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Fourier transform infrared imaging spectroscopy investigations in the pathogenesis and repair of cartilage

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Abstract

Significant complications in the management of osteoarthritis (OA) are the inability to identify early cartilage changes during the development of the disease, and the lack of techniques to evaluate the tissue response to therapeutic and tissue engineering interventions. In recent studies several spectroscopic parameters have been elucidated by Fourier transform infrared imaging spectroscopy (FT-IRIS) that enable evaluation of molecular and compositional changes in human cartilage with progressively severe OA, and in repair cartilage from animal models. FT-IRIS permits evaluation of early-stage matrix changes in the primary components of cartilage, collagen and proteoglycan on histological sections at a spatial resolution of ~6.25 μm. In osteoarthritic cartilage, the collagen integrity, monitored by the ratio of peak areas at 1338 cm^{-1}/Amide II, was found to correspond to the histological Mankin grade, the gold standard scale utilized to evaluate cartilage degeneration. Apparent matrix degradation was observable in the deep zone of cartilage even in the early stages of OA. FT-IRIS studies also found that within the territorial matrix of the cartilage cells (chondrocytes), proteoglycan content increased with progression of cartilage degeneration while the collagen content remained the same, but the collagen integrity decreased. Regenerative (repair) tissue from microfracture treatment of an equine cartilage defect showed significant changes in collagen distribution and loss in proteoglycan content compared to the adjacent normal cartilage, with collagen fibrils demonstrating a random orientation in most of the repair tissue. These studies demonstrate that FT-IRIS is a powerful technique that can provide detailed ultrastructural information on heterogeneous tissues such as diseased cartilage and thus has great potential as a diagnostic modality for cartilage degradation and repair.

Keywords: Infrared imaging; Cartilage; Collagen; Proteoglycan; Osteoarthritis

1. Introduction

Osteoarthritis (OA) is a progressively disabling disease of the joints resulting in impaired motion, severe joint pain and reduced quality of life. Approximately 11% of individuals 65 years of age or older have been estimated to have symptomatic knee OA [1]. On average, 16 to 21 million individuals in the United States are diagnosed annually with OA, and its incidence is further increasing because of an aging population [2–4]. Although OA is a common disease, a proven sensitive and accurate diagnostic method is still unavailable for the early stages of OA. Although MRI is increasingly utilized for evaluation of cartilage changes in the clinical environment [5], only with a high field strength magnet, such as that used for in vitro studies [6–8], can spatially-resolved detailed information on compositional cartilage changes be elucidated. It would be extremely beneficial, both economically and with respect to public health, if the early molecular and compositional changes in osteoarthritic cartilage could be assessed before macroscopic later-stage cartilage damage occurs.

The most prominent feature of OA is the gradual erosion of articular cartilage. Articular cartilage is a dense connective tissue, which provides a smooth, frictionless articular surface for weight bearing during joint loading and motion. The primary molecular components of cartilage are water, collagen, proteoglycan (PG), non-collagenous proteins and cells, the chondrocytes [9]. Collagen and PGs play a major role in the regulation of mechanical properties of cartilage. Collagen is a fibrillar triple helical molecule with intermolecular crosslinks.

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Type II collagen forms the structural framework of the matrix and provides tensile and shear resistance for cartilage. Aggrecan, the predominant PG in cartilage is composed of a protein core to which glycosaminoglycans (GAGs) are covalently attached. These GAGs are repeating disaccharides (chondroitin sulfate and keratan sulfate) with highly negative charges under physiological conditions. PGs contribute considerable compressive strength to the tissue, largely because of their charge. The superficial zone of cartilage contains flattened chondrocytes, the highest concentration of water, low PG content and a densely packed layer of collagen fibrils oriented parallel to the surface which helps to distribute the forces during compression [9]. In the middle, or transition zone of cartilage, the collagen fibrils are less organized and PG content increases. Rounded chondrocytes are surrounded by extracellular matrix in this zone. The deep zone contains collagen fibrils oriented perpendicular to the articular surface, the highest concentration of PGs and lowest water content. The chondrocytes are grouped in columns in this region. The characteristic changes in cartilage during osteoarthritis are damage to collagen network, increased swelling of the tissue and the loss of proteoglycan [10,11]. It is thought that the chondrocytes respond to the tissue damage by increasing proteoglycan and collagen synthesis with an attempt to repair cartilage [12,13]. The damage will ultimately progress and lead to the degradation of cartilage when repair fails.

