Combined Two-Photon Excited Fluorescence and Second-Harmonic Generation Backscattering Microscopy of Turbid Tissues

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ABSTRACT

A broad range of excitation wavelengths (730-880nm) was used to demonstrate the co-registration of two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) in unstained turbid tissues in reflection geometry. The composite TPEF/SHG microscopic technique was applied to imaging an organotypic tissue model (RAFT). The origin of the image-forming signal from the various RAFT constituents was determined by spectral measurements. It was shown that at shorter excitation wavelengths the signal emitted from the extracellular matrix (ECM) is a combination of SHG and TPEF from collagen, whereas at longer excitation wavelengths the ECM signal is exclusively due to SHG. The cellular signal is due to TPEF at all excitation wavelengths. The reflected SHG intensity followed a quadratic dependence on the excitation power and exhibited a spectral dependence in accordance with previous theoretical studies. Understanding the structural origin of signal provided a stratagem for enhancing contrast between cellular structures, and components of the extracellular matrix. The use of SHG and TPEF in combination provides complementary information that allows non-invasive, spatially localized *in vivo* characterization of cell-ECM interactions and pathology.

Keywords: Second-harmonic generation (SHG); two-photon excited fluorescence (TPEF); multi-photon microscopy; spectroscopy; tissue; biological imaging; collagen.

1. INTRODUCTION

In this paper we report the combined use of two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) for the noninvasive characterization of unstained turbid tissues. TPEF has been widely used to probe biological tissues[1]. Recently, second-harmonic generation (SHG) has been employed as a high-resolution technique for imaging membranes[2-4] and endogenous collagen[5-7], which is also known to exhibit TPEF[1;8]. This has raised the question of the origin of signal (TPEF or SHG) from biological tissues. The use of TPEF and SHG in combination has been employed for the study of stained cells[3;9] by detection of TPEF in reflection mode and SHG in transmission mode, which is not practical for *in vivo* applications. Here, we demonstrate the co-registration of TPEF and SHG in backscattering geometry for an unstained organotypic tissue model (RAFT), and for *in vivo* rabbit aorta and cornea. We employ spectral measurements in order to elucidate the origin of the image-forming signal from the various tissue constituents, and we use this information to isolate cellular and extracellular matrix signal by spectral filtering.

2. EXPERIMENTAL LAYOUT

Two-photon microscope: The two-photon imaging microscope used for this study has been described[10], and has been modified to include a SpectraPro-150 spectrograph with a 300grooves/mm grating blazed at 500nm (Acton Research Corp., Acton, MA), equipped with a high dynamic range MicroMax:512BFT CCD camera (Princeton Instruments, Trenton, NJ). The average power entering the microscope is 60mW (corresponding to 5mW at the sample site). The spectrograph slit width is set to 0.5mm. The spectra acquisition time, Δt, is 60s. Switching between imaging and spectra acquisition is achieved by changing the position of a built-in microscope mirror. Two-photon images (256X256 pixels)

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are acquired and stored, immediately followed by emission spectra acquisition from the same depth (z) into the sample. Dark noise spectra are subtracted from the acquired sample spectra.

Tissue samples: The organotypic RAFT tissue model used in the experiments consists of a basic polymerized collagen gel made up of type I rat-tail collagen and primary human neonatal dermal foreskin fibroblast cells, and it was prepared as previously described[1]. Rabbit ascending aorta and rabbit cornea were imaged fresh, immediately after excision.

3. RESULTS AND DISCUSSION

3.1 TPEF and SHG co-registration from collagen

In order to elucidate the origin of the image-forming signal from two-photon excitation of collagen we have used the RAFT tissue model, which is well characterized. The laser source was tuned to various excitation wavelengths, λ_{ex}, (730-880nm) and two-photon images and spectra were obtained from the sample surface when collagen alone (i.e., at the absence of fibroblast cells) could be visualized in the images. A representative image of collagen fibers in the RAFT, acquired for λ_{ex}=840nm, is shown in Fig. 1 (inset). Collagen emission spectra acquired from the RAFT surface for λ_{ex}=730, and 840nm (Fig. 1) exhibit a narrow peak at half the excitation wavelength with a bandwidth (FWHM) that is in accordance to the excitation laser spectral width, and intensity that follows a quadratic dependence on the laser power.

