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Depth-Dependent Anisotropies of Amides and Sugar in Perpendicular and Parallel Sections of Articular Cartilage by Fourier Transform Infrared Imaging (FTIRI)

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Abstract

Full thickness blocks of canine humeral cartilage were microtomed into both perpendicular sections and a series of 100 parallel sections, each 6 μm thick. Fourier Transform Infrared Imaging (FTIRI) was used to image each tissue section eleven times under different infrared polarizations (from 0° to 180° polarization states in 20° increments and with an additional 90° polarization), at a spatial resolution of 6.25 μm and a wavenumber step of 8 cm^{-1} . With increasing depth from the articular surface, amide anisotropies increased in the perpendicular sections and decreased in the parallel sections. Both types of tissue sectioning identified a 90° difference between amide I and amide II in the superficial zone of cartilage. The fibrillar distribution in the parallel sections from the superficial zone was shown to not be random. Sugar had the greatest anisotropy in the upper part of the radial zone in the perpendicular sections. The depth-dependent anisotropic data were fitted with a theoretical equation that contained three signature parameters, which illustrate the arcade structure of collagens with the aid of a fibril model. Infrared imaging of both perpendicular and parallel sections provides the possibility of determining the three-dimensional macromolecular structures in articular cartilage. Being sensitive to the orientation of the macromolecular structure in healthy articular cartilage aids the prospect of detecting the early onset of the tissue degradation that may lead to pathological conditions such as osteoarthritis.

Keywords

articular cartilage; FTIRI; anisotropy; parallel section; collagen; amides; sugar

Introduction

Collagen fibrils, one of the principal solid components in the extracellular matrix of articular cartilage, are structured within the tissue in a dense network that provides tensile resistance to tissue swelling (Benninghoff, 1925; Maroudas, 1975; Maroudas and others, 1980; Venn and Maroudas, 1977). The fibrillar orientation of this network has been used to subdivide the thickness (depth) of the tissue into three consecutive zones: the superficial zone (SZ), transitional zone (TZ) and radial zone (RZ). With increasing depth from the articular surface towards the cartilage/bone interface, the orientation of the collagen fibrils changes from parallel (in SZ), to random (in TZ), to perpendicular (in RZ) with respect to the articular surface. This depth-dependent structure of the collagen matrix in articular cartilage is

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important in resisting the swelling pressure created by the negatively charged proteoglycans (PG), thereby preserving the tissue's integrity. Disruption of the fibrillar network has been linked to the early stages of osteoarthritis (OA) and may represent a functional failure of the tissue. (Alhadlaq and others, 2004; Buckwalter and Mankin, 1997; Burstein and others, 2000; Burton-Wurster and others, 1993).

The depth-dependent anisotropy of the collagen fibrillar structure in connective tissues becomes evident when examined by several imaging techniques, such as microscopic MRI (μ MRI) (Nieminen and others, 2001; Xia, 1998; Xia and others, 2002), polarized (visible) light microscopy (PLM) (Arokoski and others, 1996; Rieppo and others, 2007; Xia and others, 2001), and Fourier Transform Infrared Imaging (FTIRI) (Camacho and others, 2001; Gadaleta and others, 1996; Potter and others, 2001; Xia and others, 2007). With the potential to provide quantitative and spatially-resolved information about the chemical composition of degrading tissue, FTIRI has been used extensively in cartilage studies in recent years (Bi and others, 2005; Camacho and others, 2001; David-Vaudey and others, 2005; Potter and others, 2001; Ramakrishnan and others, 2007a; Ramakrishnan and others, 2007b; West and others, 2004; Xia and others, 2008; Xia and others, 2007). Such investigations of the amide absorptions of specific infrared (IR) energies have created a better understanding of the tissue's zonal arrangement of several chemical bonds relating to collagen structure, such as amide I (C=O stretching), amide II (C-N stretching and N-H bending), and amide III (N-H and C-C vibrations), as well as PG levels, through analysis of sugar (C-C ring vibrations).

