Imaging and modeling collagen architecture from the nano to micro scale

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Abstract: The collagen meshwork plays a central role in the functioning of a range of tissues including cartilage, tendon, arteries, skin, bone and ligament. Because of its importance in function, it is of considerable interest for studying development, disease and regeneration processes. Here, we have used second harmonic generation (SHG) to image human tissues on the hundreds of micron scale, and developed a numerical model to quantitatively interpret the images in terms of the underlying collagen structure on the tens to hundreds of nanometer scale. Focusing on osteoarthritic changes in cartilage, we have demonstrated that this combination of polarized SHG imaging and numerical modeling can estimate fibril diameter, filling fraction, orientation and bundling. This extends SHG microscopy from a qualitative to quantitative imaging technique, providing a label-free and non-destructive platform for characterizing the extracellular matrix that can expand our understanding of the structural mechanisms in disease.

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1. Introduction

Collagenous tissues such as cartilage, bone, tendon and arteries perform an impressive mechanical role. Articular cartilage, for example, processes loads of many times body weight while maintaining a tough, extremely low friction bearing surface that exceeds the best efforts of engineers and materials scientists to reproduce. These properties result from a highly structured meshwork of collagen fibrils entrapping a matrix of negatively charged proteoglycans that swell against it [1]. The structure is maintained by a relatively sparse array of cells that balance the degenerative (mechanical and chemical) and repair processes. Osteoarthritis and related joint disease involves the loss of this balance and an associated degradation and dysfunction of the tissue’s structure. Eventually, this leads to the pain and disability associated with end-stage disease [2, 3]. As one of the major structural components and determinants of function in the cartilage matrix, collagen also plays a central role in the disease process [4, 5]. Collagen meshwork disruption is rarely repaired, generally progressing to form lesions [6], and is therefore of considerable importance in understanding and diagnosing both disease initiation and progression.

A recent mechano-structural model of the cartilage surface [7] has shown that the onset of collagen fibril bundling is the mechanically irreversible stage of the degradation process. Due to its sensitivity to the spatial arrangement of noncentrosymmetric/piezoelectric structures such as collagen [8–11], second harmonic generation (SHG) microscopy should be an ideal...
imaging technique for characterizing such early-stage changes. SHG microscopy is emerging as a powerful imaging modality [12], and has been applied to image collagen in cartilage [13], tendon [10, 14], bone [12], arteries [15], tumors [16], and tissue culture [17]. There remains, however, a significant gap between the ability to capture high quality images [12], and to quantitatively link these SHG images with the collagen structures that produce them. Here, we bridge this gap by combining SHG with a numerical model to relate the images (hundreds of micron scale) to the underlying collagen structure (tens to hundreds of nanometer scale), and use this to describe structural changes in early disease.

2 Methods

2.1 Sample preparation

8 normal and 8 osteoarthritic samples (1 per knee) were used in this study. Normal samples were obtained from the tibial plateaus of patients undergoing above knee amputation. Osteoarthritic samples were obtained from patients undergoing medial unicompartmental knee replacement surgery for primary antero-medial osteoarthritis (AMOA) at a specialist orthopaedic centre. AMOA is characterized by a highly repeatable pattern of disease progression, from advanced degradation anteriorly to histologically and macroscopically normal tissue posteriorly, and provides a full spectrum from early to late stage disease [18]. Patients underwent a standard unicompartmental knee replacement in which the medial tibial plateau was excised whole. Following surgery, the specimens were collected and taken fresh to the pathology laboratory for preparation of wax embedded histological slides.

Each tibial plateau was consistently sectioned along an oblique line through the centre of full thickness cartilage loss and the most posterior point of the transition from fibrillated to visibly normal tissue in the osteoarthritic samples. A similar line was chosen for the normal samples. The sections underwent fixation in 10% formalin for 12 h, and were then decalcified for 24 h in nitric acid before wax embedding. For SHG microscopy, 10 μm sections were cut using a Leica RM2135 microtome (Leica Microsystems, Germany) and heat adhered onto glass slides with a number 1 coverslip. Sections were cut in the same orientation in each joint to allow comparison. For atomic force microscopy, sections were mounted on silicon wafers and treated with trypsin to remove non-collagenous proteins and enable imaging of the collagen structure. Regions from the deep zones of normal tissue, and the histologically normal (Mankin [19] grade <4) tissue from osteoarthritic samples were taken for imaging. The osteoarthritic samples described herein were therefore at a very early state of degradation.

