Nonlinear Optical Microscopy Assessment of Tissue Structure and Chondrocyte Viability of Articular Cartilage

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NONLINEAR OPTICAL MICROSCOPY ASSESSMENT OF TISSUE STRUCTURE AND CHONDROCYTE VIABILITY OF ARTICULAR CARTILAGE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
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Master of Science
Bioengineering

by
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Accepted by:
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ABSTRACT

Articular cartilage functions to protect the ends of bones by providing a surface that can withstand compressive forces and minimize friction during movement. Collagen fibers form the organizational backbone of the extracellular matrix (ECM) in cartilage. Proteoglycans within the ECM function to retain water and provide the tissue with the swelling pressure needed to withstand compressional forces. Chondrocytes, the only type of cell found in articular cartilage, produces these collagen fibers and proteoglycans to maintain the tissue structure and function. Significant injuries to articular cartilage can damage the chondrocytes and disrupt their ability to maintain homeostasis in the tissue. Therefore, chondrocyte viability is an important factor in assessing the severity of cartilage injury or the progression of degenerative joint diseases.

Assessing chondrocyte viability typically involves dye-labeling their cellular and ECM structures. Unfortunately, these methods disrupt the metabolism of the chondrocytes and cannot be used in patients or for longitudinal studies. Using nonlinear optical imaging methods, specifically two-photon excitation fluorescence (TPEF) and second-harmonic generation (SHG), chondrocyte viability and ECM structures can be nondestructively evaluated through the measurement of intrinsic signals from endogenous fluorophores. We have demonstrated a method that uses endogenous fluorophores as intrinsic biomarkers to assess chondrocyte viability and collagen organization without the need for histological dyes.

This thesis aims to validate our chondrocyte viability assay and extend this technology to study the structural and function changes in damaged cartilage. First, we
imaged articular cartilage from porcine femoral and tibial condyles using TPEF and SHG microscopy and analyzed the chondrocyte organization and ECM structure. We observed structural differences at different loading regions, implying that these structural differences may correlate to different mechanical properties at different locations. We then performed imaging experiments to validate our autofluorescence-based viability assay in porcine cartilage. Sensitivity and specificity analysis of our results against a calcein AM and ethidium homodimer dye-labeling viability assay confirmed the reliability and applicability of our assay to porcine cartilage. Finally, we developed longitudinal imaging methods to dynamically assess changes to tissue structure and chondrocyte viability in articular cartilage after mechanical loading. We designed a custom tissue culture/imaging chamber to follow the structural changes in the tissue up to several weeks. We also built a mechanical loading instrument that fits inside a biosafety hood to avoid tissue contamination while applying mechanical loading cycles. Preliminary experiments showed success in minimizing contamination during culturing, mechanical loading, and imaging procedures.
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CHAPTER ONE

INTRODUCTION

In this thesis, we applied nonlinear optical microscopy techniques to study the structural and cellular components of articular cartilage. We utilized two-photon excitation fluorescence techniques to measure autofluorescent signals from intrinsic fluorophores from the chondrocytes. Second-harmonic generation techniques were used to assess collagen fiber structures in the extracellular matrix of the articular cartilage tissue. Together, these nonlinear microscopy techniques allowed us to develop methods to assess tissue structure and chondrocyte viability in articular cartilage tissue.

Articular Cartilage

Articular cartilage is a specialized type of connective tissue that covers the ends of bones in joints and functions to protect the bones against compressive forces and friction during movement. The tissue is mainly composed of collagen fibers, chondrocytes, and water. Collagen fibers are the most abundant molecule in articular cartilage and is primarily responsible for the structure of the extracellular matrix (ECM). Most of the collagen within articular cartilage tissue is type II collagen. Chondrocytes are the only type of cell found within cartilage and produce collagen and other macromolecules for the maintenance of homeostasis and tissue function.

Figure 1-1 illustrates the chondrocyte and collagen fiber arrangement within articular cartilage tissues at different depths. Type II collagen fibers in the superficial
tangential zone (STZ) of the tissue are aligned parallel to the surface and provide resistance against shear forces. Chondrocytes in the STZ are flattened along the direction of the fibers. In the middle zone, the fibers become more random in their orientation, and the chondrocytes are more spherical in shape. Fibers and chondrocytes in the deep zone are perpendicular to the subchondral bone to anchor the tissue to the underlying bone.

Figure 1-1: Schematic of articular cartilage illustrating general chondrocyte and collagen organization.¹

Chondrocytes produce macromolecules such as aggrecan and hyaluronan, which are major components in proteoglycans. Proteoglycans provide cartilage tissue with an overall ionic charge that allows the tissue to retain water, primarily in the middle and deep zones. This osmotic property creates swelling pressures within articular cartilage that are responsible for the tissue’s ability to resist compressive forces.

Under normal conditions, chondrocytes synthesize structural ECM components to maintain the functional properties of cartilage. However, under pathologic conditions, chondrocytes exhibit an imbalance of anabolic and catabolic activities that lead to
degenerative changes in the cartilage matrix and surrounding tissues.\textsuperscript{2} The diseased or pathologic state is known as osteoarthritis (OA), generally described as “wear and tear” of the joint tissues.

Assessment of articular cartilage is crucial in the diagnosis of OA and other degenerative joint diseases. Current clinical methods for the evaluation of cartilage health typically involves measuring mechanical properties or macroscopic visual assessment.\textsuperscript{3} The most common method of cartilage assessment in the knee is arthroscopy, which involves a minimally invasive procedure that allows the surgeon to visually observe defects on the cartilage surface. The surgeon may also touch the articular surfaces with blunt tools to gain a subjective feel of the stiffness of the tissue.\textsuperscript{3} Medical imaging modalities, such as magnetic resonance imaging (MRI) and computed tomography (CT), may also be used to measure the thickness or macroscopic structure of cartilage and other joint tissues.\textsuperscript{4,5} More recent studies have correlated electromechanical properties to characteristics of cartilage degeneration.\textsuperscript{3} These methods provide clinicians and surgeons with valuable structural and mechanical information but lack the ability or the spatial resolution to assess chondrocyte viability.

Assessment of chondrocyte viability is a critical factor in the evaluation of articular cartilage health due to its crucial role in maintaining tissue homeostasis and function. Thus, it is important that we develop methods to provide structural and cell viability information that could be used in clinical settings in conjunction with existing cartilage assessment methods. The following thesis describes our application of
nonlinear optical microscopy techniques to assess structural features and chondrocyte
viability in articular cartilage tissue. The two microscopy techniques utilized were two-
photon excitation fluorescence (TPEF) and second-harmonic generation (SHG). The
following sections describe these optical imaging techniques and their applications in
more detail.

**Two-Photon Excitation Fluorescence**

Before discussing TPEF, we need to understand the mechanisms of single-photon
excitation. Molecules known as fluorophores can absorb light in a specific range of
wavelengths depending on their chemical structure and properties. Upon absorbing light
energy, fluorophores transition to an excited energy state. Loss of energy through the
excited vibrational states causes a shift in the fluorescence emission spectrum towards
longer wavelengths, known as the Stokes shift. After fluorophores return to ground
state, energy is emitted in the form of fluorescence and can be measured through
fluorescence spectroscopy systems. In general, the excitation light has a shorter
wavelength than the emission. The Jablonski diagram in Figure 1-2(a) demonstrates the
mechanism of excitation and fluorescence emission for single-photon excitation.

In TPEF, two photons with twice the wavelength required for excitation
simultaneously interact with a target molecule. The combined energy of the two photons
during this interaction excites the molecule with double the energy and half the
wavelength. The interaction with two photons results in nonlinear energy relationships in
the excitation-emission process, which is why TPEF is a nonlinear optical technique.
The molecule absorbs the photons and transitions to an excited energy state. After the molecule returns to ground energy state, a fluorescence photon is emitted, similar to single-photon excitation emissions. The Jablonski diagram in Figure 1-2(b) demonstrates the mechanism of excitation and fluorescence emission for TPEF.