1.1. FT-IRIS of articular cartilage

The technique of Fourier transform infrared (FT-IR) spectroscopy is a powerful tool to study the previously described changes in degenerative cartilage at the molecular level. The frequency at which a molecule absorbs infrared radiation is sensitive to conformation and can be used to obtain information on the orientation of biomolecules and the chemical compositions in tissues by evaluation of unique molecular spectral signatures of each component [14–18]. The coupling of an FT-IR spectrometer with an array detector to an optical microscope, namely Fourier transform infrared imaging spectroscopy (FT-IRIS), enables infrared imaging of histological sections of tissues at a pixel resolution of 6.25 μm in combination with microscopic visualization of the samples. Thus, FT-IRIS can be utilized to determine the relative content, molecular nature, distribution and orientation of the individual components of histological sections of tissues.

FT-IRIS has emerged as a valuable technique for the characterization of cartilage properties over the past 5 years [19–25]. The advantage of this technique in cartilage research is its ability to determine the content and spatial distribution of specific molecular components in cartilage without the requirement of sophisticated markers, and to determine the orientation of collagen fibrils when polarized FT-IRIS is used. Further, the ability to utilize just one tissue section to obtain all the aforementioned information is very convenient. In contrast, the use of standard histological techniques generally requires preparing individual tissue sections for staining each component of interest. The characteristic spectral features for the primary cartilage components, collagen and PG, have been differentiated by careful examination of model compounds [20,23], and thus enable the creation of FT-IRIS images based on the distribution of each component.

Recent studies in our group have successfully derived IR parameters that are sensitively correlated with cartilage degeneration [22,25] and with collagen fibril orientation [24]. Therefore, subtle changes in the structure or chemical composition of cartilage during cartilage degeneration can be quickly identified. This is essential when evaluating the degradation of cartilage or repair techniques and engineered cartilage tissues. In the studies described here, the previously-developed spectral parameters to assess cartilage are utilized to monitor molecular and compositional changes in human arthritic cartilage tissue and cells and to evaluate the properties of repair cartilage.

2. Materials and methods

2.1. Cartilage tissues

Normal bovine patella cartilage was obtained from the knees of mature cows purchased from Animal Parts (Scotch Plains, NJ) within 24 h post mortem. Arthritic human tibial plateaus were obtained from male and female patients 46–87 years of age during knee replacement surgery under an IRB-approved protocol and immediately brought to the laboratory for processing. Normal human cartilage was obtained from donors without documented history of joint disease immediately after death (National Disease Research Interchange, Philadelphia, PA) and was either snap-frozen in liquid nitrogen or stored immediately in DMEM solution and shipped overnight. Equine tissues utilized in our studies were part of another study under an IACUC-approved protocol from a separate experiment. A full-thickness chondral defect was created in the distal lateral trochlea of both equine stifles (knees), followed by treatment by a microfracture procedure. Tissues were harvested at 6 months post-surgery. Normal equine cartilage spatially distant from the defect was examined as a control.

2.2. Tissue processing

All tissues were processed for paraffin embedding through a series of steps that involves a process of dehydration, PG containment, and decalcification. Full-depth cartilage explants were harvested using a 5-mm diameter biopsy punch. The explants were fixed with 80% ethanol and 1% cetylpiridinium-choloride (CPC), decalcified with 10% EDTA/Tris buffer, and embedded in paraffin. Histological sections were cut at seven μm thickness perpendicular to the articular surface and mounted onto BaF_2 IR windows and glass slides for FT-IRIS and histologic analysis, respectively. All sections were deparaffinized before FT-IRIS measurements were performed.

2.3. FT-IRIS data acquisition

Transmission FT-IRIS data were acquired at 8 cm^{-1} spectral resolution using a Spectrum SpotLight FT-IR Imaging system (Perkin-Elmer, Bucks, UK). This system is comprised of an FTIR spectrometer coupled with a light microscope and allows data collection over a customer defined rectangular region at 6.25 μm pixel resolution. Polarized FT-IRIS data were collected to obtain information on collagen fibril orientation with a polarizer (0°) inserted in the light path.

2.4. FT-IRIS image processing

FT-IRIS images were created based on the vibrational absorbance for the specific molecular component of interest in cartilage using ISys software v3.1 (Spectral Dimensions, Olney, MD). The absorbance bands were baselined followed by area integration. Previous studies have correlated the content of collagen and PG with the integrated area under the protein amide I band (1598–

1710 cm^{-1}) and the infrared absorbance in the ranges of 950–1150 cm^{-1}, respectively [20,23]. The area under the infrared absorbance centered at 1338 cm^{-1} (1300–1356 cm^{-1}), a feature attributed to CH_2 side-chain vibrations, has previously been shown to decrease in intensity as the collagen denatures [22], was ratioed to the amide II band (1492–1598 cm^{-1}) to evaluate the integrity of collagen [22,25]. Images from the polarized data were created based on the area ratio of the amide I and amide II absorbances from collagen, which we have previously demonstrated is an index of collagen fibril orientation [24].