Most investigations into the depth-dependent structure of articular cartilage by light microscopies have been carried out using thin tissue sections oriented perpendicular to the articular surface; as such, a single section contains the full thickness of articular cartilage as well as the attached subchondral bone. Alternatively, articular cartilage may be sectioned parallel to the articular surface (Bayliss and others, 1983; Brocklehurst and others, 1984; Laasanen and others, 2003; Maroudas and Venn, 1977; Roberts and others, 1986; Venn, 1979). This type of thin section provides the unique opportunity to study the depth-dependent anisotropy of the collagen network from an orthogonal angle. Our preliminary study of this type of parallel sections of tendon and articular cartilage using FTIRI *at several selected tissue depths* has found complex anisotropies in the parallel sections not evident by studying the perpendicular sections (Ramakrishnan and others, 2008). For example, parallel sections of canine achilles tendon found clear anisotropy for both amide I and amide II, and parallel sections at several depths in the radial zone of articular cartilage found strong anisotropy for amide I but near isotropy for amide II. Neither of these two conclusions was able to be identified using conventional perpendicular sections. Analysis of these amide anisotropies from parallel sections allowed the schematic conception of an 'amide cone' (Ramakrishnan and others, 2008) that provided important insights into the features of these amide anisotropies.

The goal of the current investigation is to map the molecular anisotropies over ***the entire depth of articular cartilage*** using a series of sequentially numbered parallel sections at a 'step resolution' of 6 μ m. By examining the depth-dependent anisotropies of amides and sugar components in the orthogonal plane, we aimed to provide new insights into the 3-dimensional (3D) structural arrangement of collagen and distribution of proteoglycan in the extracellular matrix of articular cartilage. The increased understanding gained by such analysis may allow early detection of degradative alterations in cartilage morphology evident in pathological conditions such as osteoarthritis.

Materials and Methods

Specimen Preparation

Three blocks of canine articular cartilage were harvested from the central load-bearing region of three healthy and mature humeral heads. The animals were sacrificed for an unrelated scientific experiment. Two types of tissue sections were obtained from each osteochondral block ($\sim 1.75 \text{ mm} \times 1.75 \text{ mm} \times 2 \text{ mm}$), perpendicular sections and parallel sections (Fig 1a). The perpendicular sections were $6 \mu\text{m}$ thick and microtomed perpendicularly to the articular surface to contain the full thickness of the cartilage tissue attached to the subchondral bone. The parallel sections, also $650 \mu\text{m}$ thick, were microtomed in parallel to the articular surface. Both perpendicular and parallel sections were obtained from the same tissue block to allow for direct comparison between the depth-dependent anisotropies of amides and sugar. The non-calcified cartilage thickness in the tissue blocks was approximately $650 \mu\text{m}$ thick, allowing each tissue block to therefore provide 100 sequentially numbered parallel sections. Prior to parallel sectioning, the depth-wise trimming of one corner of the osteochondral block allowed the identification of the parallel section's orientation during the experiments. Standard tissue processing procedures using paraffin embedding were used (Xia and others, 2001) and all tissue sections were placed on "MirrIR" slides (Kevley Technologies, Chesterland, Ohio) for the FTIR experiments.

Fourier-Transform Infrared Imaging (FTIRI)

FTIRI measurements were performed using a PerkinElmer Spotlight 300 imager (Wellesley, MA), which integrates an infrared microscope with a mid-infrared spectrometer. Using the reflection mode, the tissue sections were examined in the infrared frequency range of $4000\text{--}750 \text{ cm}^{-1}$ with a spectral step of 8 cm^{-1} and a pixel size of $6.25 \mu\text{m} \times 6.25 \mu\text{m}$. For each tissue section, identical experiments were repeated 11 times, each at a different infrared polarization (from 0° to 180° in 20° increments with the addition of a 90° polarization) by varying a wire grid IR polarizer in the optical path (PerkinElmer, Wellesley, MA).

Once a tissue section was mounted on the FTIR imager, the section remained fixed on the scanning stage until all 11 experiments were completed. The background for each tissue section was also obtained (and subtracted in the subsequently data analysis). In this way, identical alignments and regions-of-interest (ROI) could be selected during each polarization angle which is critical especially when comparing different parallel sections. The perpendicular sections were aligned with the articular surface vertical to the left side of the IR microscope's field of view. The parallel sections were aligned so that the top and bottom edges, in relation to the cut-corner orientation were nearly horizontal in the microscope's field of view. A ROI of $150 \mu\text{m} \times 150 \mu\text{m}$ was chosen as close to the center of each parallel section for infrared imaging. Approximately 1100 independent FTIRI experiments were carried out from all 100 parallel sections obtained from each tissue block, which took about 50 hours of experimental time.

