Second harmonic generation imaging via nonlinear endomicroscopy

Hongchun Bao,[1] Alex Boussioutas,[2,3] Reynolds Jeremy,[2] Sarah Russell[1,2] and Min Gu[1, *]

[1]*Center for Micro-Photonics, Faculty of Engineering & Industrial Sciences, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia*
[2]*Peter MacCallum Cancer Centre, 1 St Andrews Place, East Melbourne, Victoria 3002, Australia*
[3]*Department of Medicine (RMH/WH), University of Melbourne, Western Hospital, Footscray, 3011, Australia*
**mgu@swin.edu.au*

Abstract: A compact endomicroscope is the only solution for transferring second harmonic generation (SHG) imaging into *in vivo* imaging and real time monitoring the content and structure of collagen. This is important for early diagnoses of different diseases associated with collagen change. A compact nonlinear endomicroscope using a double clad fiber (DCF) is newly employed in SHG imaging. The experiment shows the core of the DCF can maintain the linear polarization of the excitation laser beam in particular directions, and the degree of polarization of the excitation laser beam directly affects signal to noise ratio of SHG imaging. The nonlinear endomicroscope can display clear three dimensional (3D) SHG images of mouse tail tendon without the aid of contrast agents, which reveals the collagen fiber structure at different depths. The high resolution of SHG imaging from the endomicroscope shows that SHG imaging can reveal additional information about the orientation and degree of organisation of proteins and collagen fibers than two-photon-excited fluorescence imaging. Therefore SHG imaging offers endomicroscopy with additional channel of imaging for understanding more about biological phenomena.

©2010 Optical Society of America

OCIS codes: (170.2150) Endoscopic imaging; (110.2350) Fiber optical imaging; (110.6880) Three-dimensional image acquisition; (110.3080) Infrared imaging; (180.4315) Nonlinear microscopy.

References and links

1. W. R. Zipfel, R. M. Williams, and W. W. Webb, "Nonlinear magic: multiphoton microscopy in the biosciences," Nat. Biotechnol. **21**(11), 1369–1377 (2003).
2. L. Moreaux, O. Sandre, M. Blanchard-Desce, and J. Mertz, "Membrane imaging by simultaneous second-harmonic generation and two-photon microscopy," Opt. Lett. **25**(5), 320–322 (2000).
3. E. Brown, T. McKee, E. diTomaso, A. Pluen, B. Seed, Y. Boucher, and R. K. Jain, "Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation," Nat. Med. **9**(6), 796–801 (2003).
4. M. Stroh, J. P. Zimmer, D. G. Duda, T. S. Levchenko, K. S. Cohen, E. B. Brown, D. T. Scadden, V. P. Torchilin, M. G. Bawendi, D. Fukumura, and R. K. Jain, "Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo," Nat. Med. **11**(6), 678–682 (2005).
5. P. J. Campagnola, and L. M. Loew, "Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms," Nat. Biotechnol. **21**(11), 1356–1360 (2003).
6. D. A. Dombeck, K. A. Kasischke, H. D. Vishwasrao, M. Ingelsson, B. T. Hyman, and W. W. Webb, "Uniform polarity microtubule assemblies imaged in native brain tissue by second-harmonic generation microscopy," Proc. Natl. Acad. Sci. U.S.A. **100**(12), 7081–7086 (2003).
7. N. Uldbjerg, A. Malmström, G. Ekman, and U. Ulmsten, "The integrity of cervical collagen during pregnancy and labor," Gynecol. Obstet. Invest. **20**(2), 68–73 (1985).
8. R. M. Williams, W. R. Zipfel, and W. W. Webb, "Interpreting second-harmonic generation images of collagen I fibrils," Biophys. J. **88**(2), 1377–1386 (2005).
9. L. Fu, and M. Gu, "Polarization anisotropy in fiber-optic second harmonic generation microscopy," Opt. Express **16**(7), 5000–5006 (2008).
10. M. E. Llewellyn, R. P. J. Barretto, S. L. Delp, and M. J. Schnitzer, "Minimally invasive high-speed imaging of sarcomere contractile dynamics in mice and humans," Nature **454**(7205), 784–788 (2008).