The values for a specific parameter were quantitatively scaled using a corresponding color code for each FT-IRIS image where red equals higher values and blue equals lower values. For the polarized data, the collagen fibril orientation was quantitated as previously described [24]; an amide I/II ratio ≥2.7=fibrils parallel to the articular surface, an amide I/II ratio ≤1.7=fibrils perpendicular to the articular surface, and amide I/II ratios between 2.7–1.7 indicated random or mixed fibril orientation.

For the studies of chondrocytes, FT-IRIS images of collagen content, PG content and pk1338/amide II were created for human osteoarthritic cartilage samples with Mankin grade 1.5, 4 and 11.5. A straight line was drawn across the center of a random cell in the deep zone of osteoarthritic cartilage, and the pixel values for the IR parameters: collagen content, PG content and pk1338/amide II, in the extracellular matrix and the chondrocytes were then extracted from the images using ISys software. The pixel values for each IR parameter were then plotted vs. their distance to the center of the cell.

2.5. Histologic evaluation

PG content and the cartilage tissue morphology were demonstrated with Alcian blue stain and Hematoxylin and Eosin (H&E) stain, respectively. On the Alcian blue-stained histological cartilage sections, dark blue corresponds to PG, red/pink to nuclei, pale pink to cytoplasm [26]. H&E stains nuclei as blue, while cytoplasm and most other tissue structures stain as pink to red [26]. For polarized light microscopy studies, histologic sections were stained with Picrosirius red, which stains collagen type I, II and III [27]. Two cross polarizers were used such that highly ordered collagen perpendicular to the articular surface appeared bright or red, while collagen that is not ordered (non-birefringent) appears darkest. The contrast of fibrils oriented more parallel to the articular surface appeared darker than for collagen perpendicular to the articular surface. All microscopic images were acquired through a Nikon digital camera using BioQuant Nova software (Version 5.00.8 MR, R&M Biometrics, TN).

The human osteoarthritic cartilage tissues were evaluated histologically based on the Mankin grading system using Alcian Blue and H&E staining. The histological Mankin score is the standard grading system for cartilage based on structural fissuring, cell cloning, loss of proteoglycan and tidemark integrity [28,29]. Grade 0 represents normal cartilage and grade 14 represents severely degenerated cartilage. Two investigators evaluated randomized and blind-coded samples independently.

3. Results

3.1. Normal bovine patella cartilage

A representative spectrum obtained from the middle zone of cartilage is shown with the absorbance bands of interest labeled (Fig. 1A). Representative FT-IRIS images of collagen content (Fig. 1E), PG content (Fig. 1F), and collagen fibril orientation (Fig. 1G) for normal bovine patella cartilage were created to illustrate the structure and composition of cartilage samples. The zonal distribution of collagen and PG in cartilage is apparent, whereby more abundant collagen and PG are present in the middle and deep zones of cartilage. The collagen fibrils are oriented parallel to the articular surface at the superficial zone, random in the middle, and perpendicular to the surface at the deep zone. All the above FT-IRIS findings are consistent with accompanying histology outcomes (Fig. 1B–D). The boundaries of the cartilage zones can be identified based on polarized FT-IRIS determined collagen orientation, such that the superficial zone spanned the regions of the image with an amide I/II ratio ≥2.7, the deep zone spanned regions with an amide I/II ratio ≤1.7, and the middle zone spanned the region between these two, with amide I/II ratios between 2.7–1.7 [24].

3.2. Human osteoarthritic cartilage

The PG content and morphology of osteoarthritic human tibial plateau cartilage were demonstrated by Alcian blue (Fig. 2A) and H&E (Fig. 2B) stains on the histological sections, respectively. Progressively severe osteoarthritis was indicated by the increasing Mankin grades of the cartilage. FT-IRIS images were created based on PG content (Fig. 2C), collagen content (Fig. 2D), collagen fibril orientation (Fig. 2E) and the collagen molecular integrity (Fig. 2F).

