

Second harmonic generation imaging of collagen fibrils in cornea and sclera

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Abstract: Collagen, as the most abundant protein in the human body, determines the unique physiological and optical properties of the connective tissues including cornea and sclera. The ultrastructure of collagen, which conventionally can only be resolved by electron microscopy, now can be probed by optical second harmonic generation (SHG) imaging. SHG imaging revealed that corneal collagen fibrils are regularly packed as a polycrystalline lattice, accounting for the transparency of cornea. In contrast, scleral fibrils possess inhomogeneous, tubelike structures with thin hard shells, maintaining the high stiffness and elasticity of the sclera.

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1. Introduction

Collagen is the major component of connective tissues including tendon, skin, cornea and sclera. Due to its transparency to visual and near infrared light, the human eye is ideal for laser-based diagnostic and imaging applications. Therefore, the present study is focused primarily at the assembly of collagen fibrils in cornea and sclera. Why collagen predominated tissues like transparent cornea or opaque sclera are so different was an intriguing question. Figuring out the answer depends on how good we know the ultrastructure of individual collagen fibrils down to *nm* scale and the organization of the collagen fibril network up to μm scale. Due to the tiny size of the collagen fibril [1] (approx 30 *nm* in diameter in cornea, and 30 - 300 *nm* in sclera, respectively) and the extremely low contrast of the transparent tissue (efficient staining of the thick and dense corneal or scleral tissue is challenging), previous investigations mainly relied on complicated and invasive electron microscopy or x-ray diffraction[1, 2]. The invention of nonlinear laser microscopy[3] opened new opportunities to noninvasively examine the structure and function of living cells or tissues. Among different multiphoton implementations[4, 5], SHG imaging [6, 7, 8, 9, 10, 11] is particularly suitable to investigate non-centrosymmetric structures like collagen fibrils. The basic structure of collagen fibril is a triple helix composed of three protein chains, which gives collagen the intrinsic ability of SHG. Similar to two photon excited fluorescence (TPEF), SHG is produced in only a small focal volume, permitting high resolution 3D optical sectioning of thick tissues. In contrast to TPEF, SHG from collagen is an intrinsic and a coherence process. Intrinsic imaging avoids the complications of slicing and labelling, and samples can be investigated under physiological conditions. Coherent constructive or destructive interference of SHG provides extra hints to the ultrastructure of collagen fibrils and their organizations.

2. Material and method

All the experiments described in this letter were based on porcine eyes. The porcine cornea and sclera were obtained from the local slaughter house, and were excised and fixed with paraformaldehyde(4% in PBS buffer, pH 7.4). The typical thickness of the porcine sclera is more than 2 mm, which is beyond the penetration depth of the second harmonic signals. Therefore, a thin slice of sclera with a thickness less than 500 μm was manually prepared using a surgical scalpel. To avoid dehydration or shrinkage, the cornea/sclera samples were kept in a home-made glass bottom dish filled with PBS solution (pH 7.4) during the whole imaging process.

In this study, SHG imaging was performed on a upright (Zeiss LSM 510 NLO) laser scanning multi-photon microscope (Zeiss, Jena, Germany) equipped with a mode-locked femtosecond Ti:Sapphire laser (Coherent Chameleon XR, Coherent Inc, Santa Clara, USA)[12, 13], tunable from 720 to 980 nm. ($\lambda = 800$ nm). In this study, the Ti:Sapphire laser emission wavelength was set to 800 nm. Laser intensity attenuation was implemented using an Acoustic Optic Modulator (AOM, Zeiss). Either a 40 \times (N.A.= 0.8) or 63 \times (N.A.= 1.0)water immersion objective (Zeiss) was employed for focusing the excitation beam and for collecting of the backward SHG signals. The backward SHG signals were collected via the same objective and were directed by a dichroic mirror to the photomultiplier tube detector (PMT2). A Zeiss 1.4 N.A. oil im-

