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Title

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Journal

Applied optics, 46(10)

ISSN

1559-128X

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Publication Date

2007-04-01

DOI

10.1364/ao.46.001770

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Peer reviewed

High-resolution frequency-domain second-harmonic optical coherence tomography

Jianping Su, Ivan V. Tomov, Yi Jiang, and Zhongping Chen

We used continuum generated in an 8.5 cm long fiber by a femtosecond Yb fiber laser to improve threefold the axial resolution of frequency domain second-harmonic optical coherence tomography (SH-OCT) to 12 μm . The acquisition time was shortened by more than 2 orders of magnitude compared to the time-domain SH-OCT. The system was applied to image biological tissue of fish scales, pig leg tendon, and rabbit eye sclera. Highly organized collagen fibrils can be visualized in the recorded images. Polarization dependence on the SH has been used to obtain polarization resolved images. © 2007 Optical Society of America

OCIS codes: 170.4500, 170.3880, 190.4160.

1. Introduction

Optical coherence tomography (OCT) is a recently developed imaging modality based on coherence-domain optical technology. OCT takes advantage of the short coherence length of broadband light sources to perform micrometer-scale, cross-sectional imaging of biological tissues. OCT can provide imaging resolutions that approach those of conventional histopathology and can be performed *in situ* and *in vivo*. Despite its advantages, a serious limitation of OCT is the relatively low imaging contrast compared to histopathology and optical microscopy. The imaging contrast in OCT originates from the inhomogeneities of sample scattering properties. However, the linear scattering properties of pathological tissue are often optically similar to the scattering properties of normal tissue. For example, many cancers originate in the epithelium that has a thickness suitable for OCT imaging, but in their early stages when these cancers are developing through cell dysplasia, changes in the tissue morphology and the refractive index between normal and diseased tissues are very small and difficult to detect. Therefore to meet the challenges found in OCT clinical applications, imaging contrast enhancement is as important as the imaging resolu-

tion improvement. Recently, second-harmonic OCT (SH-OCT), which combines the sample structural sensitivity of second-harmonic generation (SHG) with the coherence gating of OCT to obtain molecular contrast, has been reported.^{1–6} The SH is generated only by molecules that are noncentrosymmetric, and hence contrast is a function of the molecular structure of the specimen and its orientation relative to the laser beam. In biological materials, collagen is the most abundant protein and possesses noncentrosymmetric structure.^{7,8} Because collagen is the predominant structural component of most biological tissues, as well as a strong SHG source, it is predicted that structural changes in collagen will result in the change of the second harmonic generation signal. Indeed, the experimental studies have found that the SH responded to the structural modifications of collagen, such as thermal denaturation, nonenzymatic glycation, and partial enzymatic cleavage.⁸ These modifications simulate the possible changes that may take place in several pathophysiologic conditions, including thermal injury, diabetes, aging, abnormal wound healing, and malignant transformation of dysplastic nevi.

SHG is a powerful contrast mechanism in nonlinear optical microscopy.^{9–12} SHG microscopy is known for its capability for high-resolution optical 3D sectioning of samples because signals only arise from the focal point of the objective where sufficient peak power can occur. Because SHG is a coherent process, the scattering beam may be highly forward directed. The directionality of SHG depends on the distribution and orientation of the induced dipoles within the focal volume and has been investigated.^{13,14} However,

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Received 23 June 2006; accepted 15 September 2006; posted 27 September 2006 (Doc. ID 72297); published 13 March 2007.

0003-6935/07/101770-06\$15.00/0

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because transmission images are difficult to acquire in thick samples or *in vivo* studies, SHG microscopy that uses backscattered light has been demonstrated.⁹ In comparison to SHG microscopy, SH-OCT has the advantage of decoupling the axial and transverse scans and is able to obtain two-dimensional tomographic images with a one-dimensional scan.