TPEF has several advantages over single-photon excitation fluorescence. Light with longer wavelengths can be used to excite fluorophores using TPEF principles. Fluorophores that normally require near-UV wavelengths for excitation can instead be excited with longer wavelengths and lower energy light, which significantly reduces the risk of damaging or photobleaching cells and tissues. The radiation risks are not higher than other widely-used imaging modalities, such as X-rays or CT scanners.
increasingly efficient instrumentation, faster image acquisition speeds further reduce the risk of damage by minimizing total irradiation.\textsuperscript{7}

The use of longer wavelengths allows the light to penetrate deeper into tissues.\textsuperscript{6,7,11} Current technology can image a few millimeters deep into the tissue while maintaining sufficient signal strength and spatial resolution\textsuperscript{12–14}. Optical sectioning is also made possible by utilizing a scanner component with the microscope to move the excitation focus around a focal plane at various tissue depths.\textsuperscript{6} Laser scanning two-photon excitation technology allows for the collection of well-localized autofluorescence signals with minimal signal loss from scattering effects, which is where a portion of photons reflect off particles in the medium though which they pass.\textsuperscript{6} These advantages of TPEF imaging have allowed it to become a powerful tool for nondestructive and noninvasive live tissue imaging. A key application of TPEF is in the excitation and detection of autofluorescent signals from intrinsic fluorophores.

\textbf{Intrinsic Fluorphores}

Intrinsic fluorophores are molecules with fluorescent properties and are native to biological tissues and cells. Intrinsic fluorophores have specific excitation and emission spectra, commonly referred to as autofluorescence.\textsuperscript{9} The main advantages to using intrinsic fluorophores in biological research is evident when comparing them to dye-labeling methods. Exogenous, or external, dye fluorophores are markers that target specific molecules with high specificity and fluoresce with strong intensities. However, exogenous dyes often disrupt biochemical or metabolic processes within the specimen.
and are not suitable for in vivo studies, observing dynamic biological processes, or tissues meant for transplantation.15 Dyes also have poor penetration depth when used in tissues with low diffusion permability.15 These disadvantages are minimized when using intrinsic fluorophores because the target fluorophore already exist in cells and tissues, and exciting these molecules typically does not disrupt their physiological functions.

A number of known intrinsic fluorophores are currently being used as biomarkers for a variety of studies.9,16 Table 1-1 summarizes well-known intrinsic fluorophores, their functions, their excitation and emission ranges, and how they are being used in current biological studies. Figure 1-3 shows the excitation and emission spectra of common endogenous fluorophores found in tissues.17
Table 1-1: Endogenous fluorophores found in literature.

<table>
<thead>
<tr>
<th>Intrinsic fluorophore</th>
<th>Function</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Research and diagnostic value</th>
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<tr>
<td>Collagen</td>
<td>ECM structural protein</td>
<td>330 – 340</td>
<td>400 – 410</td>
<td>Evaluation of lesions and fibrosis&lt;sup&gt;9,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Elastin</td>
<td>ECM structural protein</td>
<td>350 – 420</td>
<td>420 – 510</td>
<td>Changes in expression may be related to tumors&lt;sup&gt;9,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Keratin</td>
<td>ECM structural protein</td>
<td>355 – 405</td>
<td>420 – 480</td>
<td>Changes in expression may be related to tumors&lt;sup&gt;9,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Coenzyme in redox reactions</td>
<td>330 – 380</td>
<td>440 (bound); 462 (unbound)</td>
<td>Biomarkers for cellular metabolism&lt;sup&gt;9,11,12,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>FAD / Flavins</td>
<td>Coenzyme in redox reactions</td>
<td>350 – 370; 440 – 450</td>
<td>525</td>
<td>Biomarkers for cellular metabolism&lt;sup&gt;9,11,12,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>End product of lysosomal digestion</td>
<td>400 – 500</td>
<td>480 – 700</td>
<td>Biomarker of degenerative diseases and oxidative stress&lt;sup&gt;9,16,18,19&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aromatic Amino Acids: Phe, Tyr, Trp</td>
<td>Functional proteins</td>
<td>240 – 280</td>
<td>280 – 350</td>
<td>Changes in expression may be related to tumors&lt;sup&gt;9,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>Formation of hemoproteins</td>
<td>405</td>
<td>630 – 700</td>
<td>Biomarkers for heme and iron metabolism&lt;sup&gt;9,16&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>Cell growth, antioxidant</td>
<td>370 – 380</td>
<td>490 – 510</td>
<td>Retinol metabolism, related to vision and photosensitivity&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
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Intrinsic Fluorescence of NADH and FAD

Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) are intrinsic fluorophores and intracellular coenzymes that play important roles in cellular oxidation-reduction reactions. In cellular respiration, NADH and FAD serve as electron carriers in numerous steps during the conversion of glucose into energy in the form of adenosine triphosphate (ATP). Both coenzymes undergo conversions between their reduced forms, NADH and FADH2, and their oxidized forms, NAD+ and FAD. Among these forms, only NADH and FAD yield autofluorescent signals, each with distinguishable peak emission wavelengths at 440 nm and 525 nm, respectively.

Both NADH and FAD have emissions with minimal overlap in the visible light range. The wavelengths required to excite them fall in the near-ultraviolet (UV) range that generally pose a problem for biological tissues. Continued exposure to photons with
near-UV wavelengths damages to cells, causes photobleaching, and negatively impacts longitudinal studies.\textsuperscript{7} By using TPEF techniques, excitation light at double the wavelength can be used to effectively excite NADH and FAD without damaging the cells.

Some of the earliest work in NADH autofluorescence can be attributed to Dr. Britton Chance in the 1950s\textsuperscript{20}. Dr. Chance’s work demonstrated a relationship between NADH fluorescence intensity and glycolytic activity, which further developed our understanding about mitochondrial function, metabolism, and redox states of respiratory enzymes\textsuperscript{12,20}. With Dr. Chance’s contributions, researchers have studied cellular metabolic processes by using NADH and FAD as intrinsic biomarkers with a reduced risk of modifying the biochemical or physiological state of cells\textsuperscript{7,12,14,15}. More recent studies have demonstrated a correlation between increased FAD autofluorescence signals and cell death.\textsuperscript{15,21} In this thesis, we used NADH and FAD autofluorescence signals to further the development of a nondestructive chondrocyte viability assay for articular cartilage.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is also autofluorescent, with excitation and emission spectra that are nearly indistinguishable from the spectra of NADH. NADH and NADPH are normally collectively referred to as NAD(P)H in literature.\textsuperscript{22} FAD shares its excitation and emission spectra with other flavins and flavoproteins (FPs). TPEF techniques used in this thesis cannot differentiate between different forms of NADH and flavins. Henceforth, NADH and NADPH will
collectively be referred to as NAD(P)H. FAD and flavoproteins will be collectively referred to as FPs.

**Second-harmonic Generation Microscopy of Collagen**

The second nonlinear optical technique used in this thesis is second-harmonic generation (SHG). SHG signals are generated when two photons of the same wavelength interact with a non-centrosymmetric structure and generate a new photon with half the wavelength. Unlike TPEF, the interaction process does not involve a loss of energy because the incident photons do not undergo an absorption process. The interaction mechanism can be modeled with a virtual state that corresponds to double the illumination photon energy from the two incident photons.

SHG is widely used in biological research to image molecules with non-centrosymmetric structures such as collagen. Collagen fibrils generally consist of three polypeptide chains wound together to form short triple helical fibrils. The fibrils are crosslinked to form larger bundles of collagen fibers. Collagen exists in a wide range of tissues in various forms. Type I collagen is the most abundant type and can be found in skin, fibrocartilages, bones, and tendons. Type II can mainly be found in cartilaginous tissues like articular cartilage. Type III is a major component in skin and ligaments that usually accompanies type I collagen, and type IV can be found in basement membranes of tissues. There are at least 27 known types of collagens with specific structures and functions, but types I-III are the most abundant and most commonly studied with SHG microscopy. Distinguishing between different types of collagen fibers with SHG is
difficult and requires more advanced imaging techniques, such as varying excitation polarization orientation\textsuperscript{23}, using fluorescence lifetime imaging\textsuperscript{24}, and measuring SHG light intensity ratios.\textsuperscript{25} SHG microscopy is utilized in numerous studies to observe and quantify collagen-related changes in cancerous or diseased tissues.\textsuperscript{26}

The ECM of articular cartilage is composed primarily of type II collagen. Using SHG microscopy, we nondestructively imaged the ECM based on its collagen signals without the need for additional contrasting agents. Like TPEF, SHG imaging also benefits from well-localized signals from small volumes due to the focused light that allows for noninvasive optical sectioning at different tissue depths.\textsuperscript{6} SHG images provided excellent contrast for the ECM against the TPEF signals from intrinsic fluorophores in articular cartilage.