Data Analysis

Each FTIRI experiment resulted in a 3D hyperspectral image with two spatial coordinates (in μm) and one chemical dimension (in cm^{-1}). Using a double baseline-correction (Xia and others, 2007) in the system software from PerkinElmer, the IR absorption spectra for amides I, II, III and sugar were extracted in the following four regions: amide I ($1700\text{--}1600 \text{ cm}^{-1}$), amide II ($1600\text{--}1500 \text{ cm}^{-1}$), amide III ($1300\text{--}1200 \text{ cm}^{-1}$), and sugar ($1125\text{--}1000 \text{ cm}^{-1}$) from each 3D hyperspectral image. This method of wavenumber extraction provides four, two-dimensional (2D) absorption images at each IR polarization (Xia and others, 2007), resulting in approximately 4400 IR absorption images for all parallel sections from each tissue block.

Within perpendicular sections, the anisotropic profile for each amide and sugar was obtained by averaging from a row 8 pixels wide from the articular surface to the bone, yielding a depth-dependent profile for each chemical component with a pixel size of 6.25 μm . For each parallel section within a cartilage block, the entire 150 $\mu\text{m} \times 150 \mu\text{m}$ ROI was averaged into a single absorption value at each analyzer angle for each chemical component.

Modeling of Infrared Anisotropy

The anisotropic information of each chemical component can be combined into a new 3D anisotropy image (Xia and others, 2007), the rotational angle (of the IR analyzer), the tissue depth, and the infrared absorption value form the three axes of the anisotropy image. This 3D image may be shown as a surface image, which allows visual identification of the anisotropy of each chemical component throughout the depth of the tissue (Xia and others, 2007). In addition, one can extract a profile of the anisotropy at each depth of this 3D image (every 6.25 μm in perpendicular sections and 6 μm in parallel sections), to examine the anisotropic characteristics of the tissue at specific depths. In this project, all anisotropic profiles were fitted individually to an empirical equation (Xia and others, 2007) that resembles Malus's Law of polarized light,

$$\text{Absorbance}(r, \theta) = A(r) \cos^2(\theta - \theta_0(r)) + A_0(r) \quad \text{Eq. (1)}$$

This equation contains three fitting parameters: $A(r)$, $A_0(r)$, and $\theta_0(r)$. The $A(r)$ term represents the anisotropic portion of the infrared absorption, which equals the difference between the maximum and minimum absorptions in each anisotropic profile. The $A_0(r)$ term represents the baseline portion of the infrared absorption, which equals the difference between the minimum of the fitting and the absorption zero. The $\theta_0(r)$ term represents the angle at which the maximum of the anisotropic sinusoid occurs, which is related to the offset of the fibrillar orientation with respect to a reference direction in the experiment. Although $\theta_0(r)$ is referenced to an arbitrary direction in the instrument, the differences in $\theta_0(r)$ among all components (amides I, II, III and sugar) throughout the depth of the tissue are meaningful because at each tissue depth in parallel sections, the four chemical components were acquired from a single infrared experiment. In addition, the identification of the specimen's corner firmly registered the identical orientation of all parallel sections from the same tissue block. The fitting of this equation was done using KaleidaGraph (Synergy Software, Reading, PA).

Results

Each specimen block provided one to two perpendicular sections and approximately 100 parallel sections. Although three specimen blocks were analyzed, only data from one representative block is shown since all blocks had similar features. The visible images of the representative perpendicular and parallel sections from one specimen block are shown in Fig. 1. It is clear that the perpendicular section (Fig. 1b) shows the usual features associated with the traditional histological zones in articular cartilage (Xia, 2008). Four parallel sections (Fig. 1c) from different tissue depths illustrate the histological zones of cartilage from the same specimen block that provided the perpendicular sections. Due to the surface curvature and/or the slight misalignment of the tissue block during sectioning, the first several parallel sections missed some portion of the tissue. Most of the 100 parallel sections cut from one tissue block had the complete tissue, where the marked corner (the top right corner) could be easily identified.

