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Fiber-optic Raman Spectroscopy of Joint Tissues

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Abstract

In this study, we report adaptation of Raman spectroscopy for arthroscopy of joint tissues using a custom-built fiber optic probe. Differentiation of healthy and damaged tissue or examination of subsurface tissue, such as subchondral bone, is a challenge in arthroscopy because visual inspection may not provide sufficient contrast. Discrimination of healthy versus damaged tissue may be improved by incorporating point spectroscopy or hyperspectral imaging into arthroscopy where contrast is based on molecular structure or chemical composition. Articular joint surfaces of knee cadaveric human tissue and tissue phantoms were examined using a custom-designed Raman fiber optic probe. Fiber-optic Raman spectra were compared against reference spectra of cartilage, subchondral bone and cancellous bone collected using Raman microspectroscopy. In fiber-optic Raman spectra of the articular surface, there was an effect of cartilage thickness on recovery of signal from subchondral bone. At sites with intact cartilage, the bone mineralization ratio decreased but there was a minimal effect in the bone mineral chemistry ratios. Tissue phantoms were prepared as experimental models of the osteochondral interface. Raman spectra of tissue phantoms suggested that optical scattering of cartilage has a large effect on the relative cartilage and bone signal. Finite element analysis modeling of light fluence in the osteochondral interface confirmed experimental findings in human cadaveric tissue and tissue phantoms. These first studies demonstrate proof of principle for Raman arthroscopic measurement of joint tissues and provide a basis for future clinical or animal model studies.

Introduction

Arthroscopy is a minimally invasive surgical technique that is commonly used to treat or repair joint tissues.^{1–3} Features such as torn soft ligaments or tendons, and osteophytes can be readily visualized and treated through arthroscopy surgery. The cartilage surface of joints, also called the articular surface, is assessed for visual evidence of damage such as roughness or erosion. Techniques used in arthroscopic surgery include reshaping of roughened cartilage surfaces, repair of torn menisci, tendons or ligaments, and microfracture to induce growth of fibrous cartilage in large chondral defects. Predicting the long-term efficacy of arthroscopic interventions is a topic in clinical outcomes research, and current studies rely on patient satisfaction and joint functionality. In particular, the microfracture procedure provides significant improvements over preoperative state after 2 years and the long-term efficacy is also under study.^{4–6} Currently, arthroscopic interventions rely on

visual inspection to identify damaged sites. As a result, healthy cartilage may be removed in addition to damaged cartilage or sclerotic bone may be included as a microfracture site. For this reason, there is interest in both clinical and basic science research to couple arthroscopy with techniques that can rapidly identify damaged joint tissue to guide surgical interventions.²

Optical spectroscopy and imaging of joint tissues can show molecular signatures of damage, even if it is invisible to the eye. Structural or chemical alterations in cartilage or subchondral bone that are associated with joint tissue damage have previously been identified using optical spectroscopy to examine excised joint tissue.^{7–12} Optical coherence tomography (OCT) is well suited for measuring microstructural changes in cartilage, and can potentially identify eroded cartilage *in situ*. Fourier-transform infrared (FT-IR) and Raman spectroscopy showed loss of proteoglycan content or collagen defibrillation in damaged cartilage and decreased mineralization or increased carbonate ion substitution into the bone mineral in damaged subchondral bone.^{13–18} While most spectroscopy studies of joint tissues have been performed *in vitro* using microspectroscopy instrumentation, the techniques can be translated so they can be used in a clinical environment. Fiber-optic probes are a convenient format for coupling optical spectroscopies into an arthroscopic probe because they are compatible with the small dimensions of the arthroscope and provide the same chemical information that is available in a microscopy instrument. Fiber-optic FT-IR or OCT instruments have been developed to study changes in cartilage microstructure or chemical composition,^{19, 20} but there have been no reports of fiber-optic Raman spectroscopy in joint tissues.

In this study, fiber optic Raman spectroscopy was used to examine joint tissues of human cadaveric tissues, with an emphasis on measuring subchondral bone. There were two goals of the study. The first goal of the study was to demonstrate measurement of compositional changes in human joint tissues using fiber optic Raman spectroscopy. A fiber optic probe was designed to examine subchondral bone in human knee joint tissues, a common anatomical site for arthroscopic surgery. Based on the predicted and measured optical properties of cartilage and bone, the excitation light from a fiber optic Raman probe is expected to penetrate to the subchondral bone layer and, to a lesser extent, to cancellous bone. Thus, Raman spectra of the articular surface using a near-infrared, non-confocal fiber-optic probe are expected to contain contributions from the underlying bone tissue layers. A fiber optic probe where the collection fibers are spatially offset from the excitation fibers can maximize collection of subsurface bone signal. The Raman fiber optic probe incorporated spatially-offset illumination and detector optical fibers, and enabled detection of cartilage with contributions from subchondral bone.

The second goal of the study was to model the osteochondral interface to understand the relationship between the optical scattering properties of cartilage and collection of Raman scattering from subchondral bone. The optical properties of the osteochondral interface have contributions from the three cartilage layers (superficial, intermediate, and deep), calcified cartilage, subchondral bone and cancellous bone. The optical properties of these six layers likely will vary, but they have not yet been studied. In this study, the osteochondral interface was treated as a simplified system consisting of a uniform cartilage layer and uniform bone layer with lipid-filled marrow space. Even in this simplified model, the optical properties and tissue geometry (complex shapes of the tissue layers) affect the relative contributions of cartilage and bone in Raman spectroscopy of joint surfaces. Previous studies showed that scattering is the dominant optical property in cartilage, and we hypothesized that high optical scattering in cartilage would reduce the amount of bone that could be sampled.^{21–24} The effects of cartilage optical scattering on collection of Raman scatter were studied using finite element analysis models and experimental techniques. Planar tissue phantoms were

prepared to simulate the chemical composition and optical properties of cartilage and bone. Tissue phantoms have been used in a variety of diffuse reflectance and fluorescence studies to model photon propagation through turbid media, and they may be considered as analytical standards because the optical and chemical properties can be controlled.^{25, 26} Finite element analysis models and Raman spectroscopy of tissue phantoms verified our hypothesis that optical scattering in cartilage significantly affects recovery of Raman signal from subchondral bone. Finite element analysis models showed that thicker cartilage tissue and high optical scattering reduced the sampling volume in subchondral bone.

