# Two-photon excited spectroscopies of *ex vivo* human skin endogenous species irradiated by femtosecond laser pulses

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Received March 10, 2006

Two-photon excited spectroscopies from *ex vivo* human skin are investigated by using a femtosecond laser and a confocal microscope (Zeiss LSM 510 META). In the dermis, collagen is responsible for second harmonic generation (SHG); elastin, nicotinamide adenine dinucleotide (NADH), melanin and porphyrin are the primary endogenous sources of two-photon excited autofluorescence. In the epidermis, keratin, NADH, melanin and porphyrins contribute to autofluorescence signals. The results also show that the SHG spectra have the ability to shift with the excitation wavelength and the autofluorescence spectra display a red shift of the spectral peaks when increasing the excitation wavelength. These results may have practical implications for diagnosis of skin diseases.

*OCIS codes:* 170.6510, 170.4580, 300.6410.

When biological tissues are irradiated by intense near-infrared femtosecond laser pulses, the endogenous structural proteins with noncentrosymmetric structures can easily produce the optical second harmonic generation (SHG) signal[1−3]. The SHG process changes two near-infrared incident photons into one visible photon at half the wavelength. At the same time, the native fluorophores in the biological specimen may also emit the autofluorescence through two-photon-excited fluorescence process[4−7]. Two-photon excited process occurs when an electron is excited to a higher energy electronic state by the simultaneous absorption of two photons in the near-infrared wavelength. Two-photon excitation and SHG processes have many important advantages compared with one-photon excited techniques, such as its higher spatial resolution, deeper penetration, better back ground rejection, less photonbleaching, and less damage[8]. At present, the nonlinear optical effect of SHG and two-photon excited fluorescence have been widely used in the fields of biophysics, biology, bioengineering, material sciences and medicine[1−9].

Endogenous species are related to tissue physiological and pathological states. Endogenous species are responsible for emission spectroscopy. So, emission spectroscopy can provide useful tissue structural and biochemical information and a powerful method to identify endogenous species[10−13]. Human skin has been the subject of numerous studies using optical spectroscopy[6,7,14]. The spectral variations of the skin autofluorescence have been demonstrated and used as a noninvasive tool for disease diagnosis such as distinguishing between malignant and normal tissues[14,15]. However, there have been a limited number of studies on two-photon excited spectroscopy and the obtained spectral data of human skin irradiated by intense femtosecond laser pulses present apparently lack[6,7]. This fact prompts further investigation into two-photon excited spectroscopy from *ex vivo* human skin. In this study, two-photon excited autofluorescence spectra and SHG signal from *ex vivo* human skin are systematically investigated by the use of multi-photon microscopy. The dependences of the emission spectra on excitation wavelengths are mainly recorded and discussed.

Figure 1 presents the schematic of experimental setup. It consists of a Zeiss LSM 510 META system and a Ti:sapphire femtosecond laser (110 fs, 76 MHz), tunable from 700 to 980 nm (Mira 900-F Coherent). An acousto-optic modulator (AOM) was used to control the laser intensity attenuation. In our experiment, the average laser power at sample was maintained at 5 mW. The lens of focusing lentgh 1 m was used to expand the beam to fill the back aperture of the objective. A plan-apochromat 63× (numerical aperture NA = 1.4) oil immersion objective (Zeiss) was employed for focusing the excitation beam and collecting the backward signals. We detected all signals and spectra in the backward direction. The signals were directed by a main dichroic beam splitter (DBS) to the META detector. The emission spectra of wavelength between 377—716 nm can be easily obtained by using META detector with 32-gated photon counting module. The detector was controlled

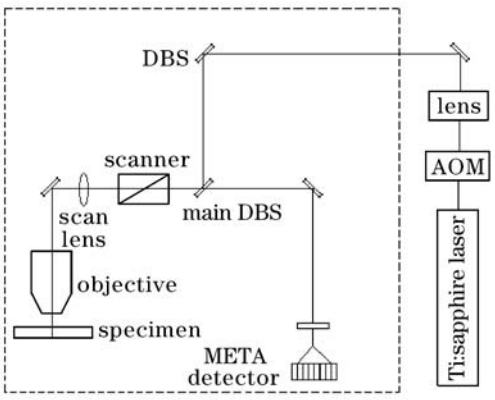


Fig. 1. Experimental setup consisting of a Zeiss LSM 510 META system and a Ti:sapphire femtosecond laser (tuning from 700 to 980 nm) pumped by a 10-W, diode-pumped laser.