Recently a time-domain (TD) SH-OCT system that utilizes the nonlinear optical effect of SHG to produce highly contrasting images of biological tissues was reported.^{1–3} This technique combines the sample structural sensitivity of SHG with the coherence gating of OCT. The images of SH-OCT can show collagen fibers that are not obvious in conventional OCT images. However, the TD SH-OCT has limited sensitivity and imaging speed. Frequency-domain (FD) OCT has been attracting much attention because of its potential higher speed and sensitivity. Recently, FD SH-OCT with an axial resolution of the order of 30 μm has been reported.^{4,6} Here we report the development of a FD SH-OCT system with an axial resolution of 12 μm .

2. Methods

The experimental setup is shown in Fig. 1. A self-starting, stretched-pulse Yb fiber laser generating femtosecond pulses centered at 1.03 μm wavelength with a 50 MHz repetition rate is used as the light source.¹⁵ The mode-locked pulses from the fiber oscillator are temporally stretched in a 10 m single mode fiber and amplified in yttrium-doped fibers pumped by two diode lasers. The output pulses are

Fig. 1. (Color online) Schematic of the FD SH-OCT experimental system. FSL, femtosecond fiber laser; HWP, half-wave plate; DM, dichroic mirror; NLC, nonlinear crystal; OBJ, objective; BS, beam splitter; CF, color filter.

Fig. 2. (Color online) Relationship between fiber length (Corning HI 780) and output spectra bandwidth. Without fiber continuum generation, the corresponding SH resolution is 30 μm . A 1 m long fiber generates continuum with corresponding SH resolution of 8 μm ; however, the continuum pulse duration broadens to 4 ps. Experimentally, the pulse duration was measured by an autocorrelator.

compressed in external grating pairs to 170 fs with an average power of 260 mW at 50 MHz repetition rate. The output spectra have an approximate Gaussian shape with a linewidth of 13.5 nm (FWHM), which allowed us to record FD SH-OCT images with 30 μm axial resolution.⁶

We need a light source with a broader spectrum to increase the axial resolution. For our study, we used a continuum generation in the fiber to broaden the spectra. Spectral broadening in a fiber is also associated with pulse broadening. However, pulse broadening is not desirable because the SH efficiency is quadratic dependent on the pump intensity. We studied the temporal and spectral broadening of our laser pulse in several fibers. In Fig. 2, we summarize the results for a Corning HI 780 fiber. For a 1 m long fiber, spectral broadening up to 80 nm was recorded, but at the same time the pulse duration increased to 4 ps. This leads to about 2 orders of magnitude decrease of SH efficiency. We also noted that the output spectra from the fiber was not smooth, but exhibited modulation. In the experiment, we selected an 8.5 cm long fiber, and the output spectra width was about 40 nm at 10% peak intensity level. The pulse duration of the continuum spectra was less than 400 fs. The 100 mW continuum output power from the fiber was incident on a 2:1 beam splitter (BS1). The more powerful beam was used in the sample arm where 20 $\times$ microscope objectives were used to focus the beam on the sample.

The average excitation power at the exit of the objective was about 50 mW, and the typical pulse energy density at the focus was about 3 mJ/cm². This energy density was much less than the tissue damage threshold estimated in the range 0.5–1.0 J/cm².^{8,16} The sample was situated on a motorized stage for lateral movement with submicrometer resolution. In this setup the interferometers for the fundamental and

second harmonic only partially overlapped. Both interferometers used commercially available spectrometers (Avantes), and the length of the reference arms was independently adjustable (Fig. 1). The recombined fundamental beams in BS1 were directed to a spectrometer with a resolution of $\delta\lambda_1 = 0.15$ nm at 1030 nm range (AvaSpec-3648). The spectrometer was equipped with a CCD camera with 3648 pixels (8×200 μm pixel). The integration time was adjustable from 10 μs to 600 s.