Experimental

Human cadaveric tissue

Two studies of human cadaveric tissue were performed. In the first study, a human arm was acquired from the University of Michigan anatomical donations program. Tissue was taken from the proximal radius specimen and examined by Raman microspectroscopy, operating at 785 nm, to collect reference Raman spectra of cartilage, subchondral bone, and cancellous bone tissues. Overlying soft tissue was dissected and the head of the proximal radius, including the articular cartilage and cancellous bone, was removed from the radius. Immediately after dissection, the proximal radius specimen was stored in phosphate buffered saline (PBS) at 4°C until examination. The specimen was examined within 3 days of dissection. Prior to examination by Raman spectroscopy, the specimen was placed onto a gauze pad, moistened with fresh PBS, and positioned on top of a gold-coated glass slide (EMF Corporation, Ithaca, NY, USA).

In a second series of experiments, one right and one left human cadaveric knee joint was examined. The goal for this set of experiments was to demonstrate fiber-optic Raman spectroscopy for a joint that is commonly treated by arthroscopic surgery. Age related compositional changes were not a factor in this experiment as the limbs were from the same donor. Soft tissue was dissected to expose the articular surfaces of femoral condyles and photographs of exposed femoral condyles were taken with a digital camera (PowerShot SD850IS, Canon, Tokyo, Japan). Visual inspection of the articular surface revealed sites of eroded or intact cartilage. Cartilage lesions, also called erosions, were assessed visually and recorded as either full thickness lesions (large surface area > 3 mm diameter with visible subchondral bone) or focal lesions (small surface area < 3 mm diameter with subchondral bone not visible). Full thickness lesions on the medial condyles and focal lesions or intact cartilage on the lateral condyle were found in both knee joints. This observation is consistent with other studies showing that cartilage in medial compartment is more susceptible to erosion.²⁷ Micro CT images of the knee joints confirmed damage to the medial compartment, as shown in Figure 2a, there is joint space narrowing (circle) and osteophyte growth (arrows) in the medial compartment of the right knee. Raman spectra were collected at two sites with intact cartilage (R and L knee, lateral condyles), two sites with full thickness lesions (R and L knee, medial condyles) and one area with a focal lesion (L knee, lateral condyle).

Clinical X-ray computed tomography (CT) of human knee

The knee joints were examined by computed tomography for joint damage in the form of osteophytes or narrowed joint space. CT scans were also used to generate meshes for finite element analysis models. Prior to CT scanning, the limb was thawed in a 4°F refrigerator overnight. CT images were collected in the sagittal, coronal and transverse planes using a high-resolution 64 slice clinical CT instrument (Siemens Somatom Sensation Cardiac 64, Siemens Healthcare, Germany). The scan parameters were: 250 mm field of view, slice thickness 0.6 mm, pitch 0.55 mm, acq. 64×0.6mm, 512×512 matrix.

Tissue phantoms

Gelatin tissue phantoms modeling the chemical and optical properties of cartilage and bone were prepared using a method described by De Grand and modified in-house.²⁸ A 10% w/v gelatin base was prepared by dissolving gelatin (Sigma Aldrich, St. Louis MO, USA) in pH 7.4 phosphate-buffered saline (Sigma Aldrich) at 37–55°C. Chemical components were incorporated into the gelatin base to approximate the chemical and optical scattering properties of bone or cartilage tissue. The bone layer was ~ 5 mm thick and consisted of 0.2 g/ml hydroxyapatite, approximately the hydroxyapatite concentration in healthy cortical bone²⁹, and 10% v/v Liposyn II throughout all tissue phantom experiments. The cartilage layer was ~ 6 mm thick and consisted of 3 mg/ml chondroitin sulfate, approximately the chondroitin sulfate concentration in healthy cartilage³⁰. 0%, 10%, or 20% was incorporated into the cartilage layer to model no cartilage, eroded cartilage, and intact cartilage, respectively. Liposyn II (Hospira Inc, Lake Forest, IL, USA) is similar to Intralipid, a pharmaceutical emulsion commonly used to approximate optical scattering in diffuse reflectance experiments because the micelles act as optical scattering centers with minimal optical absorption.^{31, 32} Bone and cartilage layers were cast sequentially and steps were taken to ensure a consistent fill level. First, the bone layer was cast into prechilled 1.3” diameter plastic Petri dishes then placed into a –20°C freezer until the layer was set. The overlying cartilage layer was then cast into Petri dishes prefilled with bone layer then placed into a –20°C freezer until the layer was set. Tissue phantoms were stored in a sealed container, to avoid moisture loss, at 4°C until examination and were used within one week of preparation.

Fiber-optic Raman spectroscopy

Raman spectra of tissue phantoms and human cadaveric tissue were collected using a near infrared system with 830 nm excitation. A Kaiser Rxn1 instrument, equipped with a 100 µm slit and a 1024x256 CCD camera, was used for all of the Raman measurements (Kaiser Optical Systems, Inc., Ann Arbor, MI USA). A custom designed hand-held, pen-like probe was used to examine knee joint tissue and tissue phantoms, shown in Figure 1. An 830 nm laser (Innovative Photonic Solutions, Monmouth Junction NJ, USA) was focused onto the end of the illumination bundle. The custom designed hand-held probe consisted of 19 (200 µm OD) illumination fibers interspersed with 38 (100 µm OD) collection fibers in a 3 mm ring, and 12 (100 µm OD) collection fibers placed in the center of the ring (FiberTech Optica Inc., Kitchener, ON Canada). The laser intensity was ~ 100 mW at the end of the probe, spread across the 19 illumination fibers. Five measurements were collected at each site, and each measurement was collected for 60s.

