a radial direction across the surface of the TM. The field-of-view for each spatial OCT image was 750µm × 750µm. For each image, the frame rate of our camera was set as 10 frames/sec and the exposure time of the camera was set to 0.025s. From equation (21), the actual frame rate of our camera could then be calculated as

\[
Frame \ Rate = \frac{1}{\frac{1}{10} + 0.025} = 8\, fps. \tag{21}
\]

A time delay of 0.08 seconds was used for our voltage ramp with a voltage increment of 0.001V. From here, the total time for an image to be scanned was given by

\[
Time\ to\ scan = \frac{Time\ Delay \times Max\ Voltage}{Voltage\ Increment \times 60sec}. \tag{28}
\]

From equation (28), we had scan times between 4 and 8 minutes because the total distance scanned for the human TM varied between 30µm and 60µm (1 micrometer of travel corresponded to 0.1V applied to the piezo). Furthermore, the amount of frames per micrometer was calculated by equation (22) given by

\[
Frames\ per\ micrometer = 8 \times \frac{0.08}{0.001} \times \frac{0.1}{1} = 64\frac{frames}{micrometer}. \tag{22}
\]

From the specified values for the image acquisition, the first spatial OCT image of the human TM was acquired. This image is shown in figure 60 along with the corresponding interferogram. The second and third spatial OCT images that we gathered on the human TM are shown in Figures 61 and 62 respectively with the interferogram for each image.
Figure 60: First spatial OCT image of the human TM with corresponding interferogram. The field of view is 750µm × 750µm.

Figure 61: Second spatial OCT image of the human TM with corresponding interferogram. The field of view is 750µm × 750µm.
Five more spatial OCT images were gathered radially along the tympanic membrane with each image taken at the very edge of the preceding image. By doing this, we could effectively image a cross section of the human tympanic membrane. Because all eight of our OCT images were gathered next to each other, when stitched together they would create a representation of the lamina propria of the tympanic membrane. We were able to successfully stitch all of our spatial OCT images of the human TM together. The resulting spatial OCT cross sectional representation of the human tympanic membrane is shown in Figure 63. This representation was created by using the interferograms for each of our spatial OCT images to identify the correct orientation for each spatial OCT image. Top views were then taken for each spatial OCT image which corresponded to the interferograms. The top views were extracted and stitched together to perfectly line up the spatial OCT images. The field-of-view of the linear representation of the human TM by spatial OCT is 0.75mm × 6mm.
Figure 63: Eight spatial OCT images of the human TM stitched together and the approximate location of this linear representation. The field of view is 0.75mm × 6mm (Uliyanov, 2012).
From the spatial OCT images that were gathered along the human TM and the linear representation of Figure 63, information on fiber size and density in different locations of the tympanic membrane could be found. In the following section, we examine fiber density as a function of location as well as fiber size and orientation. All of the spatial OCT images of the human TM are shown in Appendix D.

4.2.2. Fiber Density as a Function of Location

To determine fiber density present in the spatial OCT images that we acquired, we used the same methods that we used for the chinchilla tympanic membrane. Each OCT image had a field-of-view of 750µm × 750µm and contained hundreds of collagen fibers. Therefore, to determine collagen fiber density and size, smaller sections of the OCT images with a known field-of-view were created. With these smaller sectional images, the lamina propria characteristics could be very clearly seen.

For the first spatial OCT image that we acquired which was shown in Figure 60, we used this approach to determine the collagen fiber density and size. A 135µm × 135µm section was examined in which 18 collagen fibers were located. Fiber diameter was identified as being between 4µm and 15µm in size. Figure 64 shows how the size of a collagen fiber was determined in the first OCT image that we acquired. In the figure, the location of the first OCT image is shown on the total spatial OCT linear representation of the TM, and a 135µm × 135µm location is examined. The diameter of the collagen fiber shown in Figure 64 is calculated by

\[
\Delta x = |64.32\mu m - 57.62\mu m| = 6.7\mu m, \tag{29}
\]