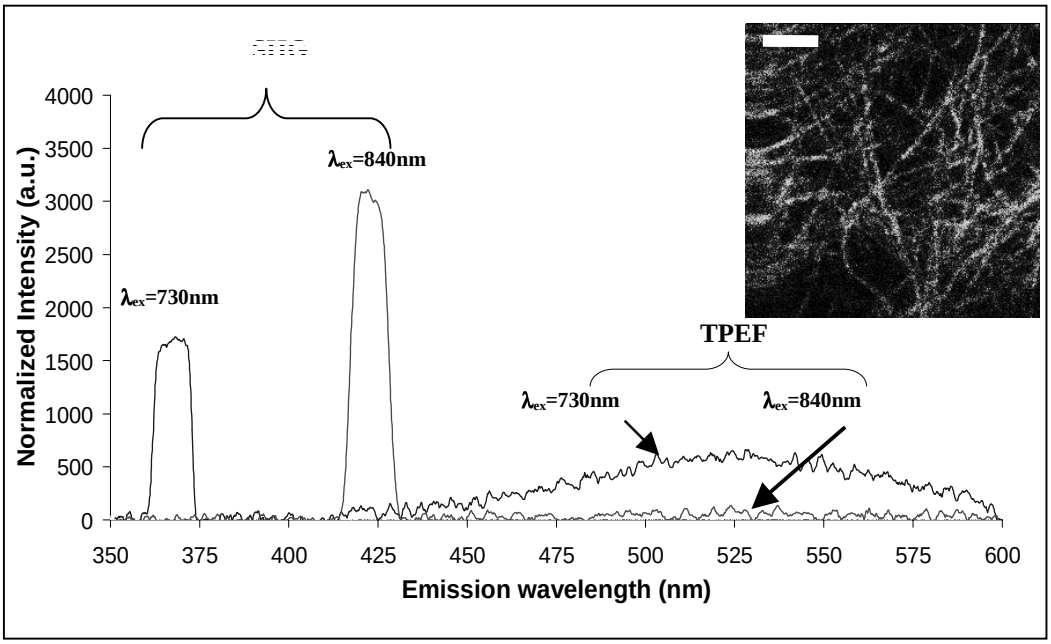

Fig. 1. Emission spectra for RAFT collagen for λ_{ex}=730, and 840nm at z=0μm for acquisition time Δt=60s. Inset shows two-photon image of collagen for λ_{ex}=840nm at z=0μm. Scale bar is 7μm.

Fig. 2 illustrates emission spectra from RAFT collagen for powers entering the microscope, P, of 20, 40, 60, and 80mW, at λ_{ex}=800nm. The inset in Fig. 2 shows a plot of the natural logarithm of the SHG intensity, $\ln(I_{SHG})$, as a function of the natural logarithm of the excitation power, ln(P). The $\ln(I_{SHG})=1.9673*\ln(P)-0.1336$ dependence demonstrated on the plot is, to a good approximation, quadratic, which is consistent with second-harmonic generation. In addition to the SHG signal, for λ_{ex}=730nm the emission spectra in Fig. 1 also display a broad peak covering the region from 410 to 600nm, with a maximum at 525nm. This spectral feature corresponds to TPEF from collagen and was not detected for λ_{ex}=840nm.