Raman microspectroscopy

Raman microspectroscopy was performed on the proximal radius specimen to obtain reference spectra of cartilage, subchondral bone and cancellous bone tissue. Raman spectra of the articular surface were collected and used as reference cartilage spectra. Cartilage was then shaved from the specimen and subchondral bone spectra were collected. The specimen was then turned over, exposing the cancellous bone surface, and cancellous bone spectra were collected. A Nikon E600 epi-fluorescence microscope (Nikon Inc., Melville, NY, USA) was modified for Raman microspectroscopy. Microscope images were collected in epi-illumination mode with 4x/0.20NA, 10x/0.50NA, and 20x/0.75NA S Fluor objectives (Nikon Inc., Melville, NY, USA). A 785 nm Kaiser Invictus laser was line focused (Kaiser Optical Systems Inc., Ann Arbor, MI, USA) and focused onto tissue specimens using a 20x/0.75NA S Fluor objective. A neutral density filter of 0.3 reduced the laser intensity at the objective to ~ 10 mW. Raman-scattered light was collected through the same 20x/0.75 NA S Fluor objective and dispersed through a spectrograph (HoloSpec f/1.8, Kaiser Optical Systems Inc., Ann Arbor, MI, USA). Raman signal was collected for 10 minutes on a deep-

depletion, back-thinned 1024x128 charge-coupled device (CCD) detector (DU401-BR-DD, Andor Technologies, Belfast, Ireland).

Raman Data Preprocessing

Raman microspectroscopy transects consisted of 1024 pixels in the spatial axis and 128 Raman spectra arranged at equidistant points along a line through the specimen. Raman data were preprocessed in Matlab (v7, The MathWorks, Natick MA, USA) using software routines developed in-house. Spectra from a neon discharge source were used to calibrate the wavelength axis, spectra from a quartz-halogen source were used to correct for variations in CCD response and standardized using a NIST-traceable intensity calibration. Dark spectra were used to correct for detector noise. Data were imported into Matlab using software developed in-house where they were corrected for cosmic ray spikes, dark current and variations in the CCD camera efficiency. Corrections for slit image curvature and tilt of the CCD relative to the spectrograph were handled using custom software routines.³³ Cosmic rays were removed from microscopy spectra manually using a software routine developed in-house.

Fiber-optic Raman transects consisted of 1024 pixels in the spectral axis and 50 spectra spread across the 128 pixel spatial axis. Fiber-optic spectra were preprocessed similarly to microspectroscopy data, except cosmic rays were removed automatically. Multiple ($n=5-10$) transects, each using a 60s integration time, were collected at each site using the fiber-optic probe. The final dimension of the accumulated acquisition was $n \times 1024 \times 128$ where n was the number of acquisitions collected at the site. The first preprocessing step was automatic remove cosmic ray spikes. To determine the pixels corresponding to spikes, 10 pixels from every x-y point in the accumulated images were examined. A median and standard deviation were calculated, and only pixel intensities within 3 times the standard deviation of the median value were used in calculating the mean image. A single averaged 1024×128 transect was calculated from the $n \times 1024 \times 128$ accumulated acquisition. Silica contributions to the measured spectra were significant because the probe uses unfiltered silica fiber optics. Silica Raman spectra were measured by reflecting the excitation laser onto a smooth aluminum surface. Silica correction was performed by using derivative subtraction on the measured data and a mean spectrum was calculated from the silica-corrected transects. The mean spectrum was baseline-corrected using a routine described by Cao et al and modified in-house.³⁴

Raman Data Processing

Baseline-corrected mean spectra were imported into GRAMS/AI[®] software (ThermoGalactic Salem NH, USA). Raman bands in the $850-1100 \text{ cm}^{-1}$ region of baseline-corrected spectra were fitted to mixed Lorentzian/Gaussian peaks to obtain band width, height and area parameters. The resulting fit was accepted if the $R^2 > 0.99$ and no negative bands were generated. Raman band assignments are presented in Table 1. Bands generated by the curvefit application were identified as arising from sulfated glycosaminoglycans, carbonated apatite mineral, types I and II collagen with possible contributions from lipids in cancellous bone.³⁵⁻³⁷ Mean spectra were intensity normalized to the 1002 cm^{-1} band for presentation in the figures.

Ratios of Raman band intensities were examined as spectroscopic measurements of compositional changes in cartilage and subchondral bone. Spectroscopic markers were used to estimate known alterations in joint tissues including tissue erosion or decreased glycosaminoglycan concentration in cartilage and decreased mineralization or increased carbonate ion substitution into the bone mineral.³⁸⁻⁴³ Table 2 shows the Raman band intensity ratios used to examine the chemical composition of bone and cartilage. We

hypothesized that the 1063 cm^{-1} : 958 cm^{-1} intensity ratio is a candidate spectroscopic marker for the relative amount of cartilage to bone because the bands are unique to cartilage or bone. We applied established spectroscopic markers of bone chemistry to subchondral bone including mineralization (958 cm^{-1} : 920 cm^{-1}) mineral crystallinity ($1/\text{FWHM } 958\text{ cm}^{-1}$) and carbonate substitution (1070 cm^{-1} : 958 cm^{-1}).⁴⁴