**Summary of the Chapters**

This thesis applied two-photon excitation fluorescence and second-harmonic generation imaging techniques in the development of an autofluorescence-based chondrocyte viability assay, and in the study of structural and function changes in damaged cartilage. Chapter II covers our study of structural differences at different locations in porcine articular cartilage. We applied TPEF and SHG microscopy to analyze the chondrocyte organization and ECM structures at different loading regions. Chapter III describes the validation of our autofluorescence-based viability assay in porcine cartilage. Sensitivity and specificity analysis of our results against a viability assay confirmed the reliability and applicability of our assay to porcine cartilage.
Chapter IV summarizes our development of longitudinal imaging methods to dynamically assess changes in articular cartilage after mechanical loading. We designed a custom tissue culture/imaging chamber to follow the structural changes in the tissue up to several weeks. We also built a mechanical loading instrument that fits inside a biosafety hood to minimize contamination while applying mechanical loading cycles. Preliminary experiments showed success in minimizing contamination during culturing, mechanical loading, and imaging procedures.
CHAPTER TWO

NONLINEAR OPTICAL MICROSCOPY OF ARTICULAR CARTILAGE

STRUCTURE

Introduction

The structure of articular cartilage is characterized by different zones in the tissue, shown in Figure 1-1. The upper layer is called the superficial tangential zone (STZ). Collagen fibers in the STZ are typically aligned parallel to the surface to provide resistance against shear forces and protect the lower layers. Chondrocytes in the STZ have a flattened shape and are aligned with the direction of the tissue fibers around them. Below the STZ is the middle zone, where collagen fibers are arranged more randomly and chondrocytes are more spherical. In the deep zone, collagen fibers and chondrocytes are aligned perpendicularly to the subchondral bone to anchor the tissue to the bone. The thickness of articular cartilage can range from 2-4 mm, with the superficial layer composing about 10-20% of the total thickness.¹ A majority of the structural details in the superficial zone can be imaged using nonlinear microscopy techniques with penetration depths up to a couple hundred microns.

In this study, we applied our SHG/AF imaging methods to observe the structural differences of articular cartilage between load-bearing regions and meniscus-covered regions in a porcine knee joint. SHG signals provided details about collagen structures, while AF signals provided information about chondrocyte morphology and organization.
In the knee joint, the fibrocartilaginous meniscus separates the femoral and tibial condyles. Most of the articular surfaces are covered by the meniscus, except for a small medial region on the condyle called the load-bearing region where the tibial and femoral articular cartilage surfaces naturally interface with each other. During movement, the region is thought to experience different loading forces compared to the meniscus-covered regions that are supported by the meniscus. As a result, the structure of the cartilage may be different to appropriately handle the different forces. We performed this imaging study to observe and compare the structural differences between these two regions.

**Materials and Methods**

*Cartilage preparation*

A porcine hind knee joint (adult Yorkshire pig) was obtained from a local meat processing company. Muscles were stripped away from the joint, and the joint cavity was opened to reveal the articular cartilage surface. Cartilage samples were harvested using 5 mm (ID) sample corers (18035-05, Fine Science Tools) and were stored in Dulbecco's phosphate-buffered saline (DPBS, Corning) at 25°C.

A total of 6 articular cartilage samples were acquired from different locations of the articular cartilage (Figure 2-1). One sample was acquired from the load-bearing region (T1) of one of the tibial condyles. Two samples were acquired from the meniscus-covered regions (T2 and T3) of the same tibial condyle. The same procedure was
performed on the same corresponding locations on the femoral condyle: one sample from
the load-bearing region (F1), and two samples from the meniscus-covered region (F2 and
F3). The chosen condyles were not determined to be the medial or the lateral condyles of
the bones. The meniscus from the chosen condyle was also saved for imaging.

For imaging, the osteochondral surfaces of the samples were glued to Petri dishes
with the articular surfaces facing upwards. Samples were handled with care to not
damage the articular surface. The samples were immersed in DPBS. Meniscus sample
was prepared in a similar manner.

Figure 2-1: Photos of articular cartilage sample locations. (A) Tibial condyle. T1 was sampled from the
load-bearing region, and T2 and T3 were sampled from the meniscus-covered region. (B) Femoral
condyle. F1 was sampled from the load-bearing region, and F2 and F3 were sampled from the meniscus-
covered region.
Custom-built 3-channel nonlinear optical microscope

The custom-built nonlinear optical microscope was equipped with three detector channels: two for TPEF of NAD(P)H and FPs, and one for the SHG signal. All 3 channels acquired images simultaneously. A femtosecond Ti:Sapphire laser (Chameleon Ultra II, Coherent Inc.) served as the excitation light source. The system was designed to pass the laser beam through a dispersion compensation system, a collimation system, and a scanning mirror system before reaching the imaging objective lens (CFI LWD Plan Fluorite, 16x, NA0.8, Nikon). The emitted fluorescence from samples was collected by this 16x objective and was separated by a dichroic mirror DM1 (FF735-Di01, Semrock) from the excitation beam. Three photomultiplier tubes, PMT1 (PMTSS, Thorlabs), PMT2 (PMT2101, Thorlabs), and PMT3 (PMT2101, Thorlabs) were used to detect TPEF and SHG signals with an appropriate set of dichroic mirrors and bandpass filters. PMT1, 2, and 3 were assigned to detect signals of FPs, NAD(P)H, and SHG, respectively. Two dichroic mirrors (FF397-Di01-25x36 and FF470-Di01-25x36, Semrock) and three bandpass filters (FF01-370/36-25, FF01-442/42-25, and FF01-607/70-25, Semrock) placed in front of the three PMTs were used to pass appropriate signals to each channel. SciScan (Scientifica, UK) written in LabVIEW 2014 32-bit (National Instruments, Austin, TX) was used to control the microscope scanning functions and image acquisition parameters.
TPEF and SHG imaging

Samples were imaged with the custom-built multi-channel TPEF / SHG microscope. The excitation laser was tuned to a wavelength of 740 nm to generate SHG signals from collagen, and to excite endogenous fluorophores NAD(P)H and FPs simultaneously in chondrocytes. The collected signals were first passed through a 720 nm low-pass filter, then separated by a 397 nm dichroic mirror. The SHG of collagen was passed through a bandpass filter of 352–388 nm and was recorded in Channel 3. The signals that passed through the 397 nm dichroic were separated again by a 470 nm dichroic mirror before reaching the fluorescence channels. The two fluorescence channels have bandpass filters at the ranges of 421 – 463 nm (Channel 2) and 572 – 642 nm (Channel 1), designed to detect NAD(P)H and FP signals, respectively. The three-channel two-photon microscope allowed us to acquire NAD(P)H, FP, and SHG images simultaneously. Appendix C features a schematic of the SHG/AF imaging filter configuration.

Image stacks up to 100 μm were acquired, consisting of 50 512x512 pixel images from the tissue surface to deeper layers at a depth interval of 2 μm/step. The thickness of the stack covered the superficial layer of the cartilage tissue. Six replicate images were acquired for each step.

Microscope settings were maintained the same when acquiring all TPEF / SHG images. PMT1 and PMT2 gains were kept at 0.657 V, and bandwidths were set to 80
Mhz. Laser power was tuned to 150-160mW by adjusting the angle of the polarizer before the beam entered the dispersion compensation system.

**Image processing**

The acquired SHG/AF images were processed using the image processing program ImageJ. The “Deinterleave” function was used to separate the raw images into their three corresponding channels. Next, the “Grouped Z Project…” function was used to average the intensities of the six replicate images for each slice in the three z-stacks. The channels were then merged using the “Merge Channels…” function to form a composite image file. Channel 1 was assigned to the red pseudocolor, channel 2 to the green pseudocolor, and channel 3 to the blue pseudocolor. A macro was written to automatically execute these operations, documented in Appendix A. Brightness and contrast levels for each channel were adjusted to improve the readability of the image. Channel 3 SHG images were saved separately for Fast Fourier Transform (FFT) analysis.

*Fast Fourier Transform (FFT) analysis in ImageJ*

SHG signals from collagen were analyzed using the built-in FFT operation in ImageJ. Two slices from each imaging location were chosen for analysis: one from the superficial layer (0 – 25 μm) and one from a lower layer (80 – 100 μm). The image slices were processed using the FFT operation to yield their Fourier transform distributions. Intensities and thresholds were adjusted to remove background signals and improve the readability of the distributions. The images were saved and cropped from 512x512 to
256x256 pixel images. Signal patterns in the FFT images were then visually identified, labeled, and evaluated with descriptions.