In general, slightly reduced collagen content and greatly reduced PG content were evident on the FT-IRIS images with progression of OA. The PG results were consistent with the Alcian blue histology, showing PG loss that began at the articular surface and was observed throughout most of the tissue with disease progression. The orientation of collagen fibrils changed dramatically at the surface of cartilage, even in the earlier stages of OA. An unusually large zone of surface parallel fibers was evident, possibly indicative of compressed matrix as a result of reduced resistance to load. By the later stages of the disease, a total lose of orientation was observed. Further cartilage changes were evident by examination of the collagen integrity parameter (Fig. 2F). The quality of matrix collagen decreased as the Mankin score increased, evidenced by a general decrease in the integrated area of the 1338 cm^{-1} absorbance (Fig. 2F). Interestingly, it appears that collagen degradation is present in the deep zone as well as in the cartilage surface in early stages of disease.

Changes in chondrocyte territorial matrix that accompany OA progression were also investigated (Fig. 3). PG, collagen and collagen integrity parameters were mapped across chondrocytes in the deep zone of cartilage, as illustrated in the FT-IRIS images of collagen amide I and in the light microscope image of Mankin grade 11.5 cartilage (Fig. 3A). In nearly normal cartilage (grade 1.5), PG content was found to be greater in the territorial matrix compared to the adjacent surrounding matrix (Fig. 3B). However, in the more diseased tissues, PG content in the territorial matrix tended to be more similar to the adjacent matrix, possibly indicative of the chondrocytes inability to make a sufficient amount of PG. In contrast, a lesser amount of collagen was present in the chondrocyte territorial matrix compared to the surrounding matrix in the nearly normal tissues, and this did not change in the more diseased tissues (Fig. 3C). However, the territorial matrix collagen integrity did appear to be reduced with increasing disease state (Fig. 3D). Together, these results suggest that although the chondrocyte continues to make a normal amount of collagen in OA, the process of collagen degradation may begin immediately as the tissue becomes more osteoarthritic.

3.3. Equine repair cartilage

As an avascular tissue, articular cartilage has limited capacity to repair itself. In the case of a full-thickness defect, when the subchondral plate is penetrated, bone marrow goes out to the subchondral surface and initiates a cellular response for tissue healing [30]. Typically, the repair cartilage formed is "fibrocartilage" which is characterized by a low GAG content and the presence of collagen in a random orientation [31,32]. FT-IRIS was utilized to evaluate the properties and quality of repair cartilage tissues after microfracture treatment, a clinically-utilized technique for treating full-thickness chondral defects that permits invasion of bone marrow into the defect [33]. It is apparent on the images that the distribution of collagen is different (Fig. 4A) and that less PG is present in the repair cartilage tissue compared to the adjacent normal cartilage (Fig. 4B). The collagen integrity parameter appears to be higher in some regions of the repair tissue compared to the adjacent normal control cartilage (Fig. 4C), which could reflect differences in the age of the collagen, whereby the repair collagen is "newer" than the adjacent normal tissue. A clear zonal distribution of collagen fibril orientation was not obvious in the repair tissue (Fig. 4D). Most fibers were randomly aligned through the full depth of cartilage. On the surface, however, collagen fibrils grew into the adjacent normal tissue and formed a well-defined superficial layer with the fibers oriented parallel to the articular surface. The above findings parallel the histology outcomes on H&E stained sections (Fig. 4E) and the results from polarized light microscopy studies on the picrosirius red-stained histology sections (Fig. 4F).