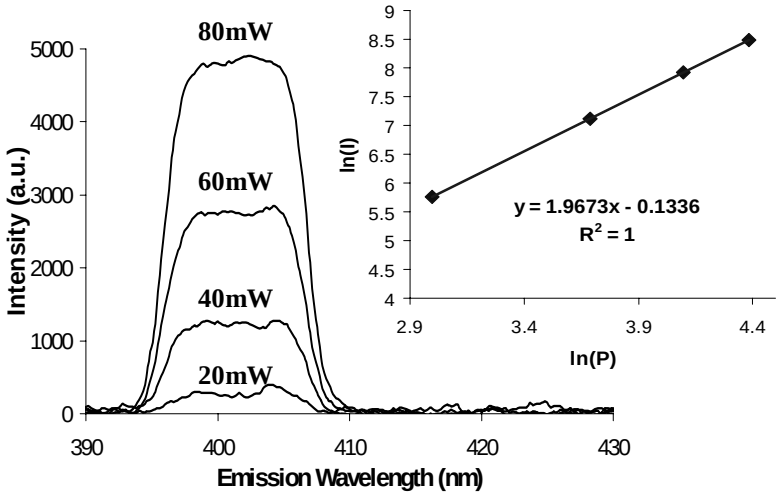

Fig. 2. Emission spectra from RAFT collagen for powers entering the microscope, P = 20, 40, 60, and 80mW, λ_{ex}=800nm, z=0μm. Spectra acquisition time was Δt=60s. Inset shows a plot of the natural logarithm of the SHG intensity as a function of the natural logarithm of the excitation power.

3.2 SHG dependence on excitation wavelength

Fig. 3 depicts a plot of the intensity of the spectral peaks registered from reflected SHG signals from collagen at various laser excitation wavelengths. The SHG intensity values have been corrected for the relative transmittance of the emission filters at the various SHG wavelengths. For the examined irradiation wavelengths, the reflected SHG signal is optimal for λ_{ex}=800nm. This SHG intensity profile is in agreement with previous theoretical studies of the reflected second-harmonic field generated in a nonlinear medium that is embedded in a linear medium[11]. The spectral dependence of the reflected SHG intensity can provide a means for obtaining quantitative tissue information[12].

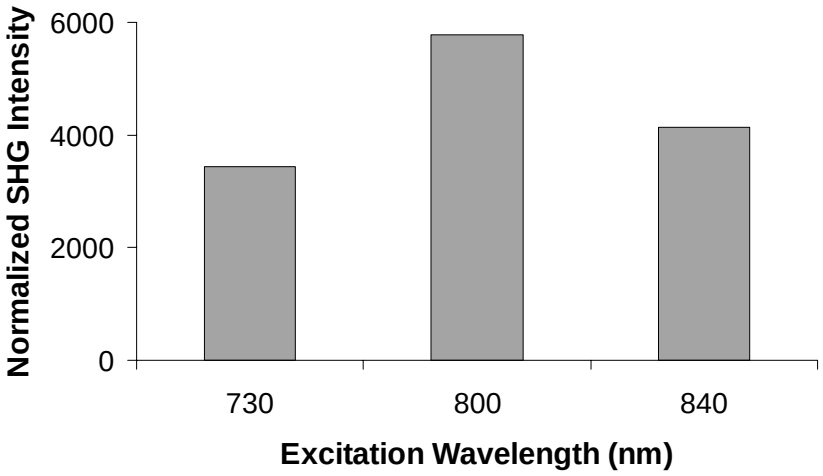

Fig. 3. Spectral dependence of reflected SHG intensity.

3.3 Separation of collagen from cellular signal

To determine the origin of the image-forming signal for the fibroblast cells in the RAFT, two-photon images and the corresponding spectra were obtained for a broad range of excitation wavelengths when both collagen and fibroblasts could be visualized in the images, and when collagen alone from the same image plane was visualized in the images. Fig. 4 shows representative images (Fig. 4(a), (b)) and spectra (Fig. 4(c)) for λ_{ex}=750nm. The emission spectrum for collagen alone exhibits a 375nm peak due to SHG, and a broad peak in the 410-600nm region with a maximum at 525nm corresponding to TPEF. The spectrum from both collagen and a fibroblast cell consists of the 375nm SHG signal, and a broad peak with a maximum at 510nm, resulting from both collagen and fibroblast autofluorescence. Subtraction of the former from the latter spectrum yields TPEF exclusively from cellular autofluorescence that peaks at 460nm and is consistent with NAD(P)H fluorescence[13-15]. For longer excitation wavelengths (>800nm) the TPEF signal was exclusively due to cellular autofluorescence, and the collagen only generated SHG (data not shown).