\[
\Delta y = |32.16\mu m - 37.52\mu m| = 5.36\mu m, \tag{30}
\]

\[
diameter = \sqrt{\Delta x^2 + \Delta y^2} = 8.58\mu m. \tag{31}
\]
Figure 64: Fiber diameter and orientation at the left of the cross sectional spatial OCT representation of the lamina propria. The diameter of the collagen fiber examined is 8.58µm, and there are 18 collagen fibers in the 135µm × 135µm location.
This process was repeated for different locations throughout the linear representation of the lamina propria that we generated in Figure 63. The collagen fiber density for an OCT image generated next to where the umbo would be located is shown in Figure 65. Additionally, we identify the number of fibers present in a 135µm × 135µm location at the outer edge of the tympanic membrane in Figure 66.
Figure 65: Fiber diameter and orientation at the center of the linear representation of the lamina propria. The diameter of the collagen fiber examined is $8.04\mu m$, and there are 17 collagen fibers in the $135\mu m \times 135\mu m$ location.
Figure 66: Collagen fiber density and diameter near the outside edge of the TM. 15 collagen fibers are identified in a 135µm x 135µm location. Fiber diameter is identified as 6.7µm.
These methods for determination of the collagen fiber density and size were used in various locations throughout the tympanic membrane to draw conclusions on the collagen fibers of the lamina propria. We identified collagen fiber density and size in each of the eight spatial OCT images that we acquired. The results are shown in Table 1. TM image number 1 corresponds to Figure 64, while TM image number 7 corresponds to Figure 66. TM image numbers 1 through 8 are results from the spatial OCT images in Figure 63 from top to bottom. In the following section, we discuss these results that we obtained, as well as the implications that they might have on hearing mechanics as a whole.

<table>
<thead>
<tr>
<th>TM Image Number</th>
<th>Fiber Density per 134µm× 134µm location</th>
<th>Fiber Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>5µm - 15µm</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>5µm - 15µm</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>5µm - 15µm</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>5µm - 15µm</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>3µm - 18µm</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>3µm - 18µm</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>1µm - 20µm</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>1µm - 20µm</td>
</tr>
</tbody>
</table>

Table 1: Collagen fiber density and size throughout the linear representation of the lamina propria.
4.3. Interpretation of Results and Discussion

Through analysis of the spatial optical coherence tomography images that we acquired, we were able to determine that fiber density, orientation, and size varied throughout the tympanic membrane. In locations closer to the umbo, fiber density was found to be higher than locations closer to the outer edge of the tympanic membrane. Fibers were more clearly defined near the umbo and had a diameter range of 5µm - 15µm. As we gathered spatial OCT images radially toward the outer edge of the TM however, radial fibers became less defined. There was a larger range of diameter size as distance from the umbo increased, and the fiber density was decreasing. Collagen fibers either thinned out or clumped together toward the outer edge of the tympanic membrane, making them more difficult to identify. Additionally, circumferential fibers became more prevalent in the outer edges of the tympanic membrane than closer to the umbo. Because circumferential fibers are located underneath radial fibers when examined in the depth of the TM, it makes sense that they became more visible closer to the edge of the TM. This is due to the thinning and clumping together of radial fibers as distance from the umbo increased.

When comparing the results that we obtained from the chinchilla tympanic membrane to the human tympanic membrane, it is very noticeable how much more defined the collagen fibers in the chinchilla tympanic membrane are than the human TM. There are a few possible reasons for this outcome. The donor for the human TM sample that we imaged was known to have mild tympanosclerosis. Tympanosclerosis causes mild calcification of the TM, which can lead to hearing loss. Because of this, the orientation, density, and size of fibers could have been altered from that of a healthy TM. Additionally, because the human TM was dissected to expose the fibers of the lamina propria, it is possible that the fibers were damaged in the dissection process causing them to be not as clearly defined as the chinchilla TM. Nevertheless, we were able to determine that the collagen fibers in
the chinchilla tympanic membrane as well as the human tympanic membrane had similar diameters and fiber densities throughout.