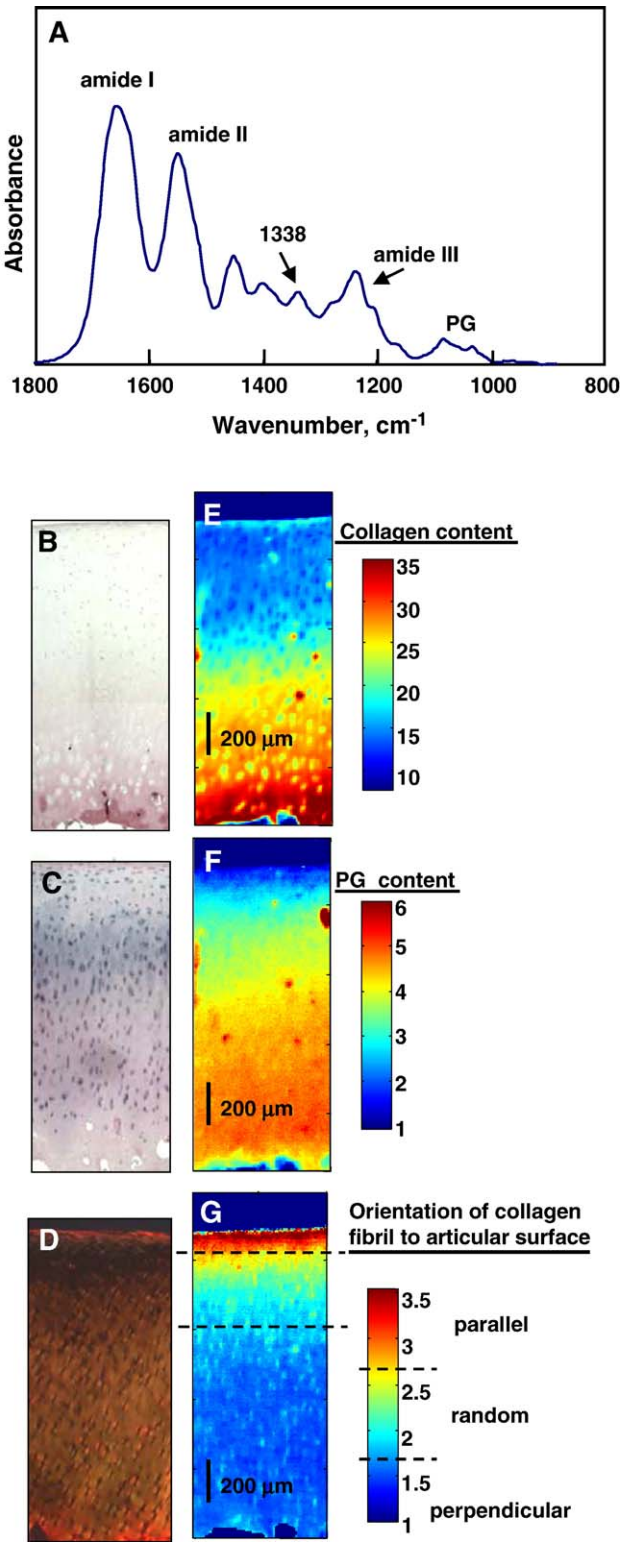


Fig. 1. A representative spectrum obtained from the middle zone of normal bovine patella cartilage with the absorbances of interest labeled (A). Histological images of normal bovine patella cartilage based on H&E for morphology (B), Alcian blue for proteoglycan (PG) content (C) and picrosirius red for collagen fibril orientation (D). The corresponding FT-IRIS images of collagen content (E), PG content (F), and collagen fibril orientation (G) are shown. The red and dark blue in the color scales indicate the highest and lowest contents for collagen or PG in E, F, and indicate collagen fibril orientation parallel and perpendicular to the articular surface in G, respectively. The boundaries of the cartilage zones were identified based on the amide I/II ratio from polarized FT-IRIS. The superficial, middle and deep zones spanned regions with an amide I/II ratio ≥2.7, between 2.7–1.7, and ≤1.7, respectively.

4. Discussion

The studies described demonstrate the application of FT-IRIS as a powerful technique to monitor progression and to evaluate repair tissue in diseases that involve cartilage degeneration. Although the data presented were all ex vivo analyses, there is great potential for in vivo analyses utilizing infrared fiber optic probe technology [34]. Identification of early alterations in cartilage that involve degradation of major or minor matrix components, such as minor collagens and small proteoglycans, all of which are required for matrix stabilization and integrity [35], would likely be feasible utilizing these techniques.

Previous reports suggested that the initial event in OA starts at or just below the articular surface, in the uppermost region of the matrix, the laminar splendens (LS) and superficial tangential zone (STZ) [36], although subchondral bone alterations were considered critical in the disease process as well [37,38]. In the current study, human articular