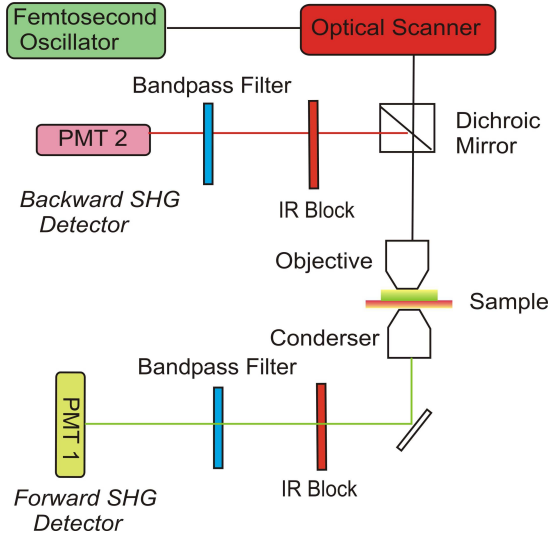


Fig. 1. Schematic drawing of the SHG imaging experimental setup

mersion condenser was employed to collect the forward (transmission) SHG signal through PMT1. Two IR beam block filters (Zeiss KP685) and a narrow bandpass filter (400/10 nm) in the forward/backward light path ensured that illumination light was filtered out and only second harmonic signals from the corneal/scleral tissue were recorded (Fig. 1). The acquisition of a single 512×512 pixel image was generally achieved within a few seconds (fast laser scan with galvanometer scanners). All the images presented in this article are single optical sections. Green fluorescent beads were used to calibrate the PMT detectors in forward and backward channels. The amplification rate of the PMT detectors was set to keep the isotropic fluorescence signals equal in both detecting directions. The optical setup and the configurations of PMT are identical for both cornea and sclera imaging. Since the collecting efficiency of backward SHG signals is highly dependent on the N.A. of the objective. N.A. 0.8 objective is used only for transmission imaging due to its poor backward light collecting efficiency. All the comparisons between forward and backward SHG imaging of cornea and sclera are conducted by the N.A. 1.0 objective.

3. Results and discussion

As shown in Fig. 2, collagen fibrils in both cornea and sclera demonstrate remarkable regularity. Most collagen fibrils run parallel to the cornea/sclera surface and share similar orientations with their neighbors. However, no significant differences between the collagen fibrils in cornea and sclera were observed, mainly due to the fact that the size and the interfibrillar spacing of the collagen fibrils are far below the diffraction limit.

One of the unique features of SHG is that the geometry of the SHG emission field reflects the size and shape of the collagen fibrils. As indicated by recent theoretical and experimental works[14, 15, 16, 17], SHG emission from the collagen fibril, unlike fluorescence signal, is highly asymmetric due to the phase matching condition:

$$|\Delta\vec{k}| \cdot l - 2\Delta\phi_g \ll \pi \quad (1)$$

where $\Delta\vec{k} = \vec{k}_{2\omega} - 2\vec{k}_\omega$ denotes the wave-vector mismatch between the fundamental (ω) and the second harmonic (2ω) light and l describes the interaction length or the axial size of the collagen fibril in the laser focal volume. Under tight focusing condition, $\Delta\phi_g$ corresponds to the phase mismatch due to the Guoy phase shift of the focused excited beam along l [14, 16].

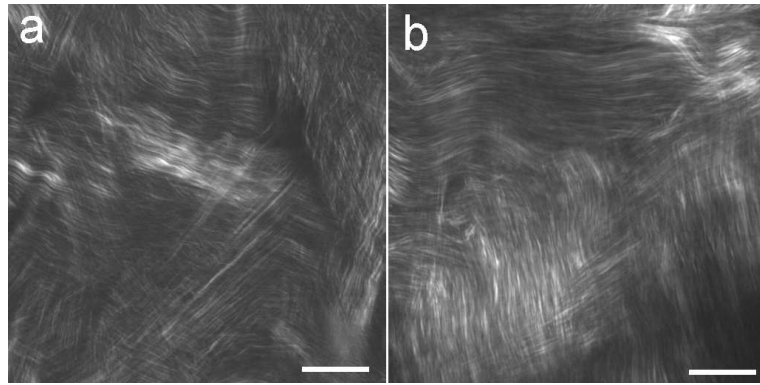


Fig. 2. SHG imaging of collagen fibrils in (a)cornea and (b)sclera. The femtosecond Ti:Sapphire laser was focused by a 40× (N.A. 0.8) water immersion objective. SHG signals were collected in the forward direction for both cornea and sclera. The image plan is parallel to the cornea/sclera surface. Bars: 20 μm

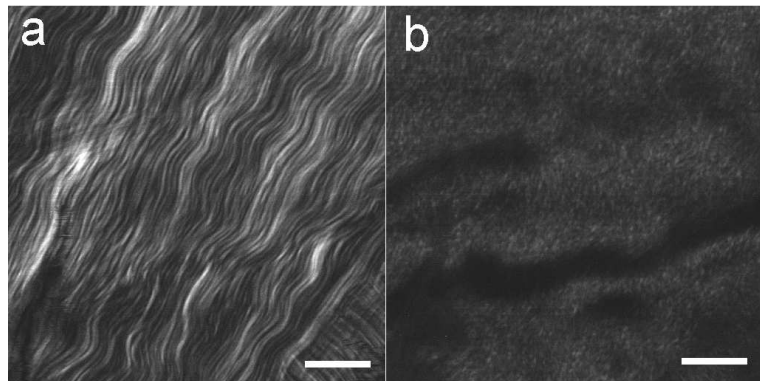


Fig. 3. SHG imaging of corneal collagen fibrils in (a)forward and (b)backward directions. The fibrillar structures resolved in (a) correspond to collagen bundles which are composed of regularly packed collagen fibrils. Objective: 63×/1.0W, Bars: 10 μm