**Results**

*Comparison of surface articular structure between different regions of femoral and tibial porcine cartilage*

After imaging the samples harvested from the locations described in Figure 2-2, we processed and merged the three channel images. Chondrocytes AF signals from NAD(P)H and FPs were assigned to green and red pseudocolors, respectively. Collagen SHG signals were assigned to the blue pseudocolor. Assessing the colored composite images, we found structural differences between the load-bearing regions (F1 and T1) and the meniscus-covered regions (F2, F3, T2, and T3) for both femoral and tibial condyle surfaces (Figure 2-2). The load-bearing regions had collagen fibers that were more densely organized than in meniscus-covered regions. Fiber structure on the surface of load-bearing regions resemble type II collagen, where the fibers are short and closely packed together. Examining the tissue structure about 80 μm below the surface, we observed a continuation of the short collagen fiber organization. In contrast, the meniscus-covered regions long, continuous fibers that were parallel to the surface. The fibers in these regions on the tibia also had wavy patterns. Deeper below the surface, we observed a transition from long and loosely organized collagen fibers to short and densely organized collagen fibers.
Chondrocyte organization was also different at the different sample locations. Chondrocytes in the load-bearing regions existed in closely-packed clusters within their lacunae, or cavities within the ECM. The dense collagen structure likely limits the chondrocytes’ ability to expand after dividing. In the meniscus-covered regions, the chondrocytes appeared scattered across the surface architecture, sitting in between the collagen fibers in lacunae that appear less defined. The cells were generally aligned with the orientation of the fibers.

Figure 2-2: Tibial (T1-T3) and femoral (F1-F3) articular cartilage SHG/AF images from superficial layers. T1 and F1 are from load-bearing regions. T2, T3, F2, and F3 are from meniscus-covered regions. Pseudocolor: Blue = Collagen; Green = NADH, Elastin; Red = FPs. Scale bar: 20 μm.
FFT analysis of collagen fiber alignment

FFT analysis revealed and confirmed patterns and structures in articular cartilage collagen fiber images that were not easily distinguishable by visual inspection. SHG channel image stacks were separated from the composite image stack. A superficial image and a deeper-layer image were chosen from each image stack for FFT processing. The chosen images, their FFT distribution, and their analyses are listed in Table 2-1. By isolating the SHG signals of the collagen fibers, we were able to visually observe the fiber structures and orientation without other overlapping intrinsic signals. The load-bearing regions had closely packed and short collagen fiber structures throughout the depth of the image stacks. The FFT distributions for load-bearing region images were generally uniform throughout the image stack, with some distinguishable fibers in the superficial layer. Spherical FFT distributions indicate uniform fiber alignment.

The meniscus-covered regions had prominent continuous collagen fiber structures on their superficial layers. These long fibers corresponded to an elliptical-shaped FFT distribution. The long axis of the elliptical shape corresponded perpendicularly to the dominant fiber alignment in its SHG image. Fibers with linear patterns were associated with narrower FFT ellipticals with shorter minor axes. Fibers with wavy patterns were associated with wider FFT ellipticals. The wave patterns added additional fiber orientations and are reflected in ellipticals with moderate minor axes. Analysis of the FFT revealed that wavy fiber patterns appeared in the meniscus-covered regions of the tibial articular cartilage samples but not in the femur samples. Examining the image
stacks of these regions showed a transition from loosely organized fibers in the superficial layers to more densely organized fibers in the deeper layers.

**Table 2-1: FFT analysis of SHG images from load-bearing and meniscus-covered regions at different depths.**

<table>
<thead>
<tr>
<th>SHG Image</th>
<th>Fast Fourier Transform (FFT)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td>F1 femoral cartilage, load-bearing region, 10 μm deep. Few collagen fibers with alignment were observed in the SHG image. The major axis of the FFT distribution corresponded to fiber alignment from the top left to the bottom right of the SHG image.</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>F1 femoral cartilage, load-bearing region, 80 μm deep. Collagen fibers appeared uniformly distributed in the SHG image. Centrally localized FFT distribution indicated no dominant fiber alignment.</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td>F2 femoral cartilage, meniscus-covered region, 14 μm deep. Distinct collagen fibers were observed in the SHG image. The major axis of the FFT distribution corresponded to the dominant fiber alignment from the bottom left to the top right of the SHG image.</td>
</tr>
<tr>
<td>Region</td>
<td>Depth</td>
<td>Observation</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>F2 femoral cartilage, meniscus-covered region</td>
<td>100 μm</td>
<td>Collagen fibers appeared uniformly distributed in the SHG image. Centrally localized FFT distribution indicated no dominant fiber alignment.</td>
</tr>
<tr>
<td>F3 femoral cartilage, meniscus-covered region</td>
<td>26 μm</td>
<td>Distinct collagen fibers were observed in the SHG image. The major axis of the FFT distribution corresponded to the dominant fiber alignment from the top left to the bottom right of the image.</td>
</tr>
<tr>
<td>F3 femoral cartilage, meniscus-covered region</td>
<td>80 μm</td>
<td>Collagen fibers appeared uniformly distributed in the SHG image. Centrally localized FFT distribution indicated no dominant fiber alignment.</td>
</tr>
<tr>
<td>T1 tibial cartilage, load-bearing region</td>
<td>14 μm</td>
<td>Few short collagen fibers were observed in the SHG image. Centrally localized FFT distribution indicated no dominant fiber alignment.</td>
</tr>
<tr>
<td>Region</td>
<td>Description</td>
<td></td>
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<tr>
<td>--------------------------------------</td>
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<tr>
<td>T1 tibial cartilage, load-bearing</td>
<td>Collagen fibers appeared uniformly distributed in the SHG</td>
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<tr>
<td>region, 80 μm deep</td>
<td>image. Centrally localized FFT distribution indicated no</td>
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<td></td>
<td>dominant fiber alignment.</td>
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<tr>
<td>T2 tibial cartilage, meniscus-</td>
<td>Distinct continuous collagen fibers were observed in the SHG</td>
<td></td>
</tr>
<tr>
<td>covered region, 25 μm deep.</td>
<td>image. FFT distribution major axis corresponded to the</td>
<td></td>
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<tr>
<td></td>
<td>dominant fiber alignment from the bottom left to the top</td>
<td></td>
</tr>
<tr>
<td></td>
<td>right of the SHG image.</td>
<td></td>
</tr>
<tr>
<td>T2 tibial cartilage, meniscus-</td>
<td>Distinct continuous collagen fibers were observed in the SHG</td>
<td></td>
</tr>
<tr>
<td>covered region, 80 μm deep.</td>
<td>image. FFT distribution major axis corresponded to the</td>
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<td></td>
<td>dominant fiber alignment from the bottom left to the top</td>
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<tr>
<td></td>
<td>right of the SHG image.</td>
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<tr>
<td>T3 tibial cartilage, meniscus-</td>
<td>Wavy collagen fibers were observed in the SHG image.</td>
<td></td>
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<tr>
<td>covered region, 20 μm deep.</td>
<td>FFT distribution major axis corresponded to the dominant</td>
<td></td>
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<tr>
<td></td>
<td>fiber alignment from the bottom left to the top right of the</td>
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<tr>
<td></td>
<td>SHG image. Wide FFT minor axis was associated with wave</td>
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<tr>
<td></td>
<td>pattern.</td>
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</tbody>
</table>
Discussion

Differences in articular cartilage structure at load-bearing versus meniscus-covered regions on each condyle has significant implications for the mechanical function of the cartilage at these locations. The meniscus is a fibrocartilage tissue that sits between femoral and tibial end bones and provides additional compression resistance and lubrication for movement. Most of the articular cartilage on the femur and tibia do not physically make contact in healthy joints because the meniscus covers a majority of the cartilage. The only exposed region, which we called the load-bearing region, is where the tibial condyle surface curves upward to meet the femoral cartilage. We acquired cartilage specifically from this region to observe the structural differences.

Load-bearing regions showed more organized collagen structures that resemble type II collagen throughout the superficial layers, implying that the region requires stronger and more rigid tissue structure than the meniscus-covered region. Compressive forces may be greater on that region without the meniscus to distribute the load. Chondrocytes in the load-bearing region, especially in the femoral sample we imaged,
were more densely populated and exist in well-defined lacunae, suggesting that the region may have more cellular activity than the meniscus-covered region does. The chondrocytes may be physiologically and metabolically different at locations with different ECM structures.