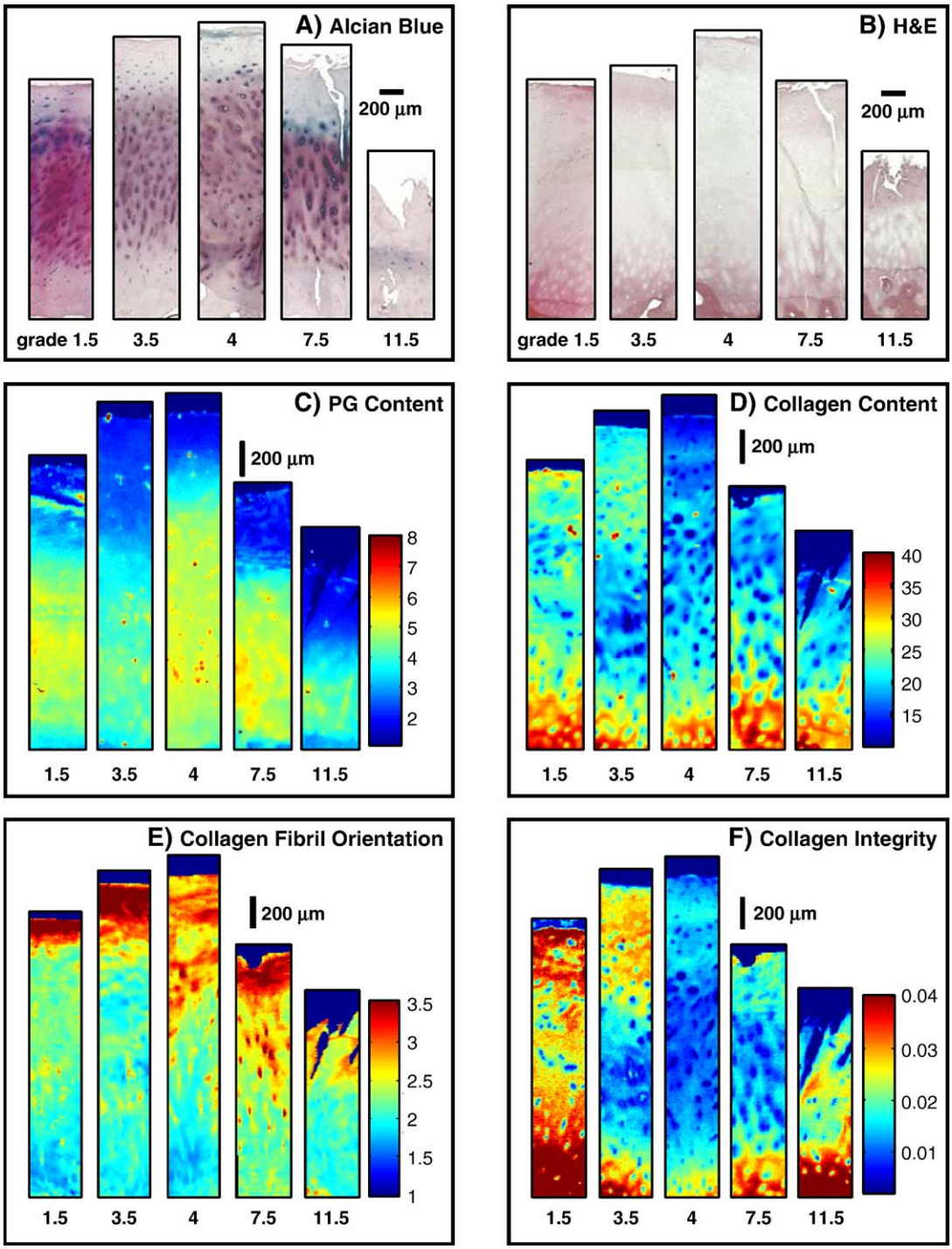
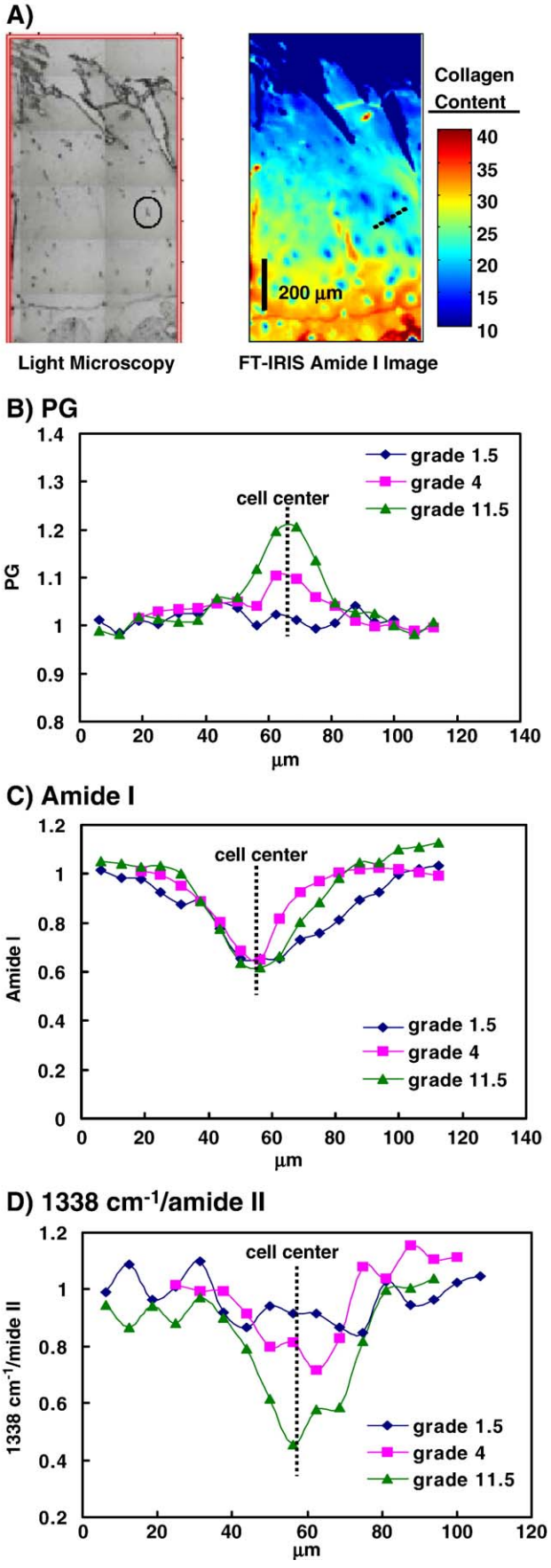