For each of the perpendicular and parallel sections, the same region of interest was imaged by all 11 FTIRI anisotropy experiments, ensured by keeping the tissue section stationary. The plots in Fig. 2 illustrate the anisotropic absorption of amides I, II, III and sugar in four parallel sections obtained from a single tissue block. The spectra show a greater peak difference (i.e., greater anisotropy) between 0° and 90° polarizations in the superficial region of the tissue (Fig. 2a) when compared to deeper tissues (e.g., Fig. 2d). Based on our established methodology (Xia and others, 2007), 2D chemical maps were extracted from the 3D hyperspectral image of raw infrared data at the four wavenumber regions corresponding to amides I, II, III and sugar, as shown in Fig. 2e. Note that these 2D images show the influence of chondrocytes on the infrared images. Since no pixel within the ROI was excluded in the analysis, the differing amounts of infrared absorption in the region of chondrocytes could contribute to the background variations in the averaged absorption measurements.

For each parallel section throughout the depth of the tissue, all 44 infrared anisotropies from the four chemical components were examined in detail. Representative samples of the anisotropic profiles are shown in Fig. 3 (amide I and amide II) and Fig. 4 (amide III and sugar), at four different depths within the tissue. The sinusoidal variations of amide anisotropies within perpendicular sections (the left columns in Fig. 3 and Fig. 4) exhibit several distinct features in their sinusoidal variations. In particular, the inversion of the sinusoids between the superficial zone and the radial zone for each amide demonstrates the perpendicular nature of the collagen fibrils between these two zones. The inversion of the sinusoids between amides I and II at identical tissue depths demonstrates the perpendicular nature of these two amide bonds. The similarity between the sinusoids of amides II and III suggests an approximately parallel arrangement of these two amide vibrations in perpendicular sections. Sugar, which we have previously considered to be isotropic (Xia and others, 2007), clearly shows weak but distinct anisotropy in the upper part of the radial zone (Fig. 4c).

Several unique features for the anisotropies of amides and sugar were also found in parallel sections. First, just as in the case with the perpendicular sections, amides I and II of the parallel sections (the right column in Fig. 3) also show opposite sinusoids between them at each tissue depth, confirming that these dipole bonds with respect to the collagen fibrils are also oriented perpendicularly in the parallel sections. Second, dissimilar to perpendicular sections, parallel sections show no clear sinusoid inversion between the superficial zone and the radial zone for each amide. Third, similar to perpendicular sections, parallel sections also show the anisotropy of amide III to largely follow the trend of amide II. Finally, the anisotropies of all amides increase with the tissue depth in perpendicular sections. In contrast, an opposite trend is seen in parallel sections where the largest anisotropies of all amides occur in the surface portion of the tissue and decrease with greater tissue depth. It is important to consider the fact that each data point in Figs. 3 and 4 came from an independent imaging experiment; the quality of the experimental data in this project is thus remarkably high.

Also shown in Figs. 3 and 4 are the fitting (the solid lines) of these experimental data using Eq.(1) (Xia and others, 2007), which contains three parameters, the anisotropic amplitude $A(r)$, the isotropic baseline $A_0(r)$ and the anisotropic orientation $\theta_0(r)$, as described previously. All experimental data were fitted individually with this equation. Fig. 5 shows the three fitting parameters for each chemical tabled and plotted for the 100 parallel sections as well as the perpendicular sections. When the anisotropy profiles are compared in this manner, several distinct features of these fitting parameters can be identified, as the following:

1. For the anisotropic amplitude $A(r)$ term, all four chemical components (three amides and sugar) had small but finite values in the superficial zone for both perpendicular and parallel sections (Fig 5a). For the perpendicular sections, the values of $A(r)$ for all four components increased significantly as a function of the tissue depth. In contrast, in the parallel sections, the values of $A(r)$ for all three amides decreased noticeably as a function of the tissue depth, while the value of $A(r)$ for sugar did not exhibit noticeable changes.
2. The isotropic baseline $A_0(r)$ term also appears to be depth-dependent (Fig 5b). However, the difference between the perpendicular section and parallel sections was small. Although we investigated the meaning of this isotropic absorption term through additional infrared-imaging of tissue sections that had different thickness (4, 6, 8, 10 μm), no conclusive features were found (data not shown). The lack of difference in the value of this isotropic baseline term between perpendicular and parallel sections could be caused by several practical factors in the experiments, for example the fluctuation of the infrared irradiation (source energy), and any residual quantity of tissue's embedding material.
3. For the anisotropic orientation $\theta_0(r)$ term, its values between amide I and amide II in the perpendicular sections have a nearly constant difference of 90° *for the entire tissue depth*. The $\theta_0(r)$ from amide III closely resembled the trends shown for amide II in the perpendicular sections. In the parallel sections, similar features could also be observed among the three amides, although only in the superficial and transitional zones. The $\theta_0(r)$ term for the parallel sections in the radial zone had considerable 'oscillations'. There is no difference in the angular term for sugar between the perpendicular and parallel sections.