Due to the thin nature of the samples, very little backscattering of forward SHG signal would be expected from within the sample when measuring in the backward direction. We would, however, expect some Fresnel reflection from the glass slide behind the sample. To limit this effect, a drop of water was placed behind (on top) the slide to create a curved and more distant reflective surface.

2.2 Experimental setup

A titanium:sapphire oscillator (Tsunami, Spectra Physics, Santa Clara, USA) laser source was used to generate \( \approx 150 \text{ fs} \) pulses with a 80 MHz repetition rate centered at 810 nm. The average power of the laser was reduced using a half-wave plate and a Glan Thompson polarizer. Images were acquired with a laser scanning microscope (Till Photonics GmbH, Munich, Germany) based on galvanometer mirrors. The focusing objective was an Olympus UPlanSApo 20X air immersion microscope objective with a numerical aperture of 0.75. The height of the objective relative to the sample was controlled using a mechanical and piezoelectric motor for coarse and fine adjustments respectively. To ensure that the back aperture of the microscope objective was filled to ensure the highest spatial resolution, the beam size was increased using a telescope.

Each region was imaged at polarizations from 0 to 180° in 10° steps. Polarization of the pump beam was controlled by a half-wave plate, placed in front of the entrance to the
microscope. A Berek compensator was placed after the half-wave plate to compensate the ellipticity induced by the dichroic mirror inside the microscope.

The SHG light in the forward direction was collected using an objective with a numerical aperture of 0.8 (40X, Olympus, Japan). In the backward direction, the SHG light was reflected by a long pass dichroic mirror (FF735-Di01-25x36, Semrock, Rochester) towards the photomultiplier tube (PMT) and filtered with a narrow bandpass filter (405 ± 10 nm, SEMRock, Rochester, NY, USA) and a 380 to 700 nm bandpass filter (SEMRock, Rochester, NY, USA), ensuring that only the wavelength of interest (SHG signal) was detected. In the forward direction, a 380 to 700 nm bandpass filter (SEMRock, Rochester, NY, USA) cut the pump beam and a narrow bandpass filter (405 ± 10 nm, SEMRock, Rochester, NY, USA) was used to transmit the wavelength of interest to the PMT. Forward and backward collection efficiencies were determined using fluorescent beads, and signals normalized to allow calculation of the forward:backward SHG ratio.

Atomic force microscopy (AFM) was performed in closed-loop operation with a SmartSPM 1000 (AIST-NT Co., Moscow, Russia) in tapping mode using a stiff (~5 N/m) cantilever operating at its first harmonic at 180 kHz (cantilever: MikroMasch NSC-12/C-130). The tip had a nominal radius of approximately 10 nm before scanning. The square scan range was 70 µm by 70 µm with 2048 by 2048 data points. The amplitude was set to 100 nm (peak to peak).

2.3 Simulation

Building on existing models [10, 20, 21] and experimental data [22, 23] from collagenous tissues, we developed a numerical model to provide a quantitative insight into collagen structure and its role in the disease process. To simulate the dependence of forward and backward SHG on tissue structure, and thus provide a quantitative interpretation of the images, we have represented the tissue as a collection of long cylindrical fibrils (Fig. 1(a)). Fibrils were aligned along the laser polarization (perpendicular to the plane in Fig. 1(a), 1(b)). Both forward and backward SHG is maximum at this alignment. Forward and backward SHG was solved for the out of plane ‘tilt’ of fibrils, which was found to produce a small increase in forward signal and a rapid decrease in backward signal. We did not observe any regions of normal forward and very low backward SHG in our images that would indicate such tilt. Our simulation therefore assumes that out of plane tilt is similar in the normal and diseased tissues.