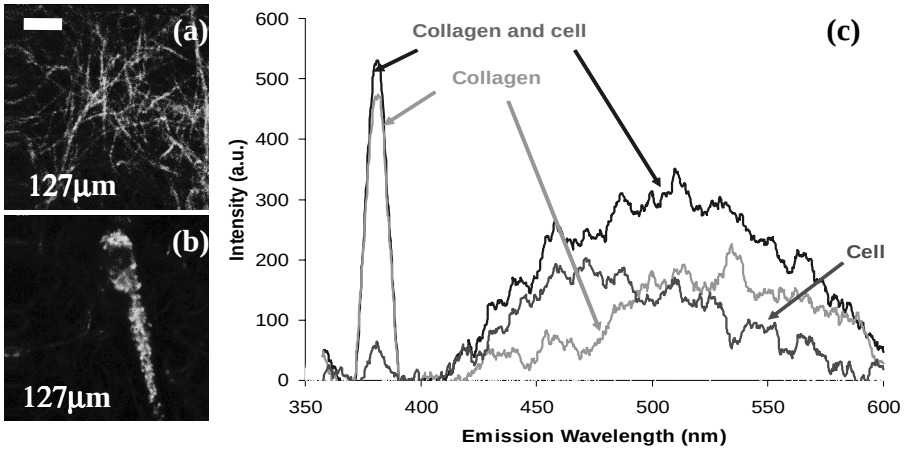

Fig. 4. Two-photon images from RAFT collagen only (a) and for collagen and cell (b) for λ_{ex}=750nm, P=57.3mW, z=127μm. Scale bar is 7μm. The corresponding emission spectra (Δt=60s) are shown in (c).

To further demonstrate the spectrally distinct origin of the image-forming signals for the cellular and extracellular matrix components in two-photon microscopy of turbid tissues, we examined a fresh sample of excised rabbit cornea, which is known to produce SHG signals[16]. The corneal epithelium consists exclusively of cells, whereas the corneal stroma is predominantly composed of layered sheets of collagen fibers. Fig. 5(a) illustrates an image of the corneal epithelium obtained for an excitation wavelength of 750nm. The corresponding emission spectrum (Fig. 5(b)) consists of a peak covering the region from 370 to 600nm, with maxima at 440 and 470nm, typical of NAD(P)H fluorescence[13;17]. Fig. 5(c) shows an image obtained from an acellular region of the stroma for λ_{ex}=800nm, at a depth of 60μm into the sample, where sheets of collagen fibers can be readily distinguished. The emission spectrum for the corneal stroma (Fig. 5(d)) is consistent with SHG, displaying a narrow peak at half the excitation wavelength (400nm).

3.4 Isolation of cellular and ECM signals by spectral filtering

Since endogenous cellular and extracellular matrix signals in two-photon microscopy of biological tissues are characteristically different, they can provide a means to isolate cell and matrix components by spectral filtering. An example of this approach is illustrated in Fig. 6 showing images obtained from the same site at a depth of ~80μm into the RAFT, for an excitation laser wavelength of 840nm, using different emission filters in front of the PMT. Shown in Fig. 6(a) is a two-photon image obtained from the RAFT using an SBG39 (320-600nm) emission filter. The image displays a fibroblast cell in a matrix of collagen fibers. Strong SHG signal is emitted from the collagen fibers as compared to the weak TPEF cellular signal. Using a band-pass filter near the maximum of the cellular fluorescence (520 nm), the cell can be imaged alone as shown in Fig. 6(b). Here, the bright SHG collagen signal has been suppressed, leaving the TPEF signal from the fibroblast cell. Similarly, the collagen matrix can be visually isolated using a band-pass filter at the second-harmonic wavelength (440nm) as shown in Fig. 6(c).