#119918 - $15.00 USD Received 12 Nov 2009; revised 28 Dec 2009; accepted 30 Dec 2009; published 11 Jan 2010

11. H. Bao, and M. Gu, "Reduction of self-phase modulation in double-clad photonic crystal fiber for nonlinear optical endoscopy," Opt. Lett. **34**(2), 148–150 (2009).
12. D. Bird, and M. Gu, "Compact two-photon fluorescence microscope based on a single-mode fiber coupler," Opt. Lett. **27**(12), 1031–1033 (2002).
13. L. Fu, X. Gan, and M. Gu, "Use of a single-mode fiber coupler for second-harmonic-generation microscopy," Opt. Lett. **30**(4), 385–387 (2005).
14. L. Fu, and M. Gu, "Double-clad photonic crystal fiber coupler for compact nonlinear optical microscopy imaging," Opt. Lett. **31**(10), 1471–1473 (2006).
15. L. Fu, X. Gan, D. Bird, and M. Gu, "Polarization characteristics of a 1×2 fibre coupler under femtosecond pulsed and continuous wave illumination," Opt. Laser Technol. **37**(6), 494–497 (2005).
16. H. Bao, J. Allen, R. Pattie, R. Vance, and M. Gu, "A fast handheld two-photon fluorescence microendoscope with a 475µmx475µm field of view for in vivo imaging," Opt. Lett. **33**(12), 1333–1335 (2008).
17. M. Goetz, C. Fottner, E. Schirrmacher, P. Delaney, S. Gregor, C. Schneider, D. Strand, S. Kanzler, B. Memadathil, E. Weyand, M. Holtmann, R. Schirrmacher, M. M. Weber, M. Anlauf, G. Klöppel, M. Vieth, P. R. Galle, P. Bartenstein, M. F. Neurath, and R. Kiesslich, "In-vivo confocal real-time mini-microscopy in animal models of human inflammatory and neoplastic diseases," Endoscopy **39**(4), 350–356 (2007).
18. R. Kiesslich, P. R. Galle, and M. F. Neurath, Atlas of endomicroscopy. Springer Medizin Verlag Heidelberg (2008).

1. Introduction

Second harmonic generation (SHG) is a process where two photons simultaneously interact with non-centrosymmetrical structures without absorption, producing radiation at exactly half of the excitation wavelength [1–3]. SHG allows for the unique ability to observe fine structural details of connective tissues such as collagen, tendon and muscle in animals and humans without the aid of contrast agents [4, 5].

The properties of SHG offer several advantages for live cell or tissue imaging. Unlike fluorescence imaging, SHG does not involve excitation of endogenous florescent molecules. Thus, it does not suffer from phototoxicity effects or photo bleaching, which limits the usefulness of fluorescence imaging [6, 7]. The excitation of SHG imaging uses near-infrared wavelengths, which allows for a large penetration depth, making it a prime candidate for analysis of thick tissue specimens with the enhanced axial depth discrimination. SHG signals have well defined polarization, so SHG polarization anisotropy can be used to determine the absolute orientation and the degree of organization of proteins in tissues [7–9]. The SHG excitation is a nonlinear process and the signal intensity is directly proportional to the square of the excitation intensity. This provides SHG imaging with intrinsic confocality and deep sectioning in complex tissues.

SHG microscopy is presently being introduced as a tool for basic biomedical research to noninvasively visualize and obtain high-resolution images of unstained collagen structures [1–5, 10]. However, to date almost all research in SHG imaging has been carried out using a conventional bench top microscope system. Such a system does not provide the adjustment flexibility needed for *in vivo* imaging, where specimens need to be mounted on the microscopy stage, limits the system's use. A biopsy is required for SHG imaging which will introduce intrusion to animal or human and variation of collagen structures because of excision from a live body. An endomicroscope system having a long flexible fiber and a miniaturized probe is necessary for translating SHG imaging into *in vivo* imaging [11–15].