Each cylinder had the same diameter $D_f$ and a scalar second-order nonlinear susceptibility $\chi^{(2)}$, constant throughout the cylinders [24]. The absolute value of $|\chi^{(2)}|$ was the same in all the cylinders, with a randomly assigned sign (red/grey and blue/black fibrils in Fig. 1(a), 1(b)). To model normal tissue, the cylinder centers were distributed randomly in the plane, with a space filling ratio $\rho$. Tissue degradation was modeled by bundling a number of fibrils, $N_b$, with the same orientation (the same sign of $\chi^{(2)}$), as indicated in Fig. 1(b). The simulated SHG signal was then composed of coherent contributions from the individual fibrils. As the SHG signal from the tissue depends on the local tissue composition and thus changes from sample to sample, $10^5$ structures were randomly generated for each value of $D_f$, $\rho$ and $N_b$, and the average signal calculated. For a given $|\chi^{(2)}|$, the average forward and backward SHG signals in a normal model tissue are completely described by $D_f$ and $\rho$. In the diseased model tissue, the signals also depend on $N_b$, the number of bundled fibrils.
To compute the SHG signal from the tissue model described above we use a vectorial approach similar to that described in Novotny [25]. In short, the electric field of the signal generated by the tissue is expressed by the Hertz vector $\vec{Z}$ [26]:

$$\vec{E} = 4k_0^2\vec{Z} + \nabla (\vec{V} \cdot \vec{Z}),$$

where $\vec{E}$ is the electric field, $k_0 = \omega_0/c$ $\omega_0$ is the laser wavelength and $c$ is the speed of light. The Hertz vector satisfies Helmholtz equation

$$\nabla^2 \vec{Z} + k^2\vec{Z} = -\frac{4\pi}{n_0} \vec{P}_{NL},$$

(1)

where $\vec{P}_{NL}$ is the nonlinear polarization vector and $n_0$ is the refraction index, assumed constant $n_0 = 1.4$ throughout the sample. Equation (1) is a set of scalar equations, each of which is solved in the far-field zone by the standard scalar Green’s functions [26].

To calculate the source term in Eq. (1), we assumed a continuous wave laser beam travelling along the $\hat{x}$ axis, with a pulse of spatial Gaussian shape polarized along the $\hat{y}$ axis (fibrils were aligned along the $\hat{y}$ axis, see Fig. 1). The nonlinear susceptibility tensor was approximated by a scalar for simplicity, and the fibril orientation distribution was therefore not modeled as it has no effect on the forward:backward SHG ratio. Under these assumptions, the second-order nonlinear polarization vector in the sample simplified to

$$\vec{P}_{NL}(\vec{r}, 2\omega_0) = \hat{\chi}^{(2)}(\vec{r}) E_0^2(\vec{r}) \left( e^{2i\omega_0 \delta(\omega - 2\omega_0)} + e^{2i\omega_0 \delta(\omega + 2\omega_0)} \right)$$

(2)

where $\delta$ is the Dirac’s delta-function and $E_0(\vec{r})$ is the complex amplitude of the Gaussian beam. The $\chi^{(2)}(\vec{r})$ in Eq. (2) is a position dependent function that is nonzero in the fibrils as described above and zero outside of them.
It should be noted that the forward:backward SHG ratio described and measured here does not translate to in-vivo measurements on thick tissue, in which backward SHG is combined with backscattered forward SHG. This experimental and theoretical setup is aimed at in-vitro application. As forward SHG dominates in thick tissue samples [7], however, our calculations based on forward SHG provides information on the collagen meshwork structures that can be probed in vivo.

3. Results and discussion

Focusing on structural changes in the osteoarthritic process, we examined cartilage from normal and osteoarthritic human tibial plateaus using SHG microscopy. To quantitatively link the microscale SHG images to the nanoscale collagen structure, we developed a numerical model of SHG in tissue. The model represents the tissue as a set of fibrils possessing second-order optical nonlinearity, $\chi^{(2)}$, and calculates their coherent contributions to the SHG signal. Using a vectorial Green’s function approach [25], these contributions were calculated in terms of fibrils’ diameter, and spatial arrangement such as filling fraction, bundling and orientation/polarization (see Fig. 2). The simulated forward and backward signals from the normal tissue were both found to depend linearly on the collagen filling fraction, allowing us to decouple the two governing parameter dependencies of the signal to predict fibril diameter.