The results that we obtained were confirmed by medical researchers as the first of their kind. The fibers of the human tympanic membrane have never been quantitatively mapped out such as in Figure 63. Images such as these are an indication that in the future, maps of the entire tympanic membrane fibrous layer can be generated. When compared to generated models of tympanic membrane displacements under pressure waves, correlations between displacement and anatomical characteristics can be made. This would be a significant advancement in the study of hearing mechanics.

There are many potential applications of quantitative maps of the tympanic membrane fibers. For example, a spatial OCT representation of the fibers in the tympanic membrane could be used to determine the best location in which an incision could be made in the TM during a surgical procedure. Additionally, spatial OCT images of the human TM could then be used post-operation as a means to track the healing process of a patient after surgery. Eventually, spatial OCT representations of the fibers in the human TM could be used as a diagnostic tool to determine why some people undergo hearing loss. If images of the TM were gathered for a person as he or she ages, changes in the histology of the tympanic membrane could be identified. This could lead to a better understanding of why people begin to lose their hearing as they age.

5. Conclusion

Based on our results we believe that our imaging system is an effective method for quantitatively imaging the collagen fibers present in the lamina propria of the tympanic membrane. Our system can also be used to image other biological tissues non-invasively by modifying some of the system components. The main limitations of our system are hardware and software related. Our hardware was mainly limited by the speed of the computer that we used for image processing.
Employing a computer with more capabilities would decrease the image acquisition time considerably. Additionally, employing a high speed camera could also improve the speed of acquisition by allowing us to scan through a sample more quickly. The analysis algorithms used to generate spatial OCT images could also benefit from being reworked by someone formally trained in writing computer code. Our group has limited coding experience and we believe that there are more efficient methods to analyze the data collected by our system. These two changes would vastly improve the efficiency and speed of our system.

Spatial domain optical coherence tomography can be a very effective means to image the internal structure of the tympanic membrane. Through analysis of OCT generated TM images, the collagen fiber size and density throughout the TM can be determined. Because of the difference in collagen fiber density throughout the TM, it is very likely that TM displacements relate to the structure and orientation of the collagen fibers. This project was a success as it met the goal of imaging and quantifying the fibers in the tympanic membrane. With additional research, our system could become a valuable clinical and research tool for labs and hospitals around the world.

5.1. Future Work

Future research will attempt to correlate TM displacements at a location under a specific frequency to the anatomical characteristics of the TM generated through spatial OCT. By examining sound induced displacements of the TM at a specific location to the fiber orientation at that location, a better understanding of the deformation patterns of the TM will result. This can lead to better understanding of hearing mechanics as well as the importance of the tympanic membrane.

The CHSLT Labs at Worcester Polytechnic Institute will continue the work we have done on this project in the future. One area that will need to be significantly improved will be the code that analyzes the video files acquired from scanning a TM. Our team has limited coding and script generating experience, which forced us to learn throughout the duration of this project how to process data and
generate useable outputs of TM models. This meant that we were only able to scratch the surface of our systems’ capabilities in terms of generating 3-dimensional tissue models. A more experienced coder could develop an analysis method that would reduce the hardware demands of our system and allow for full 3-dimensional model generation of sub-surface features using available computing platforms.

A key focus of the remaining work will be to automate the system so that it can be applied to clinical use with minimal effort being needed to operate the system. This will allow doctors and researchers to obtain high quality 3-dimensional representations of the TM with minimal effort. The results and future results generated by this system will need to be compared with other measurements made on the sound induced deformation and shape of tympanic membranes by other research groups in order to correlate the fiber orientation with the physical characteristics of the TM. This is important to allow for advances in modeling techniques of the TM and research into the hearing process as a whole.
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Appendix A. LabView Voltage Ramp

Section 1 – Upward Voltage Ramp
Section 2: Stalling the Voltage Ramp between the Upward and Downward Voltage Ramp
Section 3: Downward Voltage Ramp
Appendix B. MatLab Code

This section describes the MatLab code we developed and used to analyze the data gathered by our system. The script shown below is essential to our analysis procedure. Other scripts that are not shown are submitted with this paper and are available through WPI.