In this letter, we demonstrate SHG imaging using our recently developed compact handheld nonlinear endomicroscope which is capable of *in vivo* 3D imaging [16]. Experimental results show that the core of a double clad fiber (DCF) which the endomicroscope uses has a "polarization maintaining" feature in particular directions. Using this "polarization marinating" feature, the nonlinear endomicroscope can observe clear 3D collagen structure of a mouse tail tendon within a depth of 88 µm. High resolution SHG imaging from the endomicroscope has shown more micro-structure information of biological tissue than two-photon fluorescence imaging.

2. The nonlinear endomicroscopic system for SHG imaging

The SHG endomicroscope is schematically depicted in Fig. 1, where a passive mode-locked Ti:Sapphire laser generates an 80 MHz 100 fs optical pulse train with wavelength tuneable between 730 nm and 870 nm. 800 nm pulses can introduce high SHG signal

#119918 - $15.00 USD Received 12 Nov 2009; revised 28 Dec 2009; accepted 30 Dec 2009; published 11 Jan 2010
(C) 2010 OSA

from mouse tail tendon and are used for SHG imaging. The pulses propagate through a pair of gratings which provide a negative frequency chirp. The pulses pass through a half wave plate (λ/2 plate) and a Glen Thompson polarizer being used to control the polarization direction of the optical pulses by rotating the direction of the λ/2 plate and the Glen Thompson polarizer accordingly. The optical pulses are then coupled into the core of a 2.5 m long DCF by an objective lens (Olympus MA20X/0.4), where the chromatic dispersion of the DCF is compensated by the pre-chirp in the pulses. The pulse width at the output of the endomicroscope is 1.5 ps.

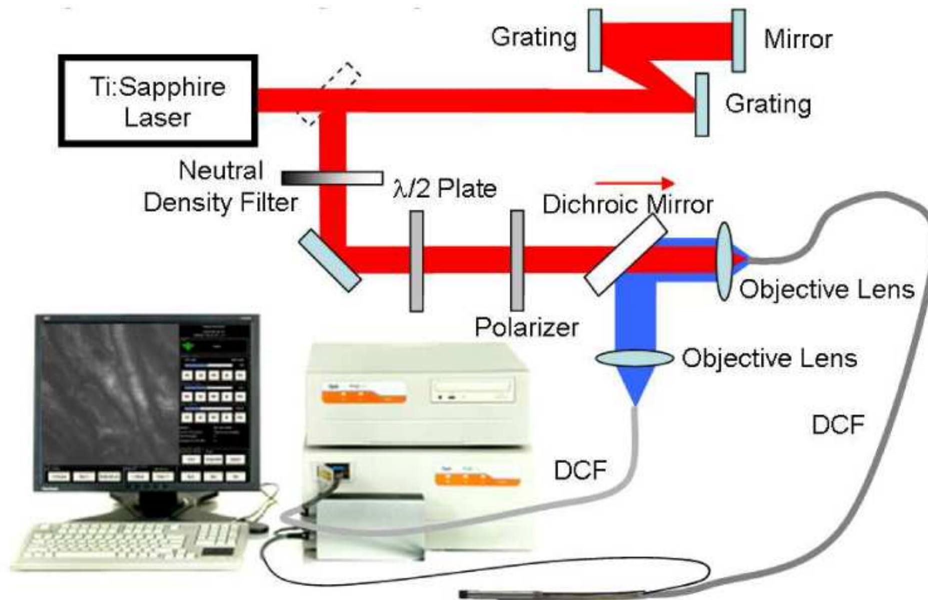

Fig. 1. Schematic representation of experimental setup for SHG imaging using the designed nonlinear endomicroscope.