In normal cartilage (Fig. 3(a), 3(b)), the mixed alignment and orientation of collagen at the submicron scale (through entanglement and cross-linking) creates the speckle pattern observed in the forward SHG image. By measuring the SHG signal as a function of laser polarization, an overall radial (bone to surface direction) alignment was observed in the mid to deep zones from which these images were taken (Fig. 4). This is consistent with the arcade architecture of the collagen in the tissue [27]. The forward:backward SHG signal ratio in normal samples of cartilage was $3.4 \pm 0.5$ (Fig. 4(a)). Based on this ratio, the numerical model predicts fibril diameters in the range of 90 to 110 nm, which is consistent with atomic force microscopy (AFM) and the literature [28]. In tissue with early-stage disease (Fig. 3(c), 3(d)), we found a number of regions of high magnitude (238% increase, $p = 0.005$ versus normal tissue), highly polarized forward SHG signal in the territorial and inter-territorial matrix. This was accompanied by a small decrease in backward signal (23%, $p = 0.026$), and thus a high forward:backward ratio, considerably higher than could be simulated with realistic parameters in a normal matrix configuration. The matrix adjacent to these regions appeared normal, yet showed slightly increased forward signal with respect to samples from normal joints (28%, $p = 0.026$), with no change in the backward signal magnitude.
Fig. 2. Simulated average forward-to-backward ratio of SHG signal from a random distribution representing healthy tissue (a) and bundled fibrils of the same polarization representing diseased tissue (b). Bundles of mixed polarization had no effect on the ratio (not shown). Dependence of forwards signal (arbitrary units) on filling fraction for normal tissue (c). Dependence of backwards signal (arbitrary units) on filling fraction for normal tissue (d).

Fig. 3. Representative forward and backward SHG images from normal ((a) and (b) respectively) and osteoarthritic ((c) and (d) respectively) cartilage with the pump beam polarized in the direction from the bone to the cartilage surface. Scale bar 50 μm. Image intensity is scaled for presentation.
Knowing that the degradation process is associated with bundling of fibrils [29], we simulated SHG signals from a bundle at the centre of the focal volume (Fig. 1(b)). Bundling fibrils with the same sign, or ‘direction’, of $\chi^{(2)}$ resulted in a rapid increase of the forward:backward SHG signal ratio (Fig. 2), mainly due to the increase of forward signal. The bundling of fibrils with mixed $\chi^{(2)}$ signs did not affect the forward:backward SHG signal ratio. This high sensitivity to fibril bundling can be understood by considering phase matching in the sample. The phase matching condition in the forward direction is automatically satisfied in the small focal volume of the tightly focused laser as $\Delta k \times L = 0$, where $L$ is the length of interaction, and $\Delta k$ is the wave vector mismatch. The forward SHG signal therefore increases upon bundling of fibrils sharing the same $\chi^{(2)}$. In the backward direction, the coherence length $L_c = \pi / \Delta k$, is on the order of 70 nm as $\Delta k = 8\pi n / \lambda$ where $n$ is the refractive index ($\approx 1.4$), and $\lambda$ is the laser wavelength (810 nm). Because the bundle diameter is larger than the backward coherence length, the signal in this direction is not strongly affected by bundling [30].

![Fig. 4. (a) Ratio of forward to backward signal from experiments in normal and diseased joints, with R1 and R2 corresponding to bright and non-bright regions respectively. (b) Representative polarization dependence of forward (black) and backward (red) signal from normal and non-bright regions of diseased samples. (c) Representative polarization dependence of forward and backward signal from bright regions in diseased samples. 0 degrees is the direction from the bone to the cartilage surface.](image)

Polarization plots (Fig. 4(b), 4(c)) indicate that bundles form on a background of normal, pseudorandom collagen meshwork structure. Backward SHG polarization remained consistent between all samples, with changes in forward SHG polarization occurring only in the small regions containing bundles. In the case of healthy tissue, or in the ‘normal’ regions of diseased tissue, there are no bundles, and the polarization dependence of the forward SHG
is the same as for the backward SHG. This is equivalent to the case of one fibril located in the focal volume. The forward:backward SHG signal ratio for a single fibril is independent of the laser polarization, as for a matrix where there is no bundle of fibrils. In the case of the regions of diseased tissue where we observed an increase of the forward:backward SHG signal ratio, we also observed that the forward signal dependence on laser polarization was more anisotropic, and that the polarization angle at which the forward signal was maximum can be different than in the backward direction. No consistent change in this polarization angle was observed. As only forward SHG is sensitive to bundling, the forward polarization dependence shows the direction of the bundles, and backwards polarization dependence shows the average orientation in the focal volume.