Finite Element Modeling of Joint Spectroscopy

Steps in the finite element modeling process are depicted in Figure 2. A clinical x-ray CT scan was recorded of a cadaveric human right knee, with a 2D slice of the knee data shown in Figure 1a, where a cross section of the knee joint is shown. The CT data was processed in the Mimics software package (Mimics 14.01 64-bit, Materialise, Leuven, Belgium) to determine the tissue type present in each voxel of the volume data set. Tissue types were assigned based on the Hounsfield unit (HU) values (the units of x-ray density used for CT data) present in the calibrated data, according to the default ranges in Mimics. The default settings in Mimics were used to assign pixels as bone (226 to 2465 HU), soft tissue (−700 to 225 HU), and adipose (−205 to −51HU). After creating 3D masks for each tissue layer type, morphological image processing and Boolean logic operators were used to refine the models into a closed model consisting of the femoral head and condyles. A tetrahedral finite element mesh was then created and labeled by region according to the assigned tissue type masks. Because CT does not provide sufficient contrast to image cartilage, morphological dilations were used to simulate a thin ($800\text{ }\mu\text{m}$) or a thick ($2000\text{ }\mu\text{m}$) cartilage layer on the surface of the femoral condyles. The thick cartilage layer simulated the thickness of intact cartilage, and thin cartilage simulated the thickness of partial erosion. A 2d slice through the thin cartilage mesh with the assigned region types is shown in Figure 2b. Even though all finite element models were calculated over the same tissue volume which encompassed the distal femur and condyles ($-14.27 < x < 73.66$, $-167.54 < y < -94.85$, and $177.38 < z < 254.23$ (all in mm)), plots in Figure 2c, 2d and Figure 2 show only the area marked by the dashed box in Figure 2b.

Tetrahedral finite element models were loaded into the NIRFAST diffuse optical tomography software (www.nirfast.org). Optical properties were assigned based on the reported optical properties of the various tissue types, and are listed in Table 3. Bone and marrow (treated as adipose) optical properties were estimated based on the method described by Alexandrakis et al using spectral data input from the Oregon Medical Laser Center (<http://omlc.ogi.edu/spectra/>).⁴⁵ For the high scattering properties of cartilage, the coefficients shown in from Fig 5B by Youn et al⁴⁶ were scaled so that the magnitude corresponded with the values reported by Beek et al.²¹ Low scattering cartilage values were taken as $1/5^{\text{th}}$ of the scattering coefficient of the high value. Four models were generated. High and low cartilage scattering level was modeled in the thick and thin cartilage thickness to simulate the effect of a lower concentration of cartilage macromolecules (proteoglycan, collagen) that may act as scattering centers. The source fiber was placed at coordinates (51.67, −130.02, 180.54, all in mm), while the measurement fiber was placed at coordinates (53.17, −130.02, 180.01, all in mm), simulating the geometry of the pen-like probe with the source fibers 1.5 mm apart from the collection fibers.

Forward modeling subroutines in NIRFAST were used to simulate the forward light fluence from the source fiber optic (of laser excitation at 830 nm) into the finite element model, as shown in Figure 2c. Forward modeling subroutines were also used to simulate the forward light fluence from the collection fiber optic into the finite element model. The forward model can be used to simulate the efficiency of light fluence toward the collection fiber (simulated for light at 901.7 nm, corresponding to the phosphate vibrational mode at 958 cm^{-1}) because the efficiency of light travelling to or from a given position in the model is commutative (in the same way that the direction of light traveling through a fiber optic cable

does not alter the transport efficiency). The collection intensity pattern is shown in Figure 2d. Scalar multiplication of the source intensity pattern (Figure 2c) and the collection intensity pattern (Figure 2d) yielded the collection efficiency from every point in the finite element model (example shown in Figure 2b). Light fluence patterns are independent of the Raman scattering cross section. The sampling patterns for each region indicated the efficiency of sampling from the cartilage, bone and adipose regions. Sampling efficiency values are listed in Table 5.

Results and Discussion

Raman microspectroscopy of human cadaveric tissue

Reference spectra of human cancellous bone (Figure 3a), subchondral bone (Figure 3b), and cartilage (Figure 3c) were collected using Raman microspectroscopy. Tissue from the proximal radius was examined because osteoarthritis damage is minimal in the elbow. Raman band assignments for spectra are presented in Table 1. Raman spectra of cartilage, subchondral bone and cancellous bone were acquired with minimal contributions from other tissues. Raman microspectroscopy of the articular surface had no spectral contributions from subchondral or cancellous bone. Cartilage spectra (Figure 3c) consist of bands from type II collagen and sulfated glycosaminoglycans. Subchondral bone spectra consist of apatite mineral and type I collagen. In the subchondral bone spectrum presented in Figure 3b there was residual signal at 1063 cm^{-1} , indicating the presence of residual cartilage tissue. In spectra of murine and equine subchondral bone collected on the same system, the 1063 cm^{-1} band was not observed.¹⁷ Cancellous bone spectra consist of apatite mineral and marrow lipids.

We focused our analysis on the $850\text{--}1100\text{ cm}^{-1}$ region because it provides the highest spectral contrast between cartilage and bone. In this region, there are three bands that are unique to cartilage or bone. A Raman band at $\sim 1063\text{ cm}^{-1}$ is unique to sulfated glycosaminoglycans in cartilage, and was used as a spectroscopic marker of cartilage.³⁵ Raman bands unique to the carbonated apatite mineral of bone were found at 958 cm^{-1} and 1070 cm^{-1} , corresponding to the phosphate ν_1 and carbonate ν_1 , and were used as spectroscopic markers of bone. Bands at 1301 cm^{-1} and 1744 cm^{-1} are unique to lipids and were attributed to marrow lipids in cancellous bone.³⁷