Single Pixel Analysis

This code isolates a single pixel in each image and graphs the intensity modulation through the entire image stack for each test. It then locates the maxima of these modulations to determine the location of surfaces or internal structures of a sample.

```matlab
clear; clc; close all;
tic

% Opens the image and isolates one pixel, manual input of pixel location and % total change in length of piezo
x=101; y=101;
num_images=3708;
total_microns=60;

%Selects video to be opened
Vid=openLVVid('TMtest4.lvvid');
Image_stack=zeros(1,num_images);
Frame_num=zeros(Vid.imageHeight,Vid.imageWidth);

%Summons each pixel in the image stack
for i=1:num_images
    Pixel = getLVVidPixel( Vid,i,x,y);
    Image_stack(1,i)=Pixel;
end

%Plot of Intensity Variation and Envelope Generation

% Measure the intensity axially along a pixel
Intensity=(Image_stack(1,:));
z=(total_microns/num_images):(total_microns/num_images):total_microns;
A=Intensity(:);
s=detrend(A);

% Filtering the acquired data using a low pass filter
dz=(total_microns/num_images); _
filter_cutoff=4.5; _
sF=my_filter(s, dz, filter_cutoff);

% Plot of intensity variation for 1 pixel
plot(z,sF,'r')
grid
xlabel('micrometers');
ylabel('Amplitude');
hold on;

% Envelope generation through Hilbert Transformation
```
H=hilbert(sF);
Envelope=abs(H);

% Filtering of the Envelope using a low pass filter
filter_cutoff2=.25;
fF=my_filter(Envelope, dz, filter_cutoff2);

% Plotting the Envelope on the graph of the intensity variation
plot(z,fF,'b')
hold on

% % Determination of the maxima of the envelope
[maxEnv, locMax]=max(fF(100:end-100));
locMax=locMax+100-1;
zpercentage=locMax/num_images;
zlocation=zpercentage*total_microns;
plot(zlocation,maxEnv, 'r*');

% Fast Fourier Transform
Fs=1/dz;
L=length(sF);
NFFT=length(sF); % NFFT = 2^nextpow2(L);
y=fft(sF,NFFT)/L; %%% FFT of s
f = Fs/2*linspace(0,1,NFFT/2);
spectrum=2*abs(y(1:NFFT/2));
figure
plot(f, spectrum)
xlabel('frequency(1/microns)');
toc

Open LVVid Code
This script opens LVvid files in MatLab and allows us to analyze them. This code is specific to LVVid files as all of our images were acquired in this format.

function [ lvvidFile ] = openLVVid( filename )

fileID = fopen(filename);

header = char(fread(fileID, 256, 'schar=>schar', 0, 'l''));
header_lines = regexp(header, '\n', 'split');
frame_properties = regexp(header_lines(1,2), ',', 'split');
frame_properties = frame_properties{1,1}(1,:);
s=dir(which(filename));
filesize=s.bytes;

lvvidFile.fileId = fileID;
lvvidFile.filesize = filesize;
lvvidFile.imageWidth = str2double(frame_properties(1));
lvvidFile.imageHeight = str2double(frame_properties(2));
lvvidFile.bytesPerPixel = str2double(frame_properties(3));
lvvidFile.lvvFrameInfoSize = str2double(frame_properties(4));
lvvidFile.imageBytes = lvvidFile.imageWidth * lvvidFile.imageHeight * 
lvvidFile.bytesPerPixel;  
lvvidFile.frameBytes = lvvidFile.imageBytes + lvvidFile.lvvFrameInfoSize;  
lvvidFile.numFrames = (lvvidFile.filesize - 256) / lvvidFile.frameBytes;