The SHG radiation pattern is mainly determined by the phase matching condition. Previous nice theoretical and experimental works have revealed that objects with the axial size on the order of the second harmonic wavelength exhibits forward directed SHG, while objects with a axial size less than $\lambda/10$ (approx. 40 nm) are estimated to produce nearly equal backward and forward SHG signals[14, 15, 17].

Electron microscopy revealed that the diameter of most corneal collagen fibrils is around 30 nm, thus backward SHG from cornea should be significant. However, as demonstrated in Fig. 3, the backward SHG from cornea was extremely weak and there was no correlation between the structures revealed by forward and backward SHG imaging. The predominant forward SHG implies that the corneal collagen fibrils are not randomly distributed. It's worth mentioning that the fibrillar structures revealed in Fig. 3 actually correspond to collagen bundles composed of many corneal collagen fibrils. In cornea, the collage fibrils ($n = 1.47$) and the extrafibrillar matrix ($n = 1.35$) have different refractive indices, thus the collagen fibrils have to be treated as individual scatters. Since the dimension of the collagen bundle (approx. 0.5 μm) is close to the second harmonic wavelength (400 nm), the phase correlation of SHG from neighboring collagen fibrils should be taken into account. The electric field of the second harmonics $E_{2\omega}$

from the ensemble of the collagen fibrils can be approximated by

$$\sum_{j=1}^n E_{2\omega}^0 e^{i\Delta\vec{k}\cdot\vec{r}_j} \quad (2)$$

where $E_{2\omega}^0$ denotes the second harmonic radiation from the individual collagen fibrils at the location of \vec{r}_j . Since $E_{2\omega}^0$ is evenly distributed in both forward and backward directions, a randomly distribution of \vec{r}_j leads to noticeable backward propagating second harmonics. However, if the collagen fibrils are regularly packed as a lattice, destructive interferences will occur in all directions except for the transmission direction, where $\Delta\vec{k}$ is close to zero. The forward predominant SHG radiation from cornea indicates the regular arrangement of the collagen fibrils, at least in the domain of the collagen bundles. Based on the directionality of the SHG radiation field, we are able to disclose the polycrystalline like structure of cornea with optical method, which is consistent with the previous electron microscopic findings and the theoretical modelling of cornea transparency[18, 19, 20].

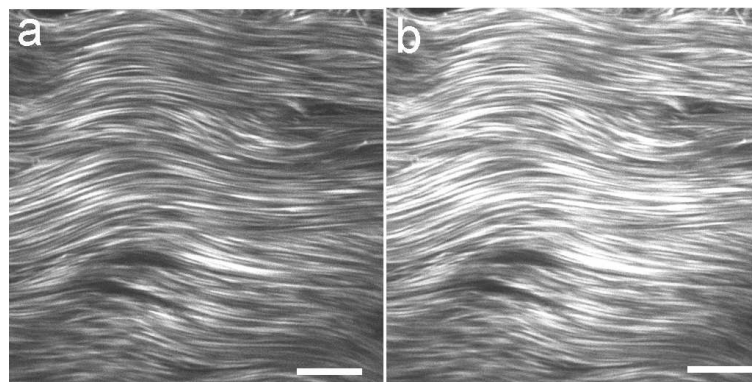


Fig. 4. SHG imaging of scleral collagen fibrils in (a)forward and (b)backward directions. In contrast to cornea, the backward SHG signals from sclera are significant. Identical structures are revealed by forward and backward SHG imaging. Objective: 63×/1.0W, Bars: 10 μm