Fiber-optic Raman spectroscopy of human cadaveric tissue

Tissue excised from the human proximal radius was also examined using a prototype fiber optic probe to determine the feasibility of measuring cartilage and subsurface bone. While the study supported the use of fiber-optic Raman spectroscopy to examine joint tissues, there was no cartilage signal observed (data not shown). The results of the study in the proximal radius indicated that another probe design would be necessary in order to better detect cartilage signal. The pen-like probe design incorporates two rings of optical fibers to collect surface measurements with less spectral contributions from underlying surfaces. As shown in Figure 1, there was minimal-to-no separation between the excitation and collection fibers in the outer ring of the probe, which enabled collection of Raman signal from the surface. The spatial separation between the excitation fibers in the outer ring and collection fibers in the inner ring enabled collection of subsurface Raman signal.^{47–49}

The pen-like probe was used as a prototype of Raman arthroscopy to examine human cadaveric knee joints from a single donor. Raman spectra were collected from femoral condyles of the left and right knee at sites with intact cartilage, focal lesions and full thickness erosions. Figure 4a shows spectra collected from the medial and lateral femur condyles of the left knee, with minimal contributions from cancellous bone. Similar to

microspectroscopy spectra, there was significant overlap in the 1200–1700 cm^{-1} spectral region because type II collagen in cartilage and type I collagen in bone have similar Raman spectra (Figure 4a).¹⁷ Signal arising from cartilage and bone were observed in spectra at sites with intact cartilage (black line, Figure 4a). In addition to finding full thickness erosions on the medial condyle and intact cartilage on the lateral condyle of both knees, there was a focal lesion on the lateral condyle of the left knee. The focal lesion provided an opportunity to examine a site with partial cartilage erosion. As expected, Raman spectra from areas with full thickness erosions contained only signal from subchondral bone (gray line, Figure 4a). Spectra of the focal lesion contained signal from cartilage with stronger contributions from subchondral bone than spectra at sites with intact cartilage (black dashed line, Figure 4a).

The region that provides the most spectral contrast between subchondral bone and cartilage was 850–1100 cm^{-1} and differences in spectra collected at sites of eroded and intact cartilage were apparent. An inset of the 900–1100 cm^{-1} spectra region is shown in Figure 4b. The Raman spectrum at sites with intact cartilage (black line) contained a band at 1065 cm^{-1} from chondroitin sulfate and the phosphate ν_1 signal at ~959 cm^{-1} was less intense. In the spectrum from the focal lesion (dashed black line), signal from bone was more evident because the phosphate ν_1 signal was more intense and a shoulder in the 1065 cm^{-1} band appeared at ~1072 cm^{-1} from carbonate ν_1 . The spectrum from a site with a full thickness lesion (gray line) did not have any cartilage signal, as evidenced by the lack of a band at ~1063 cm^{-1} . Cartilage is 2–3 mm thick in the knee joint, and these results suggest that the thickness of cartilage tissue had an effect on the recovery of subchondral bone signal. Even when cartilage was partially eroded, there was a large difference in the intensity of phosphate ν_1 signal from bone. Qualitatively, the spectra indicate an effect of cartilage thickness on subchondral bone spectra. The next step was to test if compositional changes could be quantified from fiber-optic Raman spectra.

Band intensity ratios corresponding to cartilage and bone chemical composition were calculated. Intact cartilage is expected to have higher optical scattering than eroded cartilage because it is thicker and has a higher concentration of extracellular matrix molecules, including collagen and aggrecan, which act as scattering centers. A comparison of these ratios is presented in Table 4. Intact cartilage had a large impact on the cartilage-to-bone and subchondral bone mineralization markers, as expected. The 1063 cm^{-1} : 958 cm^{-1} ratio was ~5x higher at 50 sites with intact cartilage. The thickness of cartilage tissue also affected the 958 cm^{-1} : 920 cm^{-1} intensity ratio that was used as a marker for subchondral bone mineralization. At sites with intact cartilage the 958 cm^{-1} : 920 cm^{-1} ratio was ~4x lower, because the higher optical scattering in intact cartilage translates to less 55 photons that diffuse to subchondral bone, resulting in lower collection of bone Raman scatter.

By contrast, markers of subchondral bone chemistry, crystallinity and carbonate substitution, were similar at the focal lesion and eroded cartilage sites. The carbonate substitution 60 marker reflects the rate of bone remodeling, and increased values may be a marker of early-stage response to degenerative or trauma injury.⁵⁰ This study did not show significant differences in markers of subchondral bone chemistry, but that may be attributed to the low number of sites examined. To expand these findings, future studies may include examination of human tissue or a study involving an experimental model of subchondral bone injury.⁵¹ Future validation studies include testing spectroscopic markers of cartilage-to-bone with measurements of cartilage tissue thickness.

Tissue phantoms

Multilayer planar tissue phantoms were created to examine the effect of cartilage optical scattering properties on collection of subchondral bone Raman signal. Intact cartilage is

expected to have higher optical scattering than eroded cartilage because it is thicker and has a higher concentration of extracellular matrix molecules, including collagen, proteoglycan and sulfated glycosaminoglycans, which act as scattering centers. In this study, cartilage layers were cast at similar physical thicknesses of ~ 6 mm. The bone layer was ~ 5 mm thick in all phantoms. 20% or 10% Liposyn II was incorporated into the cartilage layer to achieve a highly scattering or a moderately scattering cartilage layer, respectively. The reduced scattering coefficient μ'_s and anisotropy (g) of the cartilage layer were estimated using the equations 1 and 2, described by van Staveren for Intralipid.³²

$$\mu'_s(\lambda)=0.016\lambda^{-2.4} \quad (1)$$

$$g(\lambda)=1.1 - 0.58\lambda \quad (2)$$

Values generated from equations 1 and 2 were corrected for the concentration of Liposyn II in mL/L. For the cartilage layer with 20 % Liposyn II, the estimated μ'_s was 19.1 cm^{-1} at 830 nm, which was within the range of reported values for cartilage optical scattering of 6– 19 cm^{-1} .^{21, 23} For the cartilage layer with 10 % Liposyn II, μ'_s was 9.5 cm^{-1} at 830 nm. Thus, the cartilage layer with 20% Liposyn II modeled the optical scattering of intact cartilage and the cartilage layer with 10% Liposyn II modeled the optical scattering of partially eroded cartilage.