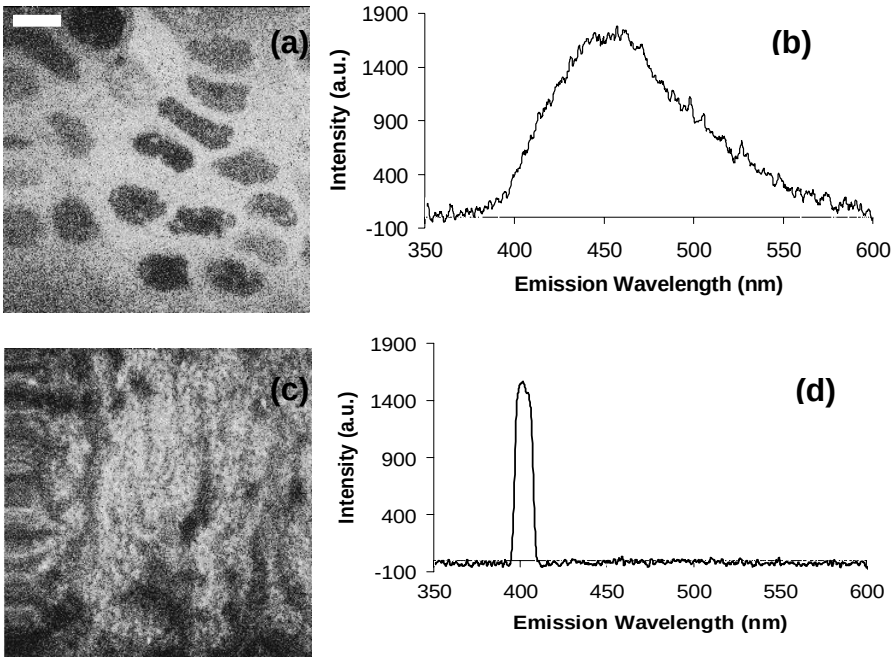

Fig. 5. Origin of signals in two-photon microscopy of the rabbit cornea. (a) Two-photon image of the corneal epithelium for λ_{ex}=750nm; and the corresponding emission spectrum (b) consisting of cellular TPEF signal. (c) Two-photon image of the corneal stroma for λ_{ex}=800nm; and the corresponding emission spectrum (d) consisting of SHG signal from collagen. Spectra acquisition time, Δt=60s. Scale bar is 6μm.

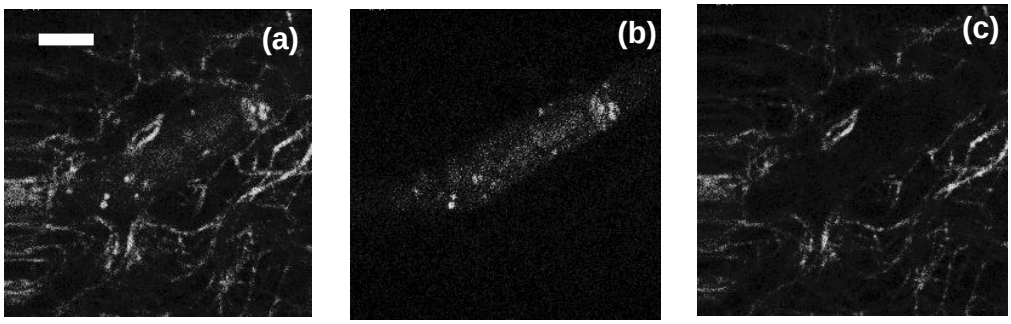

Fig. 6. Two-photon images of the same site in the RAFT acquired for λ_{ex}=840nm, at z=80μm, using three different emission filters in front of the PMT: (a) an SBG39 emission filter (320-600nm); (b) a 520/40nm band-pass filter; and (c) a 440/40nm band-pass filter. Scale bar is 6μm.