The SHG imaging technique was unable to distinguish between type I and type II collagen due to their similar SHG signals. More advanced imaging techniques would be required that involve varying excitation polarization orientation\textsuperscript{23}, using fluorescence lifetime imaging\textsuperscript{24}, and measuring SHG light intensity ratios.\textsuperscript{25}

Based on the morphology of the collagen fibers and our understanding of the general composition of menisci and articular cartilage, we can deduce the types of collagen fibers we see in our image. SHG images of the meniscus showed long, continuous collagen fibers signals throughout the acquired z-stack images (Figure 2-3). Menisci are composed primarily of type I collagen, which typically have these long fibrous characteristics.\textsuperscript{27} Then, comparing the meniscus images to the meniscus-covered region images at T2 and T3 in Table 2-1, we observed similar superficial structures. Considering that these two surfaces naturally interface with each other in the joint, this may have implications about why the meniscus-covered region has this structure on its surface. The meniscus may play a role in maintaining the surface of the articular cartilage. More studies will need to be performed to further investigate this idea.
FFT analysis of SHG images provided additional information about the differences in collagen fiber structure between load-bearing and meniscus-covered regions, and between the superficial and deeper layers. The fibers in the superficial layers were parallel to the surface, while the fibers in the deeper layers were more uniformly distributed with no dominant fiber alignment patterns. This is consistent with findings from other studies.\textsuperscript{28} The major differences between most of the SHG images were visibly obvious when examining the collagen fibers and signals. The FFT distributions provided information about smaller structural details. For example, the wavy patterns elongated the minor axes of the elliptical FFT distributions. Some images showed fibers that were aligned in different directions, making it difficult to visually determine the dominant fiber alignment direction. The FFT distributions revealed this dominant alignment in the major axes, demonstrating that this tool may be useful for future studies to map the collagen fiber architecture of articular cartilage in more detail.
Future studies should include more samples to assemble a more detailed map of articular cartilage structure. The work performed in this thesis only examined a total of 6 articular cartilage locations, with only 1 load-bearing sample and 2 meniscus-covered samples for each side of the porcine knee joint. Examining additional areas between these two regions may provide information about where and how these two regions transition in structure. Additional samples from multiple joints would also statistically support future studies, as this study only examined a single joint.

For future quantitative analyses, quantification of the densities of collagen fibers, elastin fibers, and chondrocytes can be performed. Developing a protocol to effectively obtain these metrics can be difficult and time consuming, depending on the number of images that would need to be processed and analyzed. For fiber densities, one method could use binary masks for the fiber structures, and the percentage of the image covered or left by the mask can be used as a metric. Chondrocyte counts for image stacks within a known volume provide a metric for cell density.

Conclusion

We found that the load-bearing regions of articular cartilage in the knee joint have different collagen fiber structures and chondrocyte organization compared to the meniscus-covered regions. FFT analysis of the SHG signals from the collagen fibers revealed additional differences in fiber alignment and structure between different locations and at different tissue depths. Imaging the meniscus surface showed collagen
fiber structures similar to the meniscus-covered regions. No significant differences were observed between femoral and tibial articular surface structures.
CHAPTER THREE

ASSESSMENT OF CHONDROCYTE VIABILITY IN ARTICULAR CARTILAGE
USING INTRINSIC FLUOROPHORE SIGNALS

Introduction

Chondrocytes are the only type of cell found within cartilage and produce collagen and other macromolecules, such as aggrecan, hyaluronan, and proteoglycans, for the maintenance of homeostasis and tissue function. Collagen is the primary structural component of the tissue and forms the backbone of the ECM. Proteoglycans are negatively charged macromolecules that allow the tissue to retain water to control swelling pressures responsible for compressive resistance.

Chondrocytes normally maintain these ECM components through a balance of anabolic and catabolic processes to maintain the functional properties of cartilage. However, under pathologic conditions such as osteoarthritis (OA), chondrocyte functions are disrupted, resulting in degenerative changes to the cartilage matrix, chondrocytes, and surrounding tissues. Assessment of articular cartilage is crucial in the diagnosis of OA and other joint impairments. Current clinical methods for the evaluation of cartilage health typically involves measuring mechanical properties or macroscopic visual assessment. Methods that provide clinicians with overall structural information but lack the capability or the spatial resolution to assess chondrocyte viability. Histological
staining methods also cannot be used *in vivo* because dyes disrupt biological processes and damage tissues.

Since chondrocytes play a crucial role in maintaining tissue homeostasis and function, assessment of chondrocyte viability is a critical factor in the evaluation of articular cartilage health. Thus, it is important that we develop methods to safely provide cell viability information complementary to existing cartilage assessment methods. A nondestructive and nonlabeling chondrocyte viability assay that utilizes chondrocyte intrinsic fluorophore signals for assessment had recently been demonstrated in murine articular cartilage.15 The work completed in section further developed and demonstrated this safe, intrinsic fluorophore signals-based chondrocyte viability assay in porcine articular cartilage.

**Materials and Methods**

**Cartilage preparation**

Porcine knee joints (n=3; adult Yorkshire pigs) were obtained from a local meat processing company. Under sterile conditions, the femoral and tibia heads were separated to reveal the articular cartilage. Twenty-four (24) cylindrical articular cartilage explants were harvested from the femur using 5 mm (ID) sample corers (18035-05, Fine Science Tools). The explants were rinsed with Dulbecco’s phosphate-buffered saline (DPBS, Corning) and transferred into 12-well plates with culture media. The media was composed of Dulbecco’s Modified Eagle Medium (DMEM, 1 g/L Glucose, no phenol
red, Gibco); 10% fetal bovine serum (FBS, Gibco); 1% MEM non-essential amino acids (100x, Gibco); 0.4 mM L-Proline (Alfa-Aesar); 2% Penicillin, Streptomycin, and Amphotericin B (Anti-Anti, 100x, Gibco); 1mM HEPES (Gibco); and 3mM L-Glutamine (Fisher). Appendix B outlines the culture media preparation protocol. Twelve (12) samples were cultured in incubator conditions at 37°C and 5% CO₂. The remaining 12 samples were cultured in refrigerated conditions at 4°C and normoxia. Culture media was changed three times per week.

Viability assay

We utilized a viability assay for simultaneous detection of live and dead cells by microscopy.²⁹ The dyes used in this assay were calcein AM (Biotium), ethidium homodimer (Biotium), and Hoechst 33342 (Biotium). Calcein was used to label live cells, EthD-1 was used to label dead cells, and Hoechst was used to label the nuclei of all cells. The dyes were mixed in DPBS with the following final concentrations: 2 μM Calcein AM; 8 μM EthD-1; and 10 μM Hoechst. Approximately 0.25 mL of the dye solution was used per sample, with incubation times of 20-30 minutes.

Filter configurations for SHG/AF imaging and viability assay

Samples were imaged with the custom-built multi-channel TPEF/SHG microscope. The excitation laser was tuned to a wavelength of 740 nm for SHG/AF imaging. The SHG signals were recorded in Channel 3 with a bandpass filter of 352–388
nm. Autofluorescence signals were recorded in Channels 2 and 1 the ranges of 421 – 463 nm for NAD(P)H and 572 – 642 nm for FPs, respectively.

For viability assay imaging, the excitation laser was tuned to a wavelength of 780 nm, and the filter configuration was changed. Hoechst signals were recorded in Channel 3 in the range of 417–477 nm. Calcein signals were recorded in Channel 2 in the range of 490–550 nm. EthD-1 signals were recorded in Channel 1 in the range of 580–720 nm. Appendix C features schematics of the SHG/AF imaging and viability assay imaging filter configurations.

Image stacks up to 100 μm were acquired, consisting of fifty 512x512 pixel images from the tissue surface to deeper layers at a depth interval of 2 μm/step. The thickness of the stack covered the superficial layer of the cartilage tissue. Six replicate images were acquired for each step. Microscope settings were maintained the same when acquiring all SHG/AF images. PMT1 and PMT2 gains were kept at 0.657 V, and bandwidths were set to 80 Mhz. Laser power was tuned to 150-160 mW for SHG/AF imaging. Laser power was tuned to 90-100 mW for viability assay imaging.