end

Get LVVid Single Pixel
This script is used to isolate and store single pixels into memory for each image in an image stack

function [ Value ] = getLVVidPixel( lvvidFile, frameNum, x, y )

if (frameNum < 1 || frameNum > lvvidFile.numFrames)
ex = MException('LVVid:FrameNumberInvalid', 'Frame number specified
is not available in this file');
    throw(ex);
end

frame_offset = 256 + (frameNum - 1) * lvvidFile.frameBytes;
pixel_offset = lvvidFile.lvvFrameInfoSize + (y*lvvidFile.imageWidth + 
x)*lvvidFile.bytesPerPixel;
fseek(lvvidFile.fileId, pixel_offset + frame_offset, 'bof');

switch lvvidFile.bytesPerPixel
    case 4
type = 'float=>float';
    case 2
type = 'int16=>int16';
    case 1
type = 'int8=>int8';
    otherwise
        ex = MException('Fileformat:invalid bpp', 'Invalid bits per
pixel');
        throw(ex);
end

    Value = fread(lvvidFile.fileId, 1, type, 0, 'l');

end

My Filter Code
Code developed to filter noise from our acquisition signal for a single pixel.

function signal=my_filter(signal, dz, filter_cutoff)

if nargin < 3,
    filter_cutoff = 1.5; %Hz if time is in s
end;
filter_order = 6;
[filt_num,filt_den] = butter(filter_order,filter_cutoff*2*dz);
signal= filtfilt(filt_num,filt_den,signal);
3-Dimensional Image Generation Code
This script allows for the generation of 3-dimensional images using our acquired intensity data

clear; clc; close all;
tic

% Manually set the number of images and piezo travel distance
num_images=1835;
total_microns=30;

%Selects video and reordered file
Vid=openLVVid('TMtest1.lvvid');
VidReordered=openLVVid('TMtest1.lvvid.reordered');
Image_stack=zeros(1,num_images);

Frame_num=zeros(Vid.imageHeight,Vid.imageWidth);
Frame_num(:)=NaN;

%Sets the size of the image that you want to analyze
N1=1; N2=Vid.imageWidth; dy=Vid.imageWidth;
N3=1; N4=Vid.imageHeight; dx=Vid.imageHeight;

for y=N1:N2 %Vid.imageWidth
  for x=N3:N4 %Vid.imageHeight

    %Loads the reordered stack into memory
    Image_stack=getLVVidPixelallReordered(VidReordered,y,x, Vid.imageWidth, Vid.numFrames);

    % Plot of Intensity Variation and Envelope Generation
    % Measure the intensity axially along a pixel
    Intensity=(Image_stack);
z=(total_microns/num_images):(total_microns/num_images):total_microns;
A=Intensity(:);
s=detrend(A);

    % Filtering the acquired data using a low pass filter
dz=(total_microns/num_images);
filter_cutoff=4.5;
sF=my_filter(s, dz, filter_cutoff);

    % Envelope generation through Hilbert Transformation
    H=hilbert(sF);
    Envelope=abs(H);

    % Filtering of the Envelope using a low pass filter
    filter_cutoff2=.5;
fF=my_filter(Envelope, dz, filter_cutoff2);

    % Determination of the maxima of the envelope

[maxEnv, locMax] = max(fF(100:end-100));
locMax=locMax+100-1;

Frame_num(y,x)=locMax;

if (mod(x,10) == 0)
    strcat('x=',num2str(x),',y=',num2str(y))
end
end

%Sets the axes for the generated images
Distance=dz*Frame_num;
pixel_size=6.7;
magnification=5;
Xfieldofview=(pixel_size*dx)/magnification;%
X=(pixel_size*Vid.imageHeight)/magnification;%
Yfieldofview=(pixel_size*dy)/magnification;%
Y=(pixel_size*Vid.imageWidth)/magnification;%
Dx=(Xfieldofview/dx);
Dy=(Yfieldofview/dy);
x_axis=Dx:Dx:Xfieldofview;
y_axis=Dx:Dy:Yfieldofview;