Fiber-optic Raman spectra collected from osteochondral tissue phantoms indicated that optical scattering affected the collection of subsurface bone signal. We observed that Raman spectra from a phantom with no optical scattering in the cartilage layer (solid line, Figure 5) were dominated by bone signal with no contributions from poorly-scattering chondroitin sulfate. The 1063 cm^{-1} band was the most intense in spectra from a phantom with the highest optical scattering in the cartilage layer, with minor contributions from the subsurface bone layer (short dashed line, Figure 5). In the tissue phantom with a moderate scattering cartilage layer, contributions from both cartilage and bone were observed (long dashed line, Figure 5). The relative intensity of the 1063 cm^{-1} band increased with optical scattering. The intensity ratio of the 1063 cm^{-1} band to the 958 cm^{-1} band also increased with optical scattering and supports our hypothesis that the 1063 cm^{-1} : 958 cm^{-1} intensity ratio is a good spectroscopic candidate for measuring the relative amount of cartilage-to-bone. There was no Raman signal observed from the Liposyn II. These studies support the use of tissue phantoms to model the effects of optical scattering in joint tissues on collection of Raman signal. Future tissue phantom studies include a design of experiments to study the combined effect of varying tissue thickness and chemical composition in planar phantoms. Geometrically accurate tissue phantoms can be incorporated into future tissue phantom studies to obtain information about geometry effects and test the capability of next-generation probes in a simulated knee joint

Finite Element Modeling of Light Propagation

Measurements in human cadaveric joints and tissue phantoms showed that Raman spectra collected at the articular surface using a (non-confocal) fiber-optic probe contained contributions from the underlying bone tissue. The relative bone signal varied with the cartilage thickness (in cadaveric tissue), and the scattering level (in tissue phantoms). Experimental data suggested that cartilage thickness and optical scattering affect the volume of bone that is being sampled. To demonstrate how the sampling of bone and cartilage vary

with cartilage thickness and optical scattering, finite element analysis was used to model the diffusion of light through the cartilage, bone and marrow via the osteochondral interface.

Four models were examined using the finite element analysis simulation. Normal healthy cartilage was modeled using a thick cartilage layer (2000 μm) with a high scattering coefficient. Early stage cartilage damage was modeled using the same cartilage thickness, but with a lower scattering coefficient. In early stages of damage, a reduced concentration of cartilage macromolecules (proteoglycans, collagen, sulfated glycosaminoglycans) is expected. The macromolecules act as scattering centers, and so early stages of damage should lead to reduced light scattering by cartilage. Eroded cartilage with normal macromolecular composition was modeled using a thin cartilage layer (800 μm) and a high scattering coefficient. Finally, deeply eroded cartilage with decreased macromolecular content was modeled as a thin cartilage layer with a low scattering coefficient.

The proportion of light sampled from the bone and cartilage regions are listed in Table 5a for the four different models. These models are important because they convey a sense of both the region sampled and the relative intensity expected from each region. There is no simply-defined region in which the spectrum is measured. Instead it is akin to a probability density function. Both the region sampled and the relative intensity play are of importance in understanding the problem. The key point is that geometry plays a much greater role in biomedical optical spectroscopy than typically expressed.

As expected from a conventional viewpoint on light scattering, the proportion of light sampled from cartilage is higher than the proportion of light sampled from bone in all cases. When considered individually, cartilage thickness or optical scattering was inversely proportional to the amount of light sampled from bone. For example, the amount of light sampled from the bone region increased as the cartilage layer thickness was reduced. Likewise, increased scattering in cartilage decreases the proportion of light sampled from the bone region relative to the amount of light sampled from cartilage. However, a closer examination of the absolute sampling efficiency indicates that the effects of light scattering here are much more complex than the conventional viewpoint suggests. The absolute sampling efficiency for light from cartilage and bone regions is shown in Table 5b. An unexpected observation was that cartilage optical scattering played a much greater role in thin cartilage as compared to thick cartilage. Also, cartilage is sampled more efficiently in a thin layer than in a thick layer.

Our initial hypothesis was that changes in the scattering coefficient of a thick cartilage layer would have a greater impact than corresponding changes in a thin cartilage layer. Unexpectedly, the decrease of cartilage optical scattering affected bone sampling efficiency more in the thin cartilage models than the thick cartilage models. In thin cartilage, the sampling efficiency of bone increased by more than 100% when the scattering coefficient was decreased by a factor of 5. In thick cartilage, the sampling efficiency of bone increased by only 22% for the same decrease in scattering. By examining the sampling efficiency maps we determined that increased scattering acts to confine light, preventing the field from dissipating laterally across the cartilage layer.

The lateral confinement of light by high optical scattering can be seen in Figure 6, where the rows show cartilage thickness and the columns show optical scattering. Figure 6a and 6c show the models thin and thick cartilage (respectively), both for low cartilage scattering. Figure 6b and 6d show the models with thin and thick cartilage (respectively), both for high cartilage scattering. As shown in 6b and 6d, light is constrained in the lateral direction by the high cartilage scattering coefficient. The patterns shown in Figures 6a–d denote the product of light fluence from the source and fluence from every other point back to the detector. The

figure shows the sampling efficiency and that some regions are weighted much more heavily than others. While the majority of light is sampled near the source and measurement fibers, it is not a well-defined discrete sampling volume. Despite the density being of low intensity further from the source and detector, it continues to play a substantial role even several millimeters away. These models show how non-confocal measurements in tissue do not sample a simple and clearly defined volume.