*Imaging procedure*

This section outlines the general imaging and staining procedure performed for each sample. The 5mm diameter samples were glued to custom 3D-printed sample holders designed to simplify the staining procedure. First, SHG/AF images were acquired using DPBS as the immersion liquid. Second, without moving the sample or the
objective lens, the DPBS was carefully removed from between the objective lens and the sample using a pipette. Next, approximately 0.25 mL of the viability staining mixture was applied to the surface of the sample and was incubated for 20-30 minutes. During the incubation time, the laser was tuned to 780 nm, the laser power was adjusted to 90-100 mW, and the filters configuration was swapped for viability assay imaging. After incubation, roughly 3 mL of DPBS was added on top of the sample to dilute the staining mixture and to reimmerse the objective lens and the sample. Finally, the viability assay images were acquired using the same z-stack coordinates as the SHG/AF images.

*Sensitivity and specificity analysis*

The acquired SHG/AF images were processed using the image processing program ImageJ with the procedure described in Chapter 2. Single images from various sample z-stacks with a balanced ratio of live and dead cells were selected for sensitivity and specificity analysis. A python-based graphical image annotation tool called Labelme was used to assist in the visual identification of cells. Cells in the SHG/AF images were outlined and indexed in Labelme. The outlines were then applied onto the viability assay images of the sample, serving as a mask that matched and indexed cells in both images. For live and dead cell classification, two people independently assessed the viability of the cells: one person graded the SHG/AF images, and the other graded the viability assay images. Live and dead cell classifications were then compared using contingency tables, where live cells were noted as positive while dead cells noted as negative. The viability
assay served as the gold standard, and the accuracy of SHG/AF imaging assay was evaluated by sensitivity and specificity, where:

\[
\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}
\]

\[
\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}
\]

Cells that were classified as live by both graders were counted as True Positive. Cells that were classified as dead by both graders were counted as True Negative. Cells classified as live in the viability assay images but dead in the SHG/AF images were counted as False Negative. Cells classified as dead in the viability assay images but live in the SHG/AF images were counted as False Positive. In total, 696 cells from 8 images from cultured cartilage tissues were included in this analysis. Descriptive statistics were reported as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software.

*Chondrocyte viability analysis*

SHG/AF image stacks up to 100 μm in depth with 2 μm steps were acquired for each sample. Images from every 10 μm in the stack were selected for manual chondrocyte viability assessment. An open-source, manual object counting software called DotDotGoose (American Museum of Natural History) was used to assist in counting the number of live and dead cells in each of the selected images. The total
number of live and dead cells were summed up per stack, and the viability ratio for the sample was calculated, where:

\[
Viability\ Ratio = \frac{Live\ Cells}{Total\ Cells}
\]  

(3)

The samples were categorized into different groups based on their imaging timepoint and their storage conditions. The viability ratios were averaged for each group, with 3 samples per group. Using GraphPad Prism, unpaired t-tests were performed between the two storage conditions at each imaging timepoint. Statistically significant differences were reported at \( p < 0.05 \).

**Results**

*Achondrocyte viability using SHG/AF and viability assay imaging*

A typical set of SHG/AF and viability assay images from each imaging timepoint is shown below in Figure 3-1. The imaging location in each sample was kept constant for the SHG/AF and viability assay imaging acquisitions. AF signals from the chondrocytes were directly compared to the stained chondrocytes for sensitivity and specificity analysis. Cells with stronger “green” AF signals from NAD(P)H were classified as live, while cells with stronger “red” AF signals from FPs were classified as dead. In the viability assay images, cells with strong green fluorescence from calcein AM were classified as live, while cells with strong red fluorescence from EthD-1 were classified as dead. From a set of 8 images with a total of 696 cells, the sensitivity and specificity were
0.95 ± 0.05 and 0.94 ± 0.06, respectively, demonstrating that the visual assessment of AF signals from chondrocytes was highly consistent with the calcein AM and EthD-1 staining from the viability assay. A graph of the viability ratios is shown in Figure 3-2. With an agreement of over 90%, we showed that our AF-based chondrocyte viability assay is reliable and applicable to porcine articular cartilage as a practical alternative to dye-labeling assays.
Figure 3-1: Typical set of SHG/AF and viability assay images from samples stored in 4°C and normoxia. (A-B) were from Day 3. (C-D) were from Day 5. (E-F) were from Day 7. (G-H) were from Day 10. Pseudocolor: Blue = Collagen; Green = NADH, Elastin; Red = FP. Scale bar = 40μm.
Figure 3-2: Chondrocyte viability ratios for 8 SHG/AF images with a total of 696 cells. Sensitivity and specificity were 0.95 ± 0.05 and 0.94 ± 0.06, respectively.

Effect of storage conditions on chondrocyte viability

We applied our AF-based assay to assess chondrocyte viability in articular cartilage tissues cultured under different storage conditions. Figure 3-3 summarizes our results, where we compared the viability ratios between samples cultured at 37°C and 5% CO2, and samples cultured at 4°C and normoxia. Chondrocyte viability trended downwards at a faster rate for samples cultured at 4°C and normoxia. Statistically significant differences in viability ratio between the two storage conditions were observed during Day 5 (p < 0.0005), Day 7 (p < 0.005), and Day 10 (p < 0.05) imaging timepoints. The results demonstrated that storing articular cartilage in incubator conditions slowed chondrocyte viability more effectively than in refrigerated conditions after 10 days.
Discussion

Sensitivity and specificity analysis demonstrated a strong correlation between AF signals and chondrocyte viability in porcine cartilage with over 90% agreement. Previous studies have also proven the effectiveness of this AF-based assay in murine articular cartilage.\textsuperscript{15} The results in this thesis further shows that NAD(P)H and FP intrinsic fluorophores in chondrocytes can serve as intracellular biomarkers for the assessment of cell viability in articular cartilage without the need for dye-labeling. One weakness is that only 8 image pairs were analyzed for this statistic. Additional images can be included in the sensitivity and specificity calculation to strengthen this conclusion. Future studies can further validate this assessment technique in human articular cartilage.
and eventually bring this nondestructive and nonlabeling imaging technology into clinical settings.

Combining SHG with AF imaging also provides information about collagen structures in the ECM alongside viability information. SHG imaging of the ECM provided spatial information in the lacunae that helped assess chondrocyte viability. In Figure 3-1, the green, healthy chondrocytes filled the volume of their lacuna, leaving only a small gap between the cell membrane and the ECM wall. As chondrocytes began to undergo apoptotic cell death processes, or programmed cell death, the volumes of the cells decreased and the gap between the cell and the ECM widened.\textsuperscript{30–32} Lacunae may also be empty under pathologic conditions associated with chondrocyte death.\textsuperscript{31} These dark gaps or outlines around “red” AF chondrocytes were seen in our SHG/AF images and were used to assist the assessment of chondrocyte viability. By combining SHG and TPEF microscopy techniques, we were able to observe these morphological details with great spatial resolution.

Future studies may use a higher microscope magnification to study the space between the chondrocytes and the ECM, called the pericellular matrix, more closely. The pericellular matrix contains many macromolecules produced by chondrocytes, such as perlecan, collagen, elastins, and hyaluronan, and functions to maintain the essential microenvironment for chondrocytes.\textsuperscript{28,33–36} An imbalance in this microenvironment may serve as a predictor of chondrocyte death. Click chemistry techniques may also be
applied to safely label chondrocytes and their pericellular matrices for imaging and monitoring.\textsuperscript{37,38}

The chondrocyte viability analysis under different storage conditions was a side experiment to the validation study. Cartilage was stored in refrigerated conditions to achieve a balanced ratio between live and dead cells in a shorter amount of time for the sensitivity and specificity analysis. After reviewing the literature, we found that our viability results contradict the findings in other studies that compared storage and preservation methods for osteochondral tissue. One group demonstrated slower cellular mortality in osteochondral tissue preserved at 4°C than at 37°C after 14 days.\textsuperscript{39,40} Another group developed their own preservation system, dubbed the Missouri Osteochondral Preservation System (MOPS), to enhance the quality of and the window for clinical use for osteochondral tissues. The group demonstrated that their MOPS technique could maintain 90\% chondrocyte viability after 60 days at 25°C.\textsuperscript{41,42} Other studies have also shown that engineered cartilage grafts seeded with chondrocytes had the most chondrocyte metabolic activity in incubator conditions after 56 days.\textsuperscript{43} Protocols for the harvest, transport, and preservation of osteochondral tissues in tissue banks have not yet been standardized.\textsuperscript{40} With different studies developing novel systems to improve cartilage preservation techniques, future studies related to this topic should first reproduce their results using their systems.