As the forward:backward SHG ratio is dominated by a small number of bundled fibrils within an otherwise normal matrix configuration, we argue that it is more sensitive to the collagen meshwork changes in early osteoarthritis than comparable measurements such as birefringence, x-ray scattering or spectral methods, which average the level of order in the focal volume. A comparison of SHG with common techniques is provided in Chen et al. [12]. Our interpretation of the SHG signals in normal and early-stage osteoarthritic cartilage is confirmed by AFM. Normal tissue showed a pseudorandom arrangement of collagen on the micron to tens of micron scales. AFM of diseased tissue showed small regions of highly aligned and bundled structures, particularly in the territorial matrix (see Fig. 5), within a qualitatively normal matrix structure. Regions with characteristic 'normal' and 'diseased' structures are displayed on the images.

![Fig. 5. Representative AFM height images from (a) early-stage osteoarthritic tissues, and (b) normal tissues. Osteoarthritic tissues showed regions of characteristically normal (c', random alignment) and degraded (d', kinked bundles and increased alignment) collagen meshwork structures. Zoomed-in images of normal (c) and diseased (d) regions are shown.](image)

The structural implications of the SHG-bright regions are important for disease progression. The cohesion and crosslinking in normal tissue resists the formation of bundles and cracks from the low-level damage that may result from mechanical impact or enzymatic cleavage associated with matrix turnover. In an enzymatically or mechanically weakened
matrix, however, the stress concentrations at the edge of the discontinuity will be sufficient to propagate damage, forming extended regions of fibrillar bundling as observed in the bright regions of Fig. 3. To be recognized clearly as a ‘bright’ region in the forward SHG images, a cross-sectional area in the order of 30 (for d≈120 nm) to 50 (for d≈100 nm) collagen fibrils must be disrupted and oriented according to the numerical simulations (see Fig. 2). Based on previous mechano-structural simulations [7], this level of bundling is associated with the early, mechanically irreversible degradation in the cartilage surface. The role of localized bundles in the propagation of damage can be observed in Fig. 6, which shows the transition from relatively healthy (left) to diseased (right) tissue in the deep zone of a representative sample. Here, the ‘bright’ forward SHG signals indicating localized regions of bundling and alignment become increasingly widespread from left to right in the image. This suggests both a higher number of bundling events and the accumulation of bundles to form large regions of aligned and damaged tissue.

Fig. 6. Representative image (2 x 4 mm) indicating damage progression in the deep zone of a diseased sample. Forward SHG, polarized in the direction from the bone to the cartilage surface (see arrow), shows the progression of alignment and bundling from left to right in the early stages of osteoarthritic degradation. It should be noted that forward SHG provides qualitative information showing disease progression, yet does not provide a quantitative description of collagen organization.

The requirement for bundles of the same sign of $\chi^{(2)}$, and therefore piezoelectric tensor, to produce high signals is also intriguing. Due to the $d_{15}$ component of the piezoelectric tensor in collagen [22], applied shear, bending or ‘uncrimping’ will cause a potential difference across the fibril diameter and therefore an accumulation of charge on the surface. Adjacent fibrils of the same sign of $\chi^{(2)}$ will accumulate opposite charges at their interface and attract to form rope-like fibers. Fibrils with opposite $\chi^{(2)}$ will accumulate like charges and repel. This leads us to speculate that fibril surface charge may play a role in the early degradation process by encouraging bundling in a weakened matrix and therefore contributing to the cascade of degradation.

4. Conclusions

Our study advances the application of SHG towards quantitative, label-free imaging of collagen architecture from the high tens of nanometer to hundreds of micrometer scales. We have shown that fibril diameter, filling fraction and bundling can be quantified through a numerical model. Combined polarized imaging and modeling can be used to detect and describe early degradation processes in collagenous tissue, and has provided an insight into structural mechanisms, particularly fibril bundling, driving osteoarthritis, further suggesting a piezoelectric role in these structural changes. Moreover, it has strong potential for quantifying collagen structure in a range of other applications, such as characterizing the interactions...
between cells and the extracellular matrix, remodeling processes, wound healing and regenerative medicine.

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