Results from these models suggest that an optimal design for a fiber optic probe of cartilage should incorporate additional features. Future studies should include a method for deconvolving the confounding effects of tissue thickness on the measured Raman spectra in order to provide quantitative results about cartilage and bone composition. One possible solution would be to directly measure cartilage thickness using a technique like ultrasound, MRI, or optical coherence tomography. A second solution would be to use multiple source-detector offsets to discriminate between cartilage thickness and scattering effects. These preliminary results suggest that it is possible to measure cartilage and subchondral bone composition using Raman spectroscopy. Future results should also be validated against established measurements of cartilage thickness, such as by clinical MRI. Moreover, the combined effects of cartilage thickness and optical scattering needs to be further studied.

Conclusions

Fiber-optic Raman spectroscopy of human joint tissues including cartilage, subchondral bone and cancellous bone has been demonstrated. Human cadaveric tissue and tissue phantoms studies were performed to study the effect of cartilage thickness on collection of subsurface spectra. Raman spectra collected at articular surfaces contain a mixture of signal from cartilage and subchondral bone, with minor contributions from cancellous bone. We found that the thickness and optical scattering in cartilage had a large effect on the collection of subsurface Raman signal. This effect was reflected in both the appearance of the spectra and quantitative Raman measurements of cartilage thickness and subchondral bone mineralization. Raman markers of subchondral bone chemistry did not vary between sites of eroded cartilage or focal lesion. We also created tissue simulating phantoms to act as *in vitro* models of the osteochondral interface. By varying optical scattering in the cartilage layer of tissue phantoms, we were able to simulate the effect of cartilage optical scattering on Raman spectra. In tissue phantoms models with optical scattering close to normal cartilage, minimal bone signal was observed. When optical scattering was decreased, simulating the loss of cartilage macromolecules that typically occurs in the earliest stages of cartilage damage, more bone signal was recovered. Finite element models of light fluence through the osteochondral interface were generated to estimate the relative sampling volume in cartilage and bone. Modeling studies suggest that cartilage thickness increases bone sampling volume, while cartilage optical scattering increases cartilage sampling volume. Studies in tissue phantoms and finite element models support the hypothesis that optical scattering and thickness in the cartilage layer has a large effect on photon diffusion to underlying bone layers. These studies provide preliminary data that support the further development of arthroscopic Raman optical fiber probes.

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Fig. 1.

The Raman fiber optic probe used is a custom-built pen-like probe. The pen-like probe has collection fibers adjacent to excitation fibers in the outer ring and collection fibers in the inner ring. Raman spectra collected from these probes were composed of cartilage and subchondral bone signal. Depending on the fiber configuration and thickness of joint tissues, minor-to-large contributions from lipids were also collected.

Fig. 2.

Finite element models of light fluence models were generated using a multi-step process starting with the clinical x-ray CT image of the joint. Figure 2a shows the CT image of the right distal femoral head. There are features in the CT image, such as a narrowed space between the femur and tibia and bony growths at the side of the femur, which indicate advanced osteoarthritis. Figure 2b shows a view of the femur through the same plane of the finite element model, with regions labeled by tissue type. Light blue denotes cartilage, yellow denotes bone, brown denotes marrow, and dark blue denotes the region outside the model. The arrows indicate the approximate position of the illumination ($x=51.67$, $z=178.54$ mm) and collection ($x=53.17$, $z=178.01$ mm) fibers. The dashed box indicates the subregion of the model shown in Figure 2c and 2d. Figure 2c shows light fluence from the source fiber optic at using the optical properties at 830 nm. Figure 2d shows light fluence from the measurement fiber at is shown using the optical properties at 901.7 nm (corresponding to the vibrational band at 958 cm^{-1}). The colorbar shows the $\log_{10}$ scaled fluence intensities. Larger numbers denote higher transfer efficiency from the fiber optic to the given position in the image.

Fig. 3.

Reference spectra of human cancellous bone, subchondral bone, and cartilage from the proximal radius were collected on a Raman microspectroscopy system ($\lambda = 785$ nm). Band assignments for the spectra are presented in Table 1. Cartilage spectra consist of type II collagen and chondroitin sulfate. Subchondral bone spectra contained features from carbonated apatite mineral and type I collagen matrix, and cancellous bone spectra were composed of carbonated apatite and marrow lipids.

Fig. 4.

Spectra were collected from femoral condyles of cadaveric human knee joints at sites with intact, partial erosion (also called a focal lesion), and eroded cartilage. Spectra were intensity normalized to the 1002 cm⁻¹ band. Figure 4a shows the entire spectral region. Figure 4b shows the 900–1150 cm⁻¹ region that provides the most discrimination between cartilage and bone tissues. The effect of intact cartilage on Raman spectra of the articular surface was observed by three changes. 1) In spectra from sites with intact cartilage, the OSO_3^- symmetric stretch $\sim 1063\text{--}5\text{ cm}^{-1}$ from cartilage chondroitin sulfate is evident. 2) The maximum in the 1050–1100 cm⁻¹ region shifted to $\sim 1070\text{ cm}^{-1}$, corresponding to carbonate ν_1 in bone, at sites where cartilage has eroded. 3) We also observed the relative intensity of the phosphate $\nu_1 \sim 958\text{ cm}^{-1}$ decreased at sites with intact cartilage.

Fig. 5.

Raman spectra were collected from tissue phantoms prepared using different levels of optical scattering (none, moderate, high) by incorporating different amounts of Liposyn II (0%, 10%, 20%) in the cartilage layer. Increased optical scattering decreased subsurface Raman signal, as evidenced in the 1063 cm⁻¹: 958 cm⁻¹ intensity ratio. The study in tissue phantoms supported our use of the 1063 cm⁻¹: 958 cm⁻¹ intensity ratio as a spectroscopic marker for the relative amount of cartilage to bone.