The DCF, with a core diameter of 3.6 µm and an inner cladding diameter of 105 µm, is used for the delivery of the excitation laser beam to the specimen and the collection of the SHG signal back for detection. The other end of the DCF is placed inside the endomicroscope probe. The detail structure of the probe can be found in the previous report [17, 18]. In the probe, a multiple element lens focuses the excitation laser beam delivered by the DCF into the specimen. The numerical aperture (NA) of the lens is 0.35. The lens well corrects the aberration within the field of view and realizes diffraction limited resolution [16–18], which reaches better than 1 µm and 10 µm of lateral and axial resolutions. A micro-scanner inside the probe scans the DCF line by line and moves the lens with the DCF backward and forward for 3D imaging. The excited SHG signal is collected through the lens and the cladding of the DCF, separated from the excitation laser beam by a dichroic mirror and coupled to another DCF by another objective lens (Olympus MA20X/0.4) which sends the SHG signal into the SHG endomicroscope detection box. Inside the detection box, a photomultiplier tube (PMT) is employed to detect the SHG signal after it is filtered by a 400/9 nm bandpass filter to ensure that only the SHG signal is detected. The endomicroscope's electronics and the host CPU synchronize the endomicroscope system and display SHG images with a speed of 2 frames per second.

3. Degree of polarization of the excitation laser beam delivered by the nonlinear endomicroscope

The DCF that the nonlinear endomicroscope uses has a circular core and it should not be polarization maintaining. As a linearly polarized excitation laser beam is coupled into the

#119918 - $15.00 USD Received 12 Nov 2009; revised 28 Dec 2009; accepted 30 Dec 2009; published 11 Jan 2010
(C) 2010 OSA

core of the DCF, we found that the degree of polarization, defined as $\gamma = (I_{max} - I_{min})/(I_{max} + I_{min})$ by measuring the maximum (I_{max}) and minimum (I_{min}) intensity of the output laser beam, changes by varying the polarization direction of the incident linearly polarized laser beam. Figure 2 shows the measured degree of polarization of the output laser beam at wavelength 800 nm from the fiber probe as a function of the incident polarization direction. The degree of polarization γ of the excitation laser beam from the output of the probe is changed between 0.35 and 0.96 for different incident polarization directions. The perfect circular structure of the core of the DCF should not have such features. However, minor deviations in the symmetry of the DCF during fabrication causes the 2.5 m DCF "polarization maintaining" along two directions: 0° and 90°. Since the DCF is only 2.5 m long and free running, polarization maintaining of the DCF is stable during imaging.

The degree of polarization of the excitation laser beam from the nonlinear endomicroscope directly affects the signal to noise ratio of SHG imaging. The SHG intensity and the background noise of SHG imaging of a mouse tail tendon fiber by the nonlinear endomicroscope are measured. The signal to noise ratio (SNR), defined as the ratio of the SHG intensity to the background noise, is also displayed in Fig. 2. The SNR of SHG imaging has a similar trend as the degree of polarization γ of the excitation laser from the endomicroscope. SHG imaging has a high SNR as the output laser beam is close to be linearly polarized, and the SNR of SHG imaging reduces while γ of the output laser beam decreases.