Chondrocyte viability assessment methods can be improved by using automated programs to identify and classify chondrocytes in SHG/AF images. Deep learning
algorithms have recently been developed to eliminate the need for manual counting of chondrocytes, reducing the amount of time and errors in counting.\textsuperscript{44,45} These algorithms have demonstrated accuracies of over 90\%, and they are designed to only improve as they analyze and learn from more images. Additional studies can help train these deep learning algorithms to recognize and classify chondrocytes in porcine articular cartilage.

\textbf{Conclusion}

We validated in porcine articular cartilage a chondrocyte viability assay that utilized intrinsic fluorophore signals from nonlinear microscopy techniques. Correlating autofluorescence with chondrocyte viability, we observed that live cells emit stronger “green” NAD(P)H AF, and dead cells emit stronger “red” FP AF. NAD(P)H and FPs can serve as biomarkers for the assessment of chondrocyte viability in porcine cartilage, and SHG/AF assessment methods are a nondestructive and nonlabeling alternative to dye-labeling methods.
CHAPTER FOUR

DEVELOPMENT OF LONGITUDINAL IMAGING METHODS FOR DAMAGED ARTICULAR CARTILAGE

Introduction

Articular cartilage serves to protects our bones and joints against forces and friction from everyday movement and activities. Normal mechanical loading from these activities has been shown to be beneficial to cartilage health, as dynamic compression can stimulate matrix production.\(^2,5,46\) Overloading, or when the joints experience high mechanical forces or injuries, can lead to degenerative joint diseases like osteoarthritis (OA). Overloading causes fractures and other defects in the joint tissues, particularly articular cartilage, which eventually develops into OA through inflammatory processes and tissue degeneration.\(^46\) There are currently no available treatments to restore full joint functionality for people with OA. However, studies have been performed to understand the mechanical limits of cartilage tissue and the processes that are involved in the progression of cartilage damage into OA.\(^47,48\) Studies have also been performed to determine minimum mechanical impact to cause microstructural damage.\(^48\) SHG microscopy techniques have also been used to study collagen fiber structures in damaged cartilage.\(^49\) However, few studies have dynamically observed changes in damaged articular cartilage over time.
In this study, we developed methods for longitudinal imaging of damaged articular cartilage using nonlinear microscopy techniques \textit{in vitro}. We applied mechanical loading forces to the surface of porcine cartilage samples to create microcracks in the tissue. Using SHG/AF imaging, we were able to observe structural changes in the ECM and viability changes in the chondrocytes. We developed a custom culture / imaging chamber and built a small mechanical loading device that allowed us to minimize contamination throughout the culturing, mechanical loading, and imaging processes.

\textbf{Materials and Methods}

\textit{Cartilage preparation}

A porcine knee joint was obtained from a local meat processing company. Under sterile conditions, the femoral and tibia heads were separated to reveal the articular cartilage. Six cylindrical articular cartilage explants were harvested using 5 mm (ID) sample corers (18035-05, Fine Science Tools) from load-bearing regions on the tibia. The explants were rinsed with phosphate-buffered saline (PBS) solution, transferred into custom 3D-printed chambers with culture media, and stored in incubators at 37°C and 5% CO₂. Each chamber housed two samples: one as a control, and one with a mechanically induced injury. The media was composed of Dulbecco’s Modified Eagle Medium (DMEM, 1 g/L Glucose, no phenol red, Gibco); 10\% fetal bovine serum (FBS, Gibco); 1\% MEM non-essential amino acids (100x, Gibco); 0.4 mM L-Proline (Alfa-Aesar); 2\% Penicillin, Streptomycin, and Amphotericin B (Anti-Anti, 100x, Gibco); 1
mM HEPES (Gibco); and 3 mM L-Glutamine (Fisher). Culture media was changed three
times per week.

**Culture / Imaging Chamber Design**

A custom chamber was designed to allow for culturing and imaging of cartilage
tissue over the course of several weeks. Figure 4-1 shows a SolidWorks model of the
chamber. The chamber has 3 components: the base, the interchangeable insert, and the
lid. The base has a square-shaped slot in the middle of the cavity for the interchangeable
insert to attach. The purpose of the cavity is to hold the culture media solution. On the
lip of the base is a groove that is designed to hold an O-ring gasket. Around the top of
the base are ridges for a screw-cap design that matches with the lid. When closed, the
screw-cap design with the O-ring allows for an air-tight seal that prevents solution from
leaking and contamination from occurring during the imaging process. When the
chamber is in the incubator, the lid may be loosened to allow for gas exchange to occur
while still maintaining a sterile environment.

The lid has an opening in the middle where a coverglass is attached by a silicon-
based glue. The insert design shown in Figure 4-1 has 2 sample slots for 5 mm diameter
cartilage samples, but this design may be easily changed depending on the sample size or
shape. When the chamber is closed for imaging, the distance between the insert and the
coverglass was designed for working distances of less than 3 mm.
Chambers were 3D printed using Prusa i3 MK3S+ (Prusa Research) and Ender-3 V2 (Creality) systems. PETG and ABS filaments were used for their water and heat resistance properties.

![SolidWorks model of the culture / imaging chamber](image)

**Figure 4-1: SolidWorks model of the culture / imaging chamber.**

**Mechanical Loading Instrument Design**

The mechanical loading instrument was built to fit within a sterile biosafety hood. A 25 mm motorized translation stage (KMTS25E, Thorlabs) was mounted vertically in the z-direction to an aluminum breadboard. Attached to the stage was a micro load cell (5kg, ATO Automation) with a 2 mm diameter ball-point indentation tip. Electric signals from the load cell were sent to a load cell transmitter (ATO Automation) to amplify the
voltage before being sent to the data acquisition device (USB-608, NI Instruments). Data acquisition was handled by a local laptop computer with LabVIEW 2021 (NI Instruments). Motor control was also performed using APT Software within LabVIEW 2021. A LabVIEW code for mechanical loading was written to perform simultaneous motor movements and data acquisition. Load cell calibration was performed using known weights and measuring the output voltage to generate a calibration curve. Screenshots of the mechanical loading LabVIEW code can be found in Appendix D.

The system was designed with displacement control and cyclical loading functions. For the study, 5 cycles of roughly 35 N of force were applied in 4 second intervals to the surface of the articular cartilage sample. A graph of the loading forces can be seen in Figure 4-2. This was achieved by applying a displacement of 1.1 mm and incrementing the displacement by 0.02 mm per cycle. Deformation occurs after loading due to the viscoelastic properties of articular cartilage. The displacement increments allowed us to apply a consistent loading force of 35 N for each of the 5 cycles.
Figure 4-2: Graph of the cyclical loading pattern applied to the sample. An average force of 35 N was applied over 5 cycles in 4 second intervals.

*SHG/AF Imaging Procedure*

Samples were imaged with the custom-built multi-channel TPEF / SHG microscope. The excitation laser was tuned to a wavelength of 740 nm with a laser power of 150-160 mW for SHG/AF imaging. The SHG signals were recorded in Channel 3 with a bandpass filter of 352–388 nm. Autofluorescence signals were recorded in Channels 2 and 1 the ranges of 421 – 463 nm for NAD(P)H and 572 – 642 nm for FPs, respectively. Image stacks up to 50 μm were acquired, consisting of 25 512x512 pixel images from the tissue surface to deeper layers at a depth interval of 2 μm/step. Six (6) replicate images were acquired per step. Microscope settings were maintained the same while acquiring all SHG/AF images. PMT1 and PMT2 gains were kept at 0.657 V, and bandwidths were set to 80 Mhz.
Landmarks, such as microcracks, on the tissue surface were identified as locations of interest for imaging. Image stacks were sequentially acquired along one axis at the location of interest. In other words, after the first image stack completed, the sample stage was moved in one direction to the next imaging location adjacent to the first stack. Some overlap was kept between the adjacent imaging locations to allow for stitching during image processing. Image stacks shared the same z-coordinates and depth. The sample stage was only moved in either the x- or y-direction. The same locations of interest were imaged 3, 5, and 7 days after the injury.

Image Processing

The acquired SHG/AF images were processed using the image processing program ImageJ with the procedure described in Chapter 2. The built-in Stitching plugin was used to stitch images in a sequential order. Depending on the quality of the images, overlapping regions between each image stack were either calculated automatically by the plugin or determined manually. Brightness and contrast levels were carefully adjusted for each channel to balance the signal levels from each image after stitching.