via a standard high-end Pentium PC and linked to the electronic control system via an ultrafast small computer systems interface (SCSI). In this study, the specimens of excised human skin were taken from Affiliated Xiehe Hospital of Fujian Medical University, stored in liquid nitrogen (−196 °C) before they were used. The skin samples were excided perpendicular to the epidermal layer so that each section comprised a complete transverse cross-section of the epidermal and dermal layers, and each sample was sandwiched between the microscope slide and a piece of cover glass.

To investigate the two-photon excited spectroscopy from human dermis, the emission spectrum covering from 377 to 716 nm was recorded in the dermis at 800-nm excitation wavelength, as shown in Fig. 2. As can be seen, the whole spectrum can be divided into two apparently different ranges according to the spectral shape. The first range has only one peak with a narrower width. Through the Gaussian curve fitting analysis, the peak is located at 400 nm, which is nearly half the 800-nm wavelength of the light entering the specimen. If the excited light with 110-fs pulse width has a full width at half maximum (FWHM) bandwidth of approximately 15 nm, the second harmonic for Gaussion beam will have a FWHM of $1/\sqrt{2}$ of the fundamental or approximately 10 nm. The obtained spectral bandwidth was measured to be 9.8 nm, which is consistent with the above expectation. This is the characteristic of the nonlinear optical effect called SHG. Collagen is the most widespread of structural proteins in higher vertebrates. As demonstrated in previous works, its unique triple-helix structure and extremely high level of crystallinity make it exceptionally efficient in generating the second harmonic of incident light[1−5,16]. Collagen in the human dermis displays the mesh morphological structure, as shown in Figs. 3(a) and (b) (393 and 404 nm, central wavelength at about 400 nm). We further performed two control measurements. Firstly, the dependence of SHG signal from collagen on the excitation light intensity was investigated. Using the method of linear curve fitting, the fitted log-log intensity relation shows a slope of $1.98 \pm 0.07$ (data not shown here). The obtained quadratic dependence of SHG signal on the incident intensity is consistent with the theory of SHG[17]. Secondly, the excitation wavelength dependence of SHG from human dermis was examined, increasing toward the 790—830 nm region, as depicted in Fig. 4. The SHG spectra exhibit the ability to shift with the excitation wavelength. The dependence may have practical implications for tissue study because SHG can always be distinctly separated from other emission.

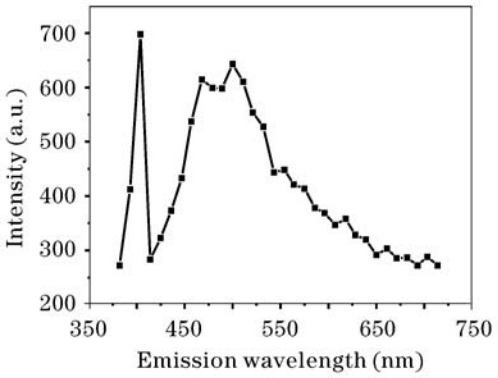


Fig. 2. Emission spectrum covering from 377 to 716 nm in dermis at 800-nm excitation wavelength.

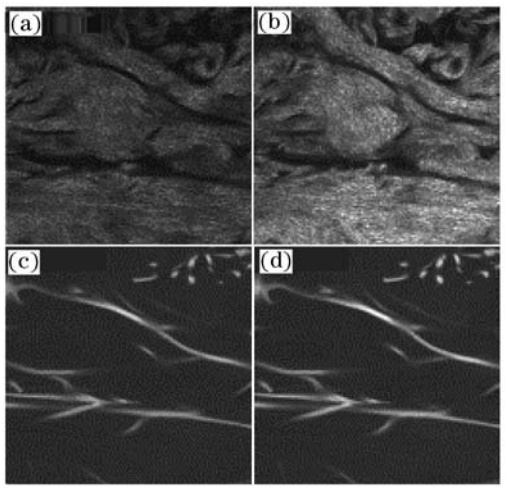


Fig. 3. Four representative two-photon images according to different wavelengths of 393 (a), 400 (b), 489 (c), and 500 nm (d) in the dermis.