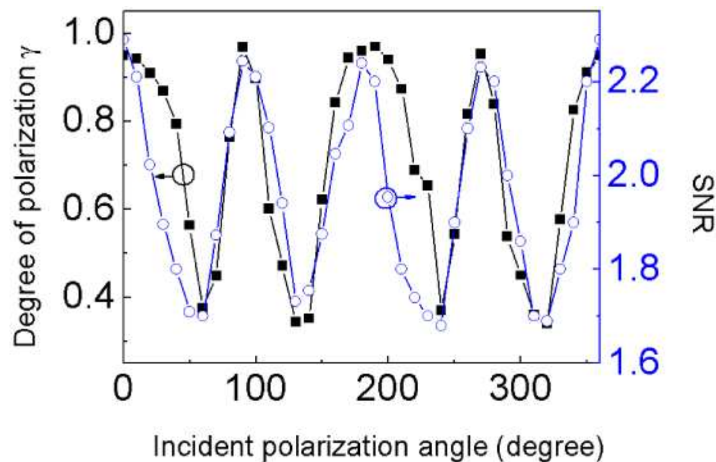

Fig. 2. Degree of polarization of the excitation laser beam and signal to noise ratio of SHG imaging as a function of the polarization direction of the input linearly polarized laser beam

4. Three-dimensional SHG imaging by nonlinear endomicroscopy

The nonlinear endomicroscope permits 3D anatomical and functional imaging of organs with a large field of view and high resolution [9]. Figure 3 is 3D SHG images of a mouse tail tendon obtained by the nonlinear endomicroscope with axial distance of 8 µm, where polarization direction of incident laser beam is 0°, aligning with the "polarization maintaining" direction of the DCF. The size of the images is 475 µm × 475 µm. Figure 3 shows clear structural details of collagen in the mouse tail tendon at different depth. This nonlinear endomicroscope can observe the collagen structure of a mouse tail tendon 88 µm under the surface.

#119918 - $15.00 USD Received 12 Nov 2009; revised 28 Dec 2009; accepted 30 Dec 2009; published 11 Jan 2010
(C) 2010 OSA

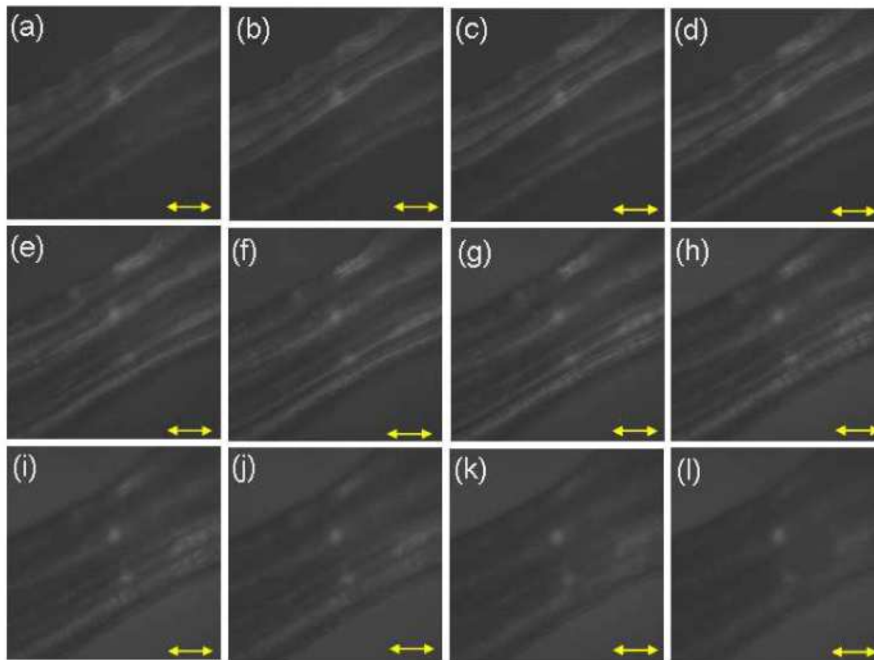

Fig. 3. SHG images of a mouse tail tendon with an axial step of 8 µm. The image size is 475 µm × 475 µm. The excitation wavelength is 800 nm. The excitation power is 30 mW on the sample. Arrows are the polarization directions of the excitation laser beam

5. SHG imaging and two-photon excited fluorescence imaging by nonlinear endomicroscopy

The nonlinear endomicroscope can observe two-channel of images – a SHG image and a two-photon excited fluorescence (TPF) image, by injecting a mouse with fluorescein and switching between the filter for SHG imaging and the filter for TPF imaging, which is a 3 mm thick BG18 glass filter (Schott Pty Ltd). Figure 4 reveals high resolution two-channel images of a mouse tail tendon.