The backscattered SH signal from the sample was reflected by a dichroic mirror and directed to beam splitter BS2. In the reference arm, a type I β -barium borate (BBO) crystal 0.1 mm thick was used to generate a reference second harmonic signal without spectral narrowing.¹⁷ A dichroic mirror and a delay line were used before the reference SH signal was combined with the sample SH signal in BS2. A variable neutral density filter was included in the reference arm to vary the reference signal. In both arms of the SH interferometer, half-wave plates (HWP2) were used to control the SH polarization. The BS2 transmitted 80% of the sample signal and reflected 20% of the reference signal. The output from BS2 after passing a color filter that blocks fundamental radiation was coupled to a fiber connected to the input of a spectrometer. The spectrometer was equipped with a 1200 line/mm grating and a 2048 pixels (14×56 μm pixel) thermoelectric cooled CCD camera (AvaSpec-2048TEC). The integration time varied from 2 ms to 60 s. The resolution of the spectrometer for the 515 nm light was $\delta\lambda_2 = 0.15$ nm. In order to decrease the mechanical vibrations and thus use longer integration time, the experimental setup was built on an optical table isolated from floor motion using a pneumatic suspension system.

Compared with other tomographic modalities, one of the advantages of OCT techniques is their high resolution. The axial resolution $l = 0.44\lambda^2/\Delta\lambda$ is determined by the bandwidth of the light source, $\Delta\lambda$. The lateral resolution is determined by the size of the focal spot used in the experiment. In the case of a Gaussian beam, the lateral resolution is defined as $w_0 = 2\lambda/(\pi \times \text{NA})$, where NA is the numerical aperture of the illumination optics. To achieve high lateral resolution, tight focusing is used in an OCT system. However, focusing has some effects on the OCT measuring range. Complementary depth of focus, $\text{DOF} = 2\lambda/(\pi(\text{NA})^2)$, defines a depth over which lateral resolution is considered constant. Thus higher lateral resolution is achieved with optics with high

NA but at the expense of the depth range. Because the SH efficiency has a square dependence on the fundamental intensity, it benefits from focusing. For a given Gaussian fundamental pump intensity, both axial and lateral resolution of SH-OCT are improved compared to fundamental radiation OCT, and they scale as $1/\sqrt{2}$ of the fundamental radiation resolution. For FD OCT, the measuring range, $\Delta z = (\lambda^2/\delta\lambda)/4n$, is determined by the resolution of the spectrometer, $\delta\lambda$, where λ is the wavelength of the radiation, and n is the refractive index of the sample.¹⁸ In practice, it is desirable to match the DOF and measuring range. However, the efficiency of SHG has square dependence on the intensity of the fundamental radiation; therefore, strong focusing of the laser beam is used. Because of this, the measuring range of a SH-OCT system is usually longer than the sample focusing optics DOF. The scanning of both focusing optics and reference delay may be used to effectively cover the whole measuring range but at the expense of the speed of image recording.

For our experimental setup, the measuring ranges in the air determined by the spectrometer resolution were 0.44 mm for the SH and 1.77 mm for the fundamental radiation, respectively. However, the DOF of the sample focusing optics was about 0.27 mm with an estimated transverse resolution of about 10 μm . The relative position of both working ranges in the sample was adjusted by tuning the reference arm length in the respective interferometers.

3. Results and Discussion

Using continuum radiation generated in the fiber, SH-OCT images of fish scales from salmon and pig leg tendon were recorded. Figure 3 shows the image of the fish scales obtained simultaneously with both fundamental and second harmonic interferometers. For these images, 50 mW of fundamental power was incident on the sample. The SH spectrometer CCD integration time was set at 20 ms, and the averaging of 50 A-scans was used. The CCD integration time of the fundamental spectrometer was set at 0.8 ms and the averaging of 50 A-scans. In Fig. 3, the left-hand side shows an OCT image of fish scales obtained by the fundamental beam. Boundaries between different layers can be clearly identified. For clarity, the SH image is displayed on the right-hand side. The polarization of the SH is parallel to the fundamental beam polarization. The SH-OCT image on the right-hand side shows layerlike distribution of collagen

Fig. 3. (Color online) Fish scale OCT and SH-OCT images. On the left-hand side is the fish scale OCT image; on the right-hand side, the SH-OCT image is shown. The length of both images is 3 mm, and the depth of the fundamental image is 0.46 mm, and the depth of the SH image is 0.27 mm. The acquisition time for the SH-OCT image was 10 min.