Although the trends of these fitting parameters seem complicated at this moment, it will become clearer when we discuss the implication of these parameters with the aid of a fibril model in the following section.

Discussion

A set of complex and depth-dependent variations in the infrared anisotropy have been found in articular cartilage between both perpendicular and parallel sections, with the most representative features being for amide I and amide II. The origin of these infrared anisotropies is fundamentally due to two perpendicularities in articular cartilage, illustrated schematically in Fig 6a. First, the collagen fibrils in the superficial zone are approximately perpendicular to the fibrils in the radial zone (cf. the short lines in the perpendicular section in Fig 6a). (Note that the collagen fibrils in the superficial zone are distributed on the 2D planes of the tissue that is in parallel with the articular surface. The fibril lines in the superficial zone in the schematic drawing in Fig 6a are merely the 'projections' of the fibrillar distribution.) Second, the transition moments of amide I and amide II, each associated with a particular molecular vibration in a collagen fibril, can be considered perpendicular to each other in the context of the long axis of collagen in cartilage (cf. the drawing inside the dash-boxed insert in Fig 6a).

The meaning of the $\theta_0(r)$ term can be understood by examining the trends of both amide I and amide II in the perpendicular section (the upper plot in Fig 6b). In this plot, the $\theta_0(r)$ data in Fig 5c were modeled by a hyperbolic tangent function, which had been used in polarized (visible) light microscopy to describe the angular transition of the collagen fibrils between the superficial and radial zones in articular cartilage (Xia and others, 2001). Considering the fact that each angle in the $\theta_0(r)$ plot is from the fitting Eq.(1) to 11 independent infrared experiments pixel-by-pixel and that the hyperbolic tangent function

describes the birefringent feature of polarized (visible) light, the quality of and agreement between these two sets of curves were astounding. Also marked on this plot are the approximate zonal divisions for this type of canine humeral cartilage, which has been studied in our lab extensively for over 15 years (Xia and others, 2001). It is clear that in the perpendicular section, (1) both amide I and amide II have an approximate 90° orientational difference between the surface tissue and deep tissue, which corresponds to the fact that the fibrillar orientation between these two zones are perpendicular to each other; and (2) there is a 90° angle difference between amide I and amide II at the same tissue depth throughout the entire cartilage depth, which confirms the understanding that the bond directions of amide I and amide II are approximately perpendicular to each other with respect to the long axis of the collagen fibril. The two schematics in the upper part of Fig. 6d and Fig. 6e illustrate these fibrillar structures.

With these confirmations for the perpendicular section in mind, the $\theta_0(\mathbf{r})$ terms for amide I and amide II in the parallel sections, grouped together in the lower plot of Fig. 6b, can be examined. For the parallel sections in the first 1/3 of the tissue depth (about 0 – 200μm), there is a constant angle difference of 90° for the $\theta_0(\mathbf{r})$ terms between amide I and amide II. For the rest of the tissue depth (about 200μm – 600μm), the $\theta_0(\mathbf{r})$ terms for amide I and amide II both fluctuated by about 90°. These features can be explained as the following. For the parallel sections in the superficial zone and transitional zone, collagen fibrils predominantly have their long axes in the plane of the tissue section. We have shown recently by μMRI experiments that the distribution of the collagen fibrils in such a 2D tissue plane is not completely random (Zheng and Xia, 2009). This non-random distribution of collagen fibrils results in a finite sum for amide I and amide II in the parallel sections, which contribute to the finite $\theta_0(\mathbf{r})$ terms for both amides. The fact that a constant 90° difference exists between the two $\theta(\mathbf{r})$ terms between amide I and amide II for the first 200μm of tissue sections further support this conclusion – the residual amides are still perpendicular to each other, as shown in Fig. 6e.