Spectral filtering can also be used for the separation of signals from different extracellular matrix components such as collagen and elastin. Both collagen and elastin exhibit autofluorescence signals that overlap significantly[17;18], thus hindering their visual isolation . However, elastin lacks the non-centrosymmetric structure required for SHG, which is the only signal associated with collagen for long excitation wavelengths (>800nm). Therefore, selection of appropriate excitation wavelengths allows the isolation of the collagen and elastin signals in two-photon microscopy. This is demonstrated in Fig. 7, which illustrates two-photon images obtained from a freshly excised rabbit aorta. The rabbit aorta consists of three relatively distinct layers, which from the lumen to the outer wall of the vessel are the intima, the media, and the adventitia. The intima consists of an endothelium, and is separated from the media by a thin layer of

elastin-rich connective tissue called the lamina propria. The media is predominantly composed of elastic fibers, and the adventitia consists mainly of sheets of collagen fibers oriented in multiple directions. Fig. 7(a) shows an image acquired from a site close to the border between the adventitia and the media in the rabbit aorta sample using an SBG39 (320-600nm) emission filter, for λ_{ex}=800nm. The image displays both collagen sheets and elastic fibers as indicated by arrows. However, the bright SHG signal from the collagen fibers masks the TPEF signal from elastin. Using a 520/40nm band-pass filter, elastin can be imaged alone as shown in Fig. 7(b). Similarly, collagen sheets can be better visualized when isolated using a band-pass filter at the second-harmonic wavelength (400nm) as shown in Fig. 7(c). These findings are of great significance for the study of cell-cell and cell-extracellular matrix interactions.

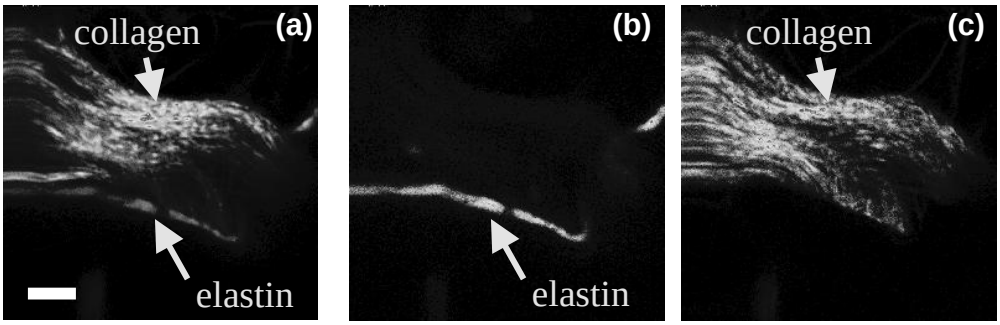

Fig. 7. Spectral filtering of the collagen and elastin components of the rabbit aortic wall for λ_{ex}=800nm. (a)Two-photon image of the rabbit aorta acquired with an SBG39 emission filter (320-600nm); (b) two-photon image from the same sample site as in (a) acquired with a 520/40nm band-pass emission filter; (c) two-photon image from the same sample site as in (a) acquired with a 400/10nm band-pass emission filter. Scale bar is 5μm.

3. CONCLUSIONS

The data presented have demonstrated the co-registration of SHG and TPEF in backscattering microscopy of turbid tissues. The origin of signal from various tissue constituents was determined with spectral measurements over a broad range of excitation wavelengths. It was shown that appropriate selection of excitation wavelengths renders the image-forming signal from various tissue constituents spectrally distinct and allows their selective visualization using spectral filtering. The combined use of TPEF and SHG in reflection geometry provides complementary information that allows non-invasive, spatially localized *in vivo* characterization of biological tissues.

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REFERENCES

1. Agarwal, A., Coleno, M. L., Wallace, V. P., Wu, W. Y., Sun, C. H., Tromberg, B. J., & George, S. C., "Two-photon laser scanning microscopy of epithelial cell-modulated collagen density in engineered human lung tissue", *Tissue Engineering*, **7**, 191-202, 2001.