Fig. 2. Alcian blue (A) and H&E (B) stained histological sections and FT-IRIS images created based on PG content (C), collagen content (D), collagen fibril orientation (E) and the molecule integrity of collagen (F) for samples of human tibial cartilage with Mankin grade 1.5, 3.5, 4, 7.5, and 11.5, respectively. Each set of FT-IRIS images utilized the same scale. The color bars represent the corresponding pixel values for each IR parameter, where red and dark blue indicate the highest and lowest contents for PG content (C), collagen (D), or collagen integrity (F), and indicate collagen fibril orientation parallel and perpendicular to the articular surface (E), respectively.

cartilage tissues with increasingly severe degeneration were investigated using FT-IRIS. Our results indicate that in addition to the surface, however, there are also molecular changes in collagen in the deeper zones of cartilage in early OA. These changes continue to proceed with the progression of OA through later stages of the disease. Specifically, we have demonstrated that a reduction in the spectral signature of the 1338 cm^{-1} peak from the collagen molecule, an indicator of type II collagen damage, is found in the early development of OA [39,40].

Morphological and functional changes of chondrocytes in osteoarthritic cartilage play an important role in the



pathogenesis of OA. OA chondrocytes display nuclear and cytoplasmic changes consistent with apoptotic cell death [41]. In OA cells, the amount of synthetic and secretory cell organelles has been shown to be reduced in the cytoplasm [42], and the structure of the chondrocyte cytoskeleton was shown to be irreversibly disrupted with loss of cytoskeletal filaments [42–44]. This may be related to our findings that the integrity of collagen in the cytoplasm of chondrocytes in the deep zone of cartilage decreased with the progression of OA, while the total amount of collagen remained stable. In contrast, the relative content of PG in the chondrocytes, although increased in normal cartilage compared to the adjacent matrix, was reduced with OA progression, most likely reflecting an inability to produce adequate PG in the osteoarthritic cartilage matrix.

One challenge of assessing a therapeutic or tissue engineering intervention is to detect the compositional and structural changes in articular cartilage repair tissue formed by various tissue engineering approaches. Although standard biochemical assays can provide average chemical composition for a whole piece of tissue, this method of assay may not provide all the required information for a heterogeneous tissue such as cartilage. This study supports the role of FT-IRIS to quickly and accurately characterize the distribution and structure of cartilage components, therefore enabling further correlation between cartilage properties and function. The repair tissue formed with a microfracture procedure contains less PG and an abnormal distribution of collagen compared to normal cartilage, which may explain the poor resultant mechanical properties often exhibited by repair tissue [31,32].

In spite of the advantageous applications of FT-IRIS in evaluating cartilage damage and repair, there are certain limitations to the use of this technique. First, the tissue sections for FT-IRIS imaging are generally dehydrated during processing. Therefore it is not possible for FT-IRIS to precisely measure the content of water in the tissue, a component that has been shown to change in the early stages of OA [45–47]. Second, FT-IRIS is an invasive technique, requiring physical removal of the tissue for data acquisition. Practically, such an assessment would only be performed in a clinical situation at a very late stage of the disease when tissue would be removed anyway, such as for knee replacement or when a debridement procedure is warranted. Thus, although FT-IRIS can detect minor changes in the chemical compositions of cartilage in the early stages of OA, this technique does not fulfill the non-invasive requirement for clinical use. It would be valuable to develop

Fig. 3. Light microscope image of cartilage sample with Mankin grade 11.5 and FT-IRIS image based on amide I (collagen) showing a typical region across a deep zone chondrocyte where data were acquired (illustrated by the dotted line) (A). IR parameters of PG content (B), collagen content (C) and collagen integrity (D) were mapped across chondrocytes in the deep zone of human tibial cartilage with Mankin grades 1.5, 4 and 11.5. In B–D, all of the curves were normalized to the pixel values of the adjacent extracellular matrix. Dashed lines indicate the cell centers.