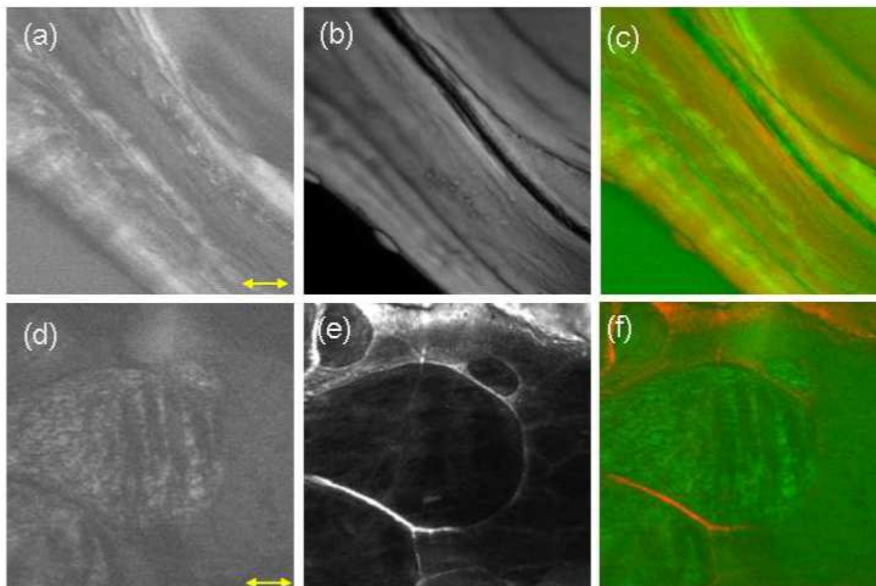

Fig. 4. Two channel images of a mouse tail tendon by the nonlinear endomicroscope. (a), (d) SHG images. (b), (e) TPF images. (c), (f) Combination of two-channel images. Red: TPF image. Green: SHG image. The size of the images: 150 µm × 150 µm. Arrows are the polarization directions of the excitation laser beam

#119918 - $15.00 USD Received 12 Nov 2009; revised 28 Dec 2009; accepted 30 Dec 2009; published 11 Jan 2010
(C) 2010 OSA

The structure of the SHG images and the TPF images is different. The TPF images show the distribution of fluorescein in the mouse tail tendon absorbed from the bloodstream. On the other hand, SHG images display non-centrosymmetrical orientation and structures of proteins in the mouse tail tendon. Therefore, SHG offers the nonlinear endomicroscope with another channel of image which helps to understand orientation and degree of organisation of proteins and collagen fiber in the tissues.

5. Conclusions

The nonlinear endomicroscope can be used for *in vivo* SHG imaging. The experimental results show that the degree of polarization of the excitation laser beam from the output of the endomicroscopy probe changes with different incident polarization directions. The circular DCF that the nonlinear endomicroscope uses shows a "polarization maintaining" feature at particular directions due to minor deviations in the symmetry of the DCF during fabrication. As a result, the SNR of the SHG images obtained by the endomicroscope changes with different incident polarization directions. With the polarization direction of the incident laser beam aligning with the "polarization maintaining" direction of the DCF, the nonlinear endomicroscope shows clear 3D collagen structures of a mouse tail tendon. Comparing with two-photon excited fluorescence imaging by the nonlinear endomicroscope, the high resolution SHG imaging offers further information about the orientation and degree of organisation of proteins and collagen fiber in biological objects.

Acknowledgements

The authors thank the Australian Research Council for its support.

#119918 - $15.00 USD Received 12 Nov 2009; revised 28 Dec 2009; accepted 30 Dec 2009; published 11 Jan 2010
(C) 2010 OSA