[X,Y]=meshgrid(x_axis,y_axis);

%Graphs the 3-dimensional plot
M=Distance(N1:N2,N3:N4);
filt2=medfilt2(M,[3 3]);
filt2(filt2>30)=NaN;
filt2(filt2<10)=NaN;
surfl(X,Y,filt2)
shading interp
colormap(gray);
xlabel('x axis (microns)');
ylabel('y axis (microns)');
zlabel('depth (microns)');
toc

Get LVVid Pixel All Script
This script works the same as the single pixel variant described above but it brings all pixels into memory instead of a chosen one.

function [ Values ] = getLVVidPixelall( lvvidFile,x,y )

pixel_offset = lvvidFile.lvvidFile.frameInfoSize + (y*lvvidFile.imageWidth + x)*lvvidFile.bytesPerPixel;

    switch lvvidFile.bytesPerPixel
    case 4
        type = 'float=>float';
    case 2
        type = 'int16=>int16';
    end
case 1
type = 'int8=>int8';
otherwise
    ex = MException('Fileformat:invalid bpp', 'Invalid bits per pixel');
    throw(ex);
end

Values=zeros(lvvidFile.numFrames,1);

for frameNum = 1:lvvidFile.numFrames
    frame_offset = 256 + (frameNum-1) * lvvidFile.frameBytes;
    fseek(lvvidFile.fileId, pixel_offset + frame_offset, 'bof');
    Values(frameNum,1)=fread(lvvidFile.fileId, 1, type, 0, 'l');
end

end

Get LVVid All Pixels Reordered
This script brings all of the reordered video pixels into memory.

function [ Values ] = getLVVidPixelallReordered( lvvidReorderedFile,x,y,
origWidth, origNumFrames )

%pixel_offset = lvvidFile.lvvFrameInfoSize + (y*lvvidReorderedFile.imageWidth + x)*lvvidFile.bytesPerPixel;

switch lvvidReorderedFile.bytesPerPixel
    case 4
        type = 'float=>float';
    case 2
        type = 'int16=>int16';
    case 1
        type = 'int8=>int8';
    otherwise
        ex = MException('Fileformat:invalid bpp', 'Invalid bits per pixel');
        throw(ex);
end

Values=zeros(origNumFrames,1);

    frame_offset = int64(256) + int64((y-1)*origWidth + (x-1)) * int64(origNumFrames * lvvidReorderedFile.bytesPerPixel);
    fseek(lvvidReorderedFile.fileId, frame_offset, 'bof');
    Values(:,1)=fread(lvvidReorderedFile.fileId, origNumFrames, type, 0, 'l');
end
Centered FFT

function [X,freq]=centeredFFT(s,Fs)
%this is a custom function that helps in plotting the two-sided spectrum
%s is the signal that is to be transformed
%Fs is the sampling rate

N=length(s);

%this part of the code generates that frequency axis
if mod(N,2)==0
    k=-N/2:N/2-1;  % N even
else
    k=-(N-1)/2:(N-1)/2;  % N odd
end
T=N/Fs;
freq=k/T;  %the frequency axis

%takes the fft of the signal, and adjusts the amplitude accordingly
X=abs(fft(s))/N;  % normalize the data
X=fftshift(X);  %shifts the fft data so that it is centered

LVVid Reorder
Although not shown, this script was developed with the help of Ellery Harrington of the CHSLT labs. The script reorders our data to allow for more efficient analysis, improving the analysis scheme
Appendix C. Chinchilla Tympanic Membrane Images
This appendix is a compilation of the OCT images generated after imaging a chinchilla tympanic membrane sample. All axes are in micrometer units.
Appendix D. Human Tympanic Membrane Images

This appendix is a compilation of the human tympanic membrane images generated using our OCT system. All axes are in micrometer units. The image of in the top right of each page shows the location on the TM where each image was taken.