Fig. 6. Sampling efficiency maps for thin (800 μm) cartilage layer with (A) low scattering, and (B) high scattering, and for thick (2000 μm) cartilage layer with (C) low scattering, and (D) high scattering. In the sampling efficiency maps the sampling efficiency is highest in the models with high cartilage scattering, near the source and detector fibers. The collection pattern has an increased lateral spread in the low cartilage scattering models. The colorbar at right shows the correspondence of image color to $\log_{10}(\text{sampling efficiency})$.

Table 1

Band assignments of Raman spectra collected from cartilage, subchondral bone and cancellous bone tissue. Reference Raman spectra are shown in Figure 3. Bolded assignments represent bands that unique to the tissue. For example, the phosphate ν_1 band at $\sim 958\text{ cm}^{-1}$ corresponding to apatite mineral is unique to bone tissue. Significant spectral overlap was observed in the $1200\text{--}1800\text{ cm}^{-1}$ region, and the $800\text{--}1100\text{ cm}^{-1}$ was found to provide the most contrast between cartilage and bone tissue. Raman band positions are $\pm 2\text{ cm}^{-1}$.

Raman Shift (cm^{-1})	Assignment	Component	Tissue(s)
850,875	Hydroxyproline	Collagen	Cartilage, Subchondral bone
920	Proline	Collagen	Cartilage, Subchondral bone
958	$\nu_1\text{ PO}_4^{3-}$	Apatite Mineral	Bone
1001	Phenylalanine	Collagen	Cartilage, Subchondral bone
1063	OSO₃⁻ symmetric stretch	Chondroitin sulfate	Cartilage
1067	$\nu\text{ C-C skeleton, trans}$	Lipid	Cancellous bone
1070	$\nu_2\text{ CO}_3^{2-}$	Apatite Mineral	Bone
1079	$\nu\text{ C-C skeleton, random}$	Lipid	Cancellous bone
1230–1280	Amide III	Collagen	Cartilage, Subchondral bone
1268	=CH deformation	Lipid	Cancellous bone
1300	CH₂ deformation	Lipid	Cancellous bone
1439	CH₂ deformation	Lipid	Cancellous bone
1450	CH ₂ deformation	Protein, Lipid	Cartilage and bone
1630–1690	Amide I	Collagen	Cartilage, Subchondral bone
1654	$\nu(\text{C}=\text{C})$	Lipid	Cancellous bone
1745	$\nu(\text{C}=\text{O})$	Lipid	Cancellous bone

Table 2

Raman spectroscopic markers of cartilage and bone composition were used to calculate composition of cartilage and subchondral bone in cadaveric tissue. The cartilage to bone ratio was also used in spectra from tissue-simulating phantoms.

Tissue Properties	Spectroscopic Marker
Cartilage to Bone	1063 cm^{-1} /958 cm^{-1}
Bone Mineralization	958 cm^{-1} ; 920 cm^{-1}
Bone Chemistry	<ul style="list-style-type: none">• Carbonate substitution (1070 cm^{-1}: 958 cm^{-1})• Crystallinity, 1/FWHM at 958 cm^{-1}

Optical scattering (μ'_s), absorption coefficient (μ_a), and refractive index (R_i) for bone, marrow and cartilage were calculated at 830 nm (laser excitation source frequency) and 901.7 nm (corresponding to the phosphate Raman shift of bone at 958 cm^{-1}).

Table 3

Tissue Layer	Excitation (830 nm)		Emission (901.7 nm, 958 cm^{-1})		R_i
	μ'_s (mm^{-1})	μ_a (mm^{-1})	μ'_s (mm^{-1})	μ_a (mm^{-1})	
Low scattering cartilage	0.2434	0.033	0.1704	0.033	1.33
High scattering cartilage	1.2170	0.033	0.8519	0.033	1.33
Bone	1.8214	0.0201	1.6125	0.0234	1.4
Marrow (adipose)	1.0781	0.0028	1.0318	0.0050	1.45

Table 4

Raman spectroscopic markers of cartilage and bone composition calculated for spectra collected at various sites on femoral condyles. Cartilage state, intact, completely eroded or focal lesion, was assessed visually at the time of collecting spectra and documented with digital photography. Average values are presented $\pm$ standard deviation where applicable, and N values are the number of different sites of measurement for each cartilage state. These preliminary results show differences in the Raman spectroscopic markers at sites of eroded cartilage and reflect the effect of cartilage thickness on recovery of subchondral bone signal

Cartilage State	Cartilage to Bone	Mineralization	Carbonate Substitution	Crystallinity
Intact cartilage (N=4)	0.96 ± 0.30	2.54 ± 1.0		
Focal lesion (N=1)	0.20	9.09	0.32	0.048
Eroded cartilage (N=3)	0.11 ± 0.05	8.95 ± 1.26	0.24 ± 0.08	0.052 ± 0.001

Table 5

Finite element models predicted the sampling efficiency in each cartilage, bone and marrow. Data from marrow are not shown because they accounted for less than 0.1% of the total value in all cases. The numerical sum of the sampling efficiency from every node in each region is listed, and percent of the sampling efficiency is in parenthesis. In all cases, cartilage is sampled with greater efficiency than bone. As expected, the lowest bone-to-cartilage sampling ratio was observed in the thick cartilage/high scattering model simulating intact healthy cartilage and the highest bone-to-cartilage sampling ratio was observed in thin cartilage/low scattering model simulating eroded cartilage.

Sampling Efficiency		Bone	Cartilage	Bone-to-Cartilage (ratio)
Thick Cartilage	High scattering	0.173 (3.13%)	5.361 (96.87%)	0.03
	Low scattering	0.141 (12.62%)	0.975 (87.37%)	0.14
Thin Cartilage	High scattering	1.352 (17.22%)	6.496 (82.77%)	0.21
	Low scattering	0.649 (31.52%)	1.410 (68.45%)	0.46

^aFootnote text.