Fig. 4. (Color online) Pig leg tendon OCT and SH-OCT images. On the left-hand side is the pig leg tendon OCT image; on the right-hand side, the SH-OCT image is shown. The length of both images is 1.5 mm, and the depth of the fundamental image is 0.47 mm, and the depth of the SH image is 0.27 mm. The acquisition time for the SH-OCT image was 5 min.

fibrils. Highly ordered sections of the collagen bundles in the fish scales are clearly visible. The measured axial resolution of the SH image was $12\ \mu\text{m}$, and the measured axial resolution of the fundamental image was $17\ \mu\text{m}$.

Images of pig leg tendon are shown in Fig. 4. The experimental setup was the same as for the fish scales sample. Both SH and fundamental radiation had parallel polarizations. Figure 4 is organized similarly to Fig. 3; the left-hand side is the OCT image of pig leg tendon. The upper layer is the tendon sheath while the layer underneath has tendon fiber bundles, which are made up of highly aligned collagen fibers that generate strong SH signals. The right-hand side is the SH-OCT image, which shows the distribution of collagen fibers. As a tension-bearing element in the tendon, collagen appears in parallel, cablelike bundles oriented in the same direction.

SHG depends on the orientation, anisotropy, and local symmetry properties of the tissue. SH efficiency in collagen depends on the orientation of the collagen fibrils relative to the incident electrical field polarizations.⁸ It was shown that SH intensity reaches a maximum when the collagen fibers are parallel to the beam polarization, and conversely, SH intensity is at a minimum for beam polarization perpendicular to the fibers. This can be used to obtain information about the degree of fibrillar alignment of collagen along a given axis. We used the polarization dependence of SHG to obtain information about the orderliness of fibrillar structure in some tissue samples. Because the diameter of the laser beam used in the experiment was considerably larger than the individual fibril diameter, the detected second harmonic

signal corresponds to an averaged effect over the illuminated area. The regional variation in collagen fibril orientation is not well understood, and it will require a submicrometer resolution study for better understanding.¹⁹

In the measurements, the input beam on BS1 was with vertical polarization, and the SH generated by the BBO crystal in the reference arm was, correspondingly, with horizontal polarization. A half-wave plate, HWP1, in the sample arm allowed rotation of the polarization of the fundamental radiation incident on the sample. SH polarization in both arms of the SH interferometer may be controlled independently by two half-wave plates, HWP2. We obtained images from the same area of the sample for both vertical and horizontal polarization of the fundamental beam. For each fundamental beam polarization, two SH images were recorded: one with SH polarization parallel to fundamental polarization and the other with SH polarization perpendicular to it. We used samples of fish scales and rabbit eye sclera; in both samples, polarization anisotropy of SH signal was observed. The SH signal with polarization parallel to the fundamental polarization was stronger for both vertical and horizontal fundamental radiation polarization. Figure 5 depicts images acquired from the same area of the fish scale and rabbit eye sclera with output SH polarization oriented parallel and perpendicular to the fundamental polarization. The information regarding the ordered nature of the collagen fibrils was obtained by measuring the anisotropy parameter¹¹

$$\beta = (I_{\text{par}} - I_{\text{perp}})/(I_{\text{par}} + 2I_{\text{perp}}),$$

Fig. 5. FD SH-OCT images of fish scales and rabbit eye sclera showing polarization anisotropy. Polarization of the fundamental and second harmonic radiation are (a) fish scales, perpendicular; (b) fish scales, parallel; (c) fish scales, overlay of both SH polarizations; (d) rabbit eye sclera, perpendicular; (e) rabbit eye sclera, parallel; (f) rabbit eye sclera, overlay of both SH polarizations.