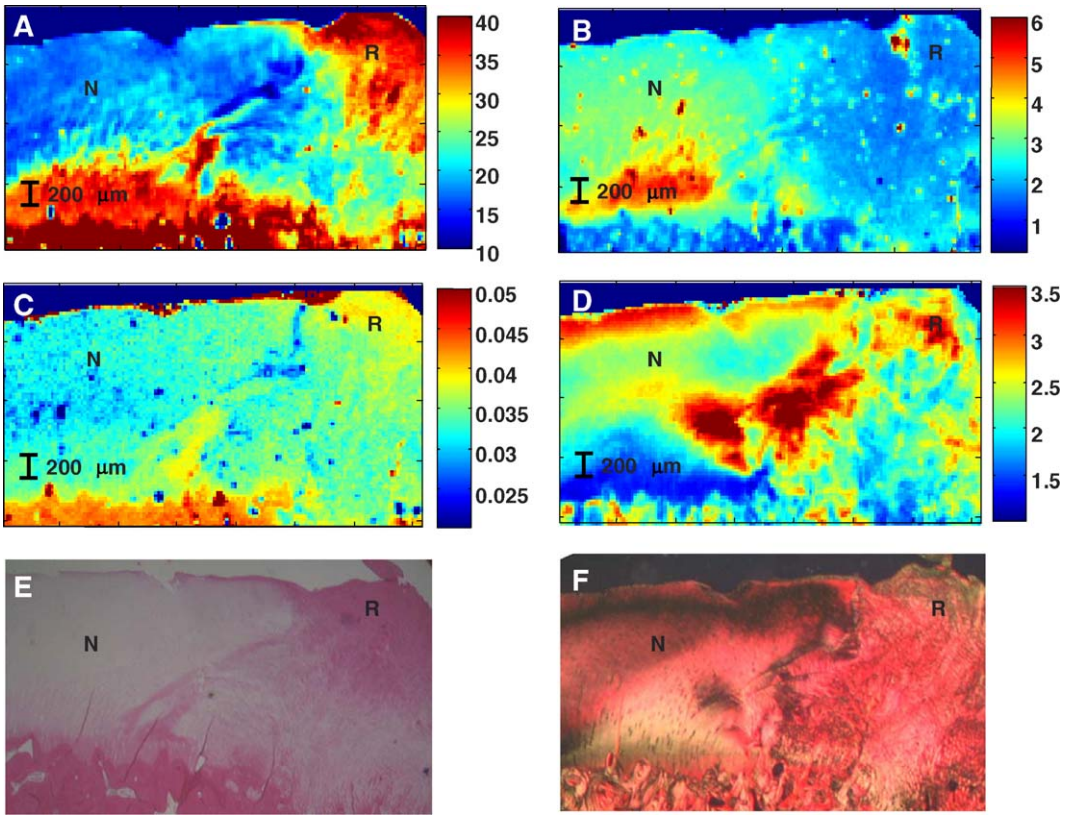


Fig. 4. FT-IRIS images created based on collagen content (A), PG content (B), collagen integrity (C), and collagen fibril orientation (D) in equine repair cartilage tissue after microfracture treatment. Color scale indicates the pixel values for the content of collagen or PG or integrity (1338 cm^{-1}/amide II), as well as the orientation of collagen fibrils (amide I/II). Histology studies on H&E stained (E) and picrosirius red stained (F) sections show the morphology of the tissue and the orientation of collagen fibrils. The repair tissue and the adjacent normal cartilage were illustrated as "R" and "N" on the images, respectively.

a non-invasive device that can incorporate detection of specific ultrastructural changes in the OA tissue while overcoming the disadvantages of ex vivo FT-IRIS analyses. This goal has been addressed in our lab with a novel design of an infrared fiber optic probe (IFOP) that enables evaluation of the chondral surface in joint diseases in a clinical setting [22,34]. With the continuing development of novel techniques for the operative treatment of osteochondral injuries [48,49], IFOP evaluations could become extremely important during arthroscopic procedures, where crucial decisions are made regarding salvaging or removing cartilage and meniscus.

In conclusion, FT-IRIS analyses can provide detailed ultrastructural information on cartilage and other biological tissues by examination of multiple parameters on just one tissue section per sample. Thus, there is great potential for FT-IRIS to be utilized as an important diagnostic tool to accurately and quickly distinguish cartilage degradation in musculoskeletal diseases including osteoarthritis and trauma injuries, and to assess repair tissue and therapeutic efficacy in these clinical conditions.

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