For the parallel sections in the radial zone of cartilage (200–600 μm), an ideal case would have the long axis of the collagen fibrils perpendicular to the plane of a tissue section, resulting a ‘dot’ on the imaging plane. Since it is well known in literature that (1) the transition moments of amide I and amide II both have finite tilting angles with respect to the axis of the helix (Fraser and MacRae, 1973), (2) the amide bonds are fixed in the peptide chains, and (3) each type-II fibril contains three identical chains in a triple helix, the signal in any finite volume of imaging element (voxel) comes from the average of a large number of bonds/molecules. Consequently, any amide bond would be distributed on the surface of an ‘amide cone’, which casts a ‘projection’ in the 2D plane of the tissue section that is undergoing the rotation in a polarization experiment (Ramakrishnan and others, 2008).

If the long axis of the fibril or fibrils is perfectly perpendicular to the section plane and when the distribution of all amides on the ‘cone’ is sufficiently symmetrical, this amide cone would have no anisotropy in a polarization experiment. In our experiment of these parallel sections, the anisotropic amplitude term $A(\mathbf{r})$, which represents the residual bond average, started at a finite value for at the superficial zone but approached zero toward the deeper tissue (the lower plot in Fig 6c), which suggest a nearly symmetrical cone for the distribution of any amide in the deeper tissue. When the anisotropy amplitude approaches the noise level (including the absorption variation from the influence of chondrocytes, as shown in Fig 2e), the fitting of the sinusoids becomes problematic, which could result in the fluctuation of the $\theta_0(\mathbf{r})$ terms for amides I–III for most of the radial zone tissue. In contrast, the anisotropic amplitude term $A(\mathbf{r})$ for the perpendicular sections (the upper plot in Fig 6c), which started at about the same values as in the parallel sections, becomes bigger and bigger when one moves into the deeper tissue, which confirms the increasing order of the collagen

orientation in the radial zone. The reason that the $A(r)$ term for amide I is bigger than that for amide II (the upper plot of Fig 6c) is due to the fact that amide I is a double bond but amide II is a single bond, consequently, the dipolar moment of amide I should be stronger than that of amide II.

Having explained the features of the $\theta_0(r)$ and $A(r)$ terms for both amide I and amide II, understanding the rest of the anisotropic parameters in Fig. 5 becomes easier. The similarities between the results of amide II and those of amide III confirm the general understanding that these two amides have a similar orientation in its molecular bonds. A subtle feature shown in the current investigation is the anisotropy of sugar that represents the absorption from the glycosaminoglycans in PGs. Although it is generally understood that sugar in articular cartilage has no infrared anisotropy (Xia and others, 2007), a close examination of the sugar data indicates that a weak but recognizable amount of anisotropy in its perpendicular sections. A curious feature of this sugar anisotropy is the fact that its anisotropic amplitude reaches its max (the peak in $A(r)$ for sugar in Fig 5a) in the middle of the tissue thickness in the regular sections. However, the parallel sections lack this amplitude peak. This anisotropy of sugar is indicative of some close interactions between the PG and collagen molecules in articular cartilage, shown recently at the 'matrix interaction domain' of collagen fibril by a X-ray diffraction study (Sweeney and others, 2008). In addition, a study of human corneal stroma (Muller and others, 2004) has found that proteoglycans formed a repeating network of ring-like structures ($\sim 45\text{nm}$) around the collagen fibrils in the cross sections. These authors suggested that the hexagonal arranged collagen fibrils are interconnected at regular distances with their next-nearest neighbors by groups of six proteoglycans, attached orthogonal to the circumference of the fibrils. This structural symmetry, if also holds in the radial zone of canine articular cartilage, could well explain the lack of sugar anisotropy in the parallel sections. It could also explain the general trends of the decreasing anisotropies for the three amides in the parallel sections when one approaches the deeper tissue.

Taken together, the anisotropic analyses of these amides and sugar in this infrared imaging project support the arcade structure of collagen fibrillar configuration in articular cartilage, where the two perpendicularities (superficial vs. radial zones, amide I vs. amide II) in articular cartilage are preserved in the parallel sections throughout the entire depth of the tissue. The finite anisotropy in parallel sections from the superficial zone confirms the recent experimental finding in μMRI (Zheng and Xia, 2009) that the fibrillar distribution in the superficial zone is not random. The diminishing anisotropy towards the deep tissue in the parallel sections implies that despite the microscopic evidences (Broom and Silyn-Roberts, 1989; Chen and Broom, 1998; Speer and Dahners, 1979; Xia and others, 2002) of orthogonal fibrils linking the main radial zone fibrils and the zigzag nature of the radial zone fibrils themselves, all orthogonal components *within any imaging voxel* ($6.25\mu\text{m} \times 6.25\mu\text{m} \times 6\mu\text{m}$ in this project) must have been sufficiently averaged out in the rotation plane to cause insignificant anisotropy for amides and sugar in the radial zone of the tissue.