where I_{par} and I_{perp} are the intensities of the SH signals parallel and perpendicular to the fundamental radiation. Using the integrated data presented in Fig. 5, we obtained a value for β of 0.65 for the fish scales sample. This value indicates a highly ordered nature of the collagen fibrils in fish scales. For rabbit sclera, we measured β around 0.1, which shows a more random distribution of collagen fibrils. Figures 5(c) and 5(f) show the images from both samples when images of both SH polarizations overlap. These polarization-resolved images display the difference in collagen fiber orientation relative to incident laser polarization. Polarization-resolved images will be critical in applications of SHG for differentiating between normal and abnormal tissue.²⁰

One important feature of the SHG process is that the geometry of the emitted SH radiation depends on the size and shape of the collagen fibrils. Theoretical studies^{13,14} have shown that SH emission is highly asymmetric due to phase-matching conditions. For objects with an axial size of the order of or larger than the SH wavelength, the SH emission is predominantly forward directed. However, for objects with an axial size of less than $\lambda/10$ (approximately 50 nm in our experiment), forward emission and backward emission are nearly equal. Recently, high-resolution SH microscopy experiments^{12,21} have confirmed these theoretical predictions. The measured tendon SH forward/backward ratio was close to 1. Because the tendon fibrils were in size comparable to or larger than the SH wavelength, the results were explained by the fibril model that assumes a thin hollow tube fibril shape.¹² Studies of collagen in sclera have confirmed the tubelike structures of sclera collagen fibrils.²¹ The SHG in such fibrils is generated in the shell, which has a thickness of less than 40 nm.

When the SH generating tissue thickness is larger than the wavelength, the SH is generated mainly in a forward direction, and a detected signal in the backward direction is a result of backscattering from underlying structures. In these cases, care must be taken when interpreting the recorded images. We assume that our samples have similar collagen structures as the ones studied in Refs. 12 and 20; therefore, detected SH signals are due mainly to backward generated SH. A qualitative support for this assumption was that the strongest backward detected SH is not correlated to the strongest SH observed in a forward direction in the samples used in this study.

In conclusion, we demonstrated FD SH-OCT using continuum generated in an optical fiber to increase threefold the axial image resolution reported.^{4,6} The acquisition time for recording an A line was by more than 2 orders of magnitude faster compared to TD SH-OCT with the same setup.^{1,2} In our experiment the average input power on the sample was about 50 mW, well below the estimated damage threshold, but still high according to the American National Standards Institute guidelines. In the measurements, the continuum pulse duration was about 400 fs. Because SH efficiency had a quadratic depen-

dence on the input intensity, a decrease of the pulse duration to 40 fs would allow the same SH intensity to be generated by input radiation of about 5 mW average power. Thus femtosecond pulses with durations in the 10–20 fs range (available by commercial lasers) are highly desirable for FD SH-OCT, because a substantial gain in SH efficiency will be achieved, and at the same time the axial resolution will be improved to the 1–2 μm range. FD SH-OCT may offer several distinct advantages for imaging ordered, or partially ordered, biological tissues. First, the nonlinear (SHG) signal from tissue tends to be a more sensitive indicator of molecular structure and symmetry changes than linear behavior. Second, coherence gating extends the capability of high-resolution detection of SHG signals at locations deep inside the sample. Third, SHG signals are produced intrinsically so imaging does not require staining the sample with dyes or fluorophores. Fourth, decoupled axial and transverse scans enable two-dimensional tomographic imaging of a sample with only one dimension moving of the probing beam, which is essential for *in vivo* endoscopic applications.

This work was supported by research grants from the National Science Foundation (BES-86924), National Institutes of Health (EB-00293, NCI-91717, RR-01192), Air Force Office of Scientific Research (FA9550-04-1-0101), and the Beckman Laser Institute Endowment.

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