Since most collagen fibrils in sclera are one magnitude bigger than those in cornea, weaker backward SHG signal from sclera is expected if assuming that scleral collagen fibrils resemble cylindrical rods. However, the striking fact demonstrated in Fig. 4 is that the intensity of the backward SHG from sclera is even comparable to the forward SHG. Since sclera is opaque, the backward SHG signals may be attributed to the backscattering of the forward SHG signals from the highly scattering scleral tissue below the focal volume. Therefore, single scleral collagen fibril immersed in the PBS buffer is imaged. Again, significant backward SHG was observed (Fig. 5). It is confirmed that the backward SHG signals are directly emitted from the scleral collagen fibril, which obviously contradicts the theoretical predication. However, as proposed by the previous investigation of tendon collagen fibrils [17], the scleral collagen fibrils seems possess inhomogeneous tubelike structures. The collagen microfibrils might be randomly arranged in the core but are well aligned in the shell (thickness < 40 nm) of the scleral fibril, generating evenly distributed forward/backward SHG. Although by optical microscopy, the shell of the scleral collagen fibril can not be directly visualized, the inhomogeneity of the scleral collagen fibril can be probed from other aspects, for example, from the mechanical characteristics of the scleral collagen fibrils. Fig. 5 illustrates the reorganization of the single scleral collagen fibril in a scleral slice which was manually dissociated from the scleral substrate with a fine surgical scalpel. The stress applied to the scleral sheet during dissection leads to enlarged interfibrillar spacing, the individual collagen fibrils now can be distinguished. As pointed out by the solid

triangles in Fig. 5, sharp bends of the collagen fibrils are commonly observed at various locations, which are the typical characteristics of tubelike, but not of rodlike structures[21]. The collagen fibril manipulation experiment provided additional evidence supporting the hypothesis of inhomogeneous, tubelike structure of scleral collagen fibrils. The shell of the sclera collagen fibrils appears thinner (tens of nm), but harder than its inner content. Most likely, the tubelike collagen fibrils, which the sclera is composed of, are beneficial to sustain the high elastic modulus of sclera, which are crucial to stabilize the curvature of cornea and to protect the eye from blunt injury.

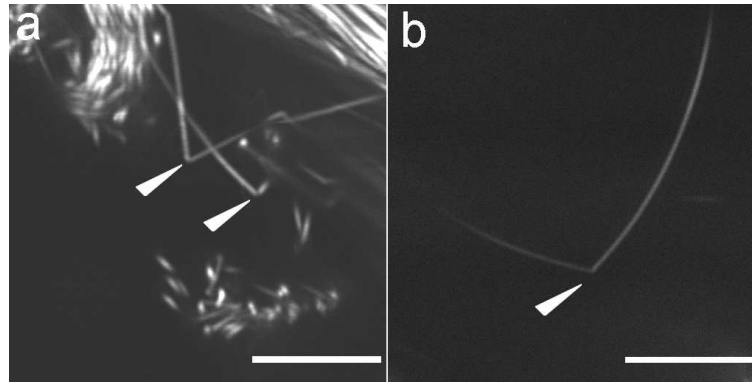


Fig. 5. SHG imaging of single scleral collagen fibril in the scleral slice which was manually dissociated from the scleral substrate. (a) and (b) were recorded at different locations. The sharp bends of the collagen fibrils are commonly observed and are indicated by the solid triangles. Objective: 63 \times /1.0W, Bars: 10 μ m

Although in term of resolution, SHG imaging can not compete with electron microscopy, the structure of cornea can be investigated under the physiological conditions. Without labelling, SHG imaging of collagen offers strong contrast and large sensing depth, which make it particularly suitable for living connective tissue studies. As a coherence excitation process, the directionality of SHG emission supplies further information about the axial dimensions and the organization of the collagen fibrils. Our experimental results suggest that the corneal collagen fibrils are regularly packed within the domains of collagen bundles, similar to a polycrystal. Constructive interference is generated mainly in the transmission direction. From the aspect of optics, enhanced forward scattering in respect to backward scattering supports the transparency of cornea. In contrast to cornea, sclera must be tough enough to stabilize the shape of cornea and to protect the eye from injury. Tubelike scleral collagen fibrils characterized by a hard shell seem more suitable in maintaining the high rupture strength of sclera. The collagen fibrils in different tissues are optimally arranged to support the tissue's distinct functions. As a rather simple and straightforward method, SHG imaging is invaluable for studying the physiology and morphology of the assembly of collagen fibrils. The generation of the individual collagen fibrils from the cultured keratocytes and the regulation and alignment mechanisms of collagen fibrils that originated from different keratocytes can be precisely followed. With the advantages of large sensing depth and high spatial resolution, SHG imaging is also useful for *in vivo* testing and evaluating of the synthetic collagen equivalents, which are among the most important bio-engineering materials. Besides the fundamental study of collagen fibrils or collagen equivalent, SHG imaging may be clinically applied for early diagnosing of corneal related diseases and accurate monitoring the tissue healing process after refractive surgery. Although forward SHG imaging seems to be unpractical for diagnostic applications, backward SHG imaging may be a sensitive method for clinical study of cornea haze or cloudiness, where the regularity of the collagen fibrils is disturbed.

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