In conclusion, to the best of our knowledge, this is the first infrared investigation of the dipolar bond anisotropies of amides and sugar in articular cartilage over the entire depth of the tissue, *based on the imaging results of parallel tissue sections*. Comparing with the use of perpendicular tissue sections, the use of parallel sections increases the workload significantly. It also requires a much higher degree of attention to experimental details. One is rewarded, however, with the unique opportunity of examining the molecular structure from an orthogonal angle perspective. Based on the results from this project, both collagen and PG macromolecules can be considered well organized and to have a sufficient symmetry for the 2/3 of the deep tissue. This lack of infrared anisotropy in the radial zone of articular

cartilage in the parallel sections could become useful when the organization of these macromolecules become disrupted in cases of clinical lesions or external loading.

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Figure 1.

(a) The schematics of the (full thickness) perpendicular sections and parallel sections of articular cartilage. (b) The visible image of a perpendicular section from the FTIRI microscope. (c) The visible images of four parallel sections at four different tissue depths from the FTIRI microscope: 18μm (superficial zone), 102μm (transitional zone), 258μm (upper radial zone), and 528μm (lower radial zone). The black boxes in (b) and (c) indicate the regions where the FTIRI experiments were carried out. The non-calcified tissue is about 650μm thick. (A.S. = articular surface; SZ = superficial zone; TZ = transitional zone; RZ = radial zone)

Figure 2.

(a) – (d) The infrared absorption spectra from four parallel sections at the depths of 18, 102, 258, and 528 μm, respectively. Each plot has two spectra from the same pixel location in the tissue, obtained when the infrared analyzer angles were at 0° and 90°. (All spectra are plotted on the same absorption and wavenumber scales.) (e) The visible and the associated chemi-maps of amide I, II, III and sugar at the same tissue depths of 18, 102, 258, and 528 μm, respectively. (Each image contains the full-size ROI as shown in Fig 1. All IR images were under the unpolarized infrared radiation.) All four IR images for the same chemical (either amide or sugar) are set to the same absorption scale of maximum and minimum. (amide I: 0.178 to 0.453; amide II: 0.071 to 0.210; amide III: 0.043 to 0.096; sugar: 0.037 to 0.083)

Figure 3.

(a – d) are the amide I and II anisotropy profiles of the perpendicular sections at the tissue depths of 18.75, 100, 256.25 and 525 μm respectively. (e – h) are the amide I and II anisotropy profiles of the parallel sections at the depths of 18, 102, 258, and 528 μm respectively. The lines on these profiles are the fittings based on Eq. (1). Although each anisotropy profile on this figure has a different vertical scale, all profiles for each type of tissue sections have the same difference in the infrared absorption values: 0.16 for all perpendicular sections and 0.06 for all parallel sections.

Figure 4.

(a – d) are the amide III and sugar anisotropy profiles of the perpendicular sections at the tissue depths of 18.75, 100, 256.25 and 525 μm respectively. (e – h) are the amide III and sugar anisotropy profiles of the parallel sections at the depths of 18, 102, 258, and 528 μm respectively. The lines on these profiles are the fittings based on Eq. (1). Although each anisotropy profile on this figure has a different vertical scale, all profiles have the same difference in the range infrared absorption value of 0.06.

Figure 5.

(a) – (c) are the depth-dependent fitting parameters in Eq. (1) for each chemical (three amides and sugar) from two types of tissue sections. (Three signature parameters can be extracted from each anisotropy profile as shown in Fig 3 and 4.)

Figure 6.

The interpretations of the Eq. (1) fitting parameters as shown in Fig 5. (a) The infrared rotation schemes for the two types of tissue section, and the schematic orientations of the amide I and amide II bonds with respect to the long axis of a collagen fibril (inside the dashed box). (b) The $\theta_0(r)$ term for both amide I and amide II in perpendicular and parallel sections. (c) The $A(r)$ term for both amide I and amide II in perpendicular and parallel sections. (d-e) The interpretation of these fitting parameters with the aid of a fibrillar structure in the plane of tissue section (the dashed rectangular).