Results

SHG/AF images of microcrack regions on the sample surfaces were acquired on Days 3, 5, and 7 after the mechanical loading injury. Stitched images from one location of interest with a long microcrack can be seen in Figure 4-3. The red circles in the image indicate regions with strong AF signals within the microcrack. In the image from Day 3
(A), red AF signals were observed in the middle of the injury. Those signals became less prominent on Days 5 and 7. In the image from Day 5 (B), green chondrocytes were observed in the microcrack that were not present in Day 3. Following the same location into Day 7 (C), we observed even more chondrocytes in the microcrack gap. Microcrack gap sizes appeared to be increasing based on visual assessment, suggesting the tissue injury may have been worsening over time. Red AF signals surrounding the microcrack corresponds to chondrocyte death that occurred around the immediate area of the injury. Chondrocytes at least 100 μm away from the microcrack remained viable. Chondrocyte viability, based on visual assessment of green AF signals, remained roughly the same across the 3 imaging timepoints.
Discussion

The results of this study demonstrated our development of our longitudinal imaging methods for articular cartilage. We tracked the same injury location on a sample across 3 imaging timepoints while maintaining sample orientation. We also successfully solved the problems we had with contamination by implementing the latest culture /
imaging chamber design and the small mechanical loading instrument. Several points of improvement, however, can be made to the imaging and mechanical loading procedures.

Chondrocytes near the damaged region in Day 5 (B) and Day 7 (C) images appeared to be moving out of their lacunae and into the damaged region. This was likely caused by the deterioration of the ECM along the microcrack injury. The gap of the microcrack also appeared to have widened over the course of the experiment. To obtain more details about the microcrack injury region, a higher microscope magnification can be used in future experiments to view the interactions more closely. Future analyses can also be performed on the width of the microcracks to quantitively determine the rate at which the microcrack gap widens. Conversely, a larger field of view (FOV) may be used to view the overall damage on the cartilage surface. A 2 mm diameter indentation tip was used to apply the loading force, but the image FOV was roughly 300x300 μm. The tip may have caused different types of damages or responses in other parts of the tissues that were missed due to our small FOV.

Closer evaluation of collagen fiber structures could also be performed in future studies. Using SHG imaging, we can compare the collagen networks between normal and damaged tissue. Studies suggest that notable differences in collagen alignment and network patterns can be observed. Proteoglycan content near the damage site may also change, as a result. Due to time constraints, we did not examine signals from the SHG channel alone to study the changes in the collagen fiber network.
A series of image stacks were stitched together to give us images similar to the ones seen in Figure 4-3. However, the sample stage was only moved in direction to obtain the sequential images due to time constraints. Imaging in a grid pattern rather than a linear pattern would greatly provide a greater FOV of the articular cartilage surface structure after injury. We attempted to develop a LabVIEW code that would allow us to automatically move to the next imaging location and trigger the image acquisition. This project seemed feasible and would have allowed us to automatically acquire image stacks in a grid pattern, significantly reducing time spent in the lab. The project could not be completed, however, due to incompatible LabVIEW versions between the SciScan software and the motor control functions.

Automation improvements could be made to the mechanical loading instrument, as well. The current system operates as a displacement-control system. Software development can be performed to add force-control functionalities to the system that would allow us to consistently apply a desired force without having to guess-and-check the amount of force applied per distance traveled. Implementation of a force-control function would also improve the applicability of the system to other projects.

The translation stage is another point of improvement. The stage has a maximum displacement of 25mm, a maximum velocity of 2.4 mm/s, and a maximum acceleration of 4.5 mm/s². In relative terms, the motor movement is slow and is not be able to significantly shorten the time between loading cycles. Replacing the motor with one that
has higher accelerations and velocities, such as a voice coil motor, would give us the ability to more accurately mimic physiological loading intervals for future studies.

Future studies may also examine different responses between low and high mechanical loads on the tissue, and ultimately determine mechanical thresholds for certain types of damage to specific parts of the cartilage. Studies could measure the shear stresses or strain rates that are required to rupture the collagen fiber network and cause the microcracks that we observed in this study. We did not provide a biomechanical analysis of the damage, but such analysis could provide a better quantitative understanding of the mechanical limits of articular cartilage.

Conclusion

In conclusion, we developed longitudinal imaging methods to dynamically assess damaged porcine articular cartilage after mechanical loading. We successfully designed and 3D-printed a custom tissue culture/imaging chamber to follow the structural changes in the tissue up to several weeks. We also developed a mechanical loading instrument small enough for the inside of a sterile biosafety hood to minimize tissue contamination risks while applying mechanical loading cycles. Preliminary experiments showed success in acquiring images at the same injury location over a several days, and in controlling the level of contamination during culturing, mechanical loading, and imaging procedures.
Appendix A

Image Processing Macro for ImageJ

```java
name = getTitle();
selectWindow(name);
run("Deinterleave", "how=3");
selectWindow(name + " #3");
run("Grouped Z Project...", "projection=[Average Intensity] group=6");
selectWindow(name + " #3");
close();
name3 = getTitle();
selectWindow(name + " #2");
run("Grouped Z Project...", "projection=[Average Intensity] group=6");
selectWindow(name + " #2");
close();
name2 = getTitle();
selectWindow(name + " #1");
run("Grouped Z Project...", "projection=[Average Intensity] group=6");
selectWindow(name + " #1");
close();
name1 = getTitle();
run("Merge Channels...", "c1=["+name1+"] c2=["+name2+"] c3=["+name3+"] create");
```
Appendix B

Culture Media Preparation Protocol

Materials

- Cell Media – DMEM w/ Sodium Pyruvate
  - Stored in fridge at 4°C
  - + Low Glucose, - Phenol Red, - L-Glutamine
- Penicillin-Streptomycin / Anti-anti
  - Stored in Freezer -20°C
  - 10,000 µg/mL each component, or 100x working concentration
- Non-essential amino acids
  - Stored in fridge at 4°C
  - 100x working concentration
- Fetal Bovine Serum (FBS)
  - Stored in -80°C Freezer
  - Qualified, Standard
- Proline, powder
- L-Glutamine, powder, 200mg per 500mL solution
- 1M HEPES Solution
- Bottle-top Vacuum Filter System

Procedure

1. Thaw out FBS and Penicillin-Streptomycin in a warm bath.
2. Set up the vacuum line with the filter system. Keep the vacuum OFF.
3. Obtain a 500ml bottle of DMEM media from the fridge.
4. Add half of the final desired concentration/amount of DMEM to a clean beaker.
5. Add powder reagents to the DMEM in the beaker. Mix until fully dissolved.
6. With the vacuum OFF, add the DMEM/reagents mixture to the top of the filter system.
7. Add 10% of final concentration of FBS.
8. Add 1% of final concentration of Penicillin-Streptomycin (or Anti-Anti).
9. Add 1% of final concentration of Non-essential AA.
10. Apply vacuum to filter system. Add the remaining DMEM volume.
11. Filter through and store in fridge.
12. Warm to 37°C before each use.
13. Wipe down and sanitize the bottle with 70% rubbing alcohol when transferring from the bath to the sterile hood.

Final Quantities - 500mL

- 430 mL DMEM
- 50 mL FBS (10%)
- 10 mL Pen-strep (2%)
- 5 mL Non-essential AA (1%)
- 5 mL HEPES (1%)
- 200 mg L-Glutamine (if using glutamine-free DMEM) (3mM)
- 23 mg L-Proline (0.4mM)
Appendix C

Microscope Filter Configurations

Figure C-1: Filter Configuration for SHG/AF Imaging.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Wavelength</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel 3</td>
<td>352 – 388 nm</td>
<td>Collagen</td>
</tr>
<tr>
<td>Channel 2</td>
<td>421 – 463 nm</td>
<td>NADH, Elastin</td>
</tr>
<tr>
<td>Channel 1</td>
<td>572 – 642 nm</td>
<td>Flavins</td>
</tr>
</tbody>
</table>

Figure C-2: Filter Configuration for Viability Assay Imaging.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Wavelength</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel 3</td>
<td>417 – 477 nm</td>
<td>Hoechst</td>
</tr>
<tr>
<td>Channel 2</td>
<td>490 – 550 nm</td>
<td>Calcein</td>
</tr>
<tr>
<td>Channel 1</td>
<td>&gt; 580 nm</td>
<td>EthD-1</td>
</tr>
</tbody>
</table>
Appendix D

LabVIEW Code for Mechanical Loading

Figure D-1: LabVIEW code for cyclical mechanical loading.
REFERENCES