2. Campagnola, P. J., Wei, M. D., Lewis, A., & Loew, L. M., "High-Resolution Nonlinear Optical Imaging of Live Cells by Second Harmonic Generation", *Biophysical Journal*, **77**, 3341-3349, 1999.

3. Moreaux, L., Sandre, O., Blanchard-Desce, M., & Mertz, J., "Membrane Imaging by Simultaneous Second-Harmonic Generation and Two-Photon Microscopy", *Optics Letters*, **25**, 320-322, 2000.

4. Moreaux, L., Sandre, O., & Mertz, J., "Membrane Imaging by Second-Harmonic Generation Microscopy", *Journal of the Optical Society of America B-Optical Physics*, **17**, 1685-1694, 2000.

5. Campagnola, P. J., Clark, H. A., Mohler, W. A., Lewis, A., & Loew, L. M., "Second-Harmonic Imaging Microscopy of Living Cells", *Journal of Biomedical Optics*, **6**, 277-286, 2001.

6. Georgiou, E., Theodossiou, T., Hovhannisyan, V., Politopoulos, K., Rapti, G. S., & Yova, D., "Second and third optical harmonic generation in type I collagen, by nanosecond laser irradiation, over a broad spectral region", *Optics Communications*, **176**, 253-260, 2000.

7. Williams, R., Zipfel, W. R., & Webb, W. W., "Multiphoton microscopy in biological research", *Current Opinion in Chemical Biology*, **5**, 603-608, 2001.

8. Masters, B. R. & So, P. T. C., "Multi-Photon Excitation Microscopy and Confocal Microscopy Imaging of in Vivo Human Skin: a Comparison", *Microscopy & Microanalysis*, **5**, 282-289, 1999.

9. Moreaux, L., Sandre, O., Charpak, S., Blanchard-Desce, M., & Mertz, J., "Coherent Scattering in Multi-Harmonic Light Microscopy", *Biophysical Journal*, **80**, 1568-1574, 2001.

10. Dunn, A. K., Wallace, V. P., Coleno, M., Berns, M. W., & Tromberg, B. J., "Influence of Optical Properties on Two-Photon Fluorescence Imaging in Turbid Samples", *Applied Optics*, **39**, 1194-1201, 2000.

11. Le Calvez, A., Freysz, E., & Ducasse, A., "Second harmonic field generated in reflection by an inhomogeneous nonlinear polarization", *Optics Communications*, **145**, 135-140, 1998.

12. Zoumi, A., Yeh, A., & Tromberg, B. J., "Structural and functional origin of contrast in multi-photon microscopy of thick tissues", *Proc. Natl. Acad. Sci. USA*, In submission.

13. Piston, D. W., Masters, B. R., & Webb, W. W., "Three-dimensionally resolved NAD(P)H cellular metabolic redox imaging of the *in situ* cornea with two-photon excitation laser scanning microscopy ", J*ournal of Microscopy*, **178**, 20-27, 1995 .

14. Masters, B. R., So, P. T., & Gratton, E., "Multiphoton excitation fluorescence microscopy and spectroscopy of in vivo human skin", *Biophysical Journal*, **72**, 2405-2412, 1997.

15. Schilders, S. P. & Gu, M., "Three-Dimensional Autofluorescence Spectroscopy of Rat Skeletal Muscle Tissue Under Two-Photon Excitation", *Applied Optics*, **38**, 720-723, 1999.

16. Fine, S. & Hansen, W. P., "Optical Second Harmonic Generation in Biological Systems", *Applied Optics*, **10**, 2350-2353, 1971.

17. Richards-Kortum, R. & Sevick-Muraca, E., "Quantitative optical spectroscopy for tissue diagnosis", *Annual Review of Physical Chemistry*, **47**, 555-606, 1996.

18. Katz, A. and Alfano, R. R., “Hot Topics. Optical Biopsy-Detecting Cancer with Light”, http://www.ieee.org/organizations/pubs/newsletters/leos/feb96/katz.html.