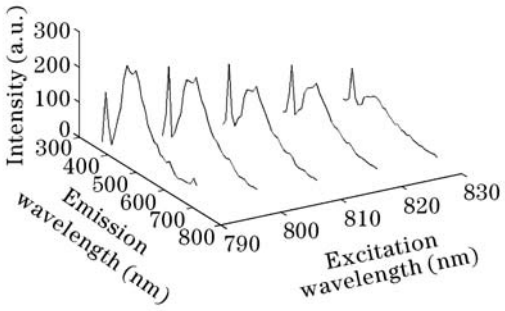


Fig. 4. Emission spectra obtained from dermis at various laser irradiation wavelengths.

The emission spectrum of the other range in Fig. 2 shows a broad peak with a monotonously decaying tail. This is the property of the autofluorescence spectrum. It can be seen from Fig. 2 that there are four distinct peaks at 470, 500, 575, and 618 nm. It was demonstrated in previous works that nicotinamide adenine dinucleotide (NADH), elastin, melanin, and porphyrins emit this band[4,6,13−16,18]. Thus, we believe that NADH, elastin, melanin and porphyrin contribute to the four peaks, respectively. Specifically, NADH fluorescence is responsible for the 470-nm peak. The 500-nm peak corresponds to the elastic fluorescence and elastin in the human dermis exhibits the morphology of thick ropes, as presented in Figs. 3(c) and (d) (489 and 500 nm, central wavelength about at 500 nm). And the 575- and 618-nm peaks are associated with the presence of melanin and porphyrin, respectively. It is worthy noting that the autofluorescence spectra show a red shift of the spectral peaks when increasing the excitation wavelength. As presented in Fig. 4, the central wavelength of autofluorescence is at about 493 nm (by the Gaussian curve fitting analysis) for 790-nm excitation wavelength and 500 nm for 830-nm excitation wavelength. This change may be because that with an increase of the excitation wavelength, the contribution of the flavin adenine

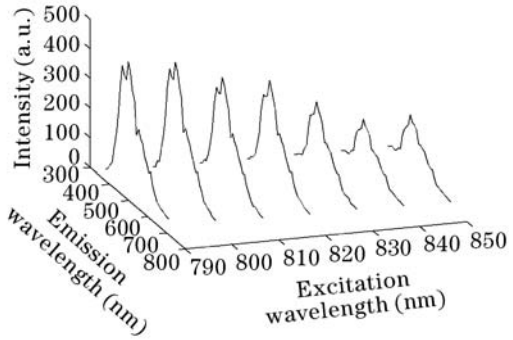


Fig. 5. Emission spectra acquired from epidermis at different excitation wavelengths.

dinucleotide (FAD) signal, peaking at 530 nm, becomes significant[19].

The dependence of the dermal autofluorescence signals on excitation intensity was also investigated. We measured the autofluorecence intensity in the wavelength range of 425—716 nm as a function of the incident intensity. The result of linear curve fitting gives a slope of $2.08 \pm 0.03$ like that of SHG (data not shown here). This result suggested that the obtained autofluorescence signal indeed originated from the two-photon excited fluorescence process.

In the epidermis, the emission spectra were acquired using seven different excitation wavelengths, the results are shown in Fig. 5. It can be seen from Fig. 5 that the emission spectra originating from the epidermis exhibit a wide peak with a long tail, which are different from those in the dermis. Similar to the autofluorescence spectra of dermis, there are four distinct peaks at 470, 500, 575, and 618 nm. According to the previous work[6,7,13−16,18], NADH, keratin, melanin, and porphyrin are responsible for the four peaks, respectively. It is also found that the central wavelength of autofluorescence shifts towards long wavelength when increasing the excitation wavelength. The phenomenon may be due to the contribution of FAD signal. Moreover, the dependence of the epidermal autofluorescence signal on the excitation intensity was also investigated. We measured the autofluorecence intensity in the wavelength range of 382—716 nm as a function of the incident intensity. The result of linear curve fitting gives a slope of $2.11 \pm 0.08$. This further indicates that the autofluorescence signal in the epidermis indeed arises from the two-photon excitation process.

In conclusion, when the femtosecond laser pulse irradiated the *ex vivo* human skin, the two nonlinear physical processes, namely two-photon excited autofluorescence and SHG, occur simultaneously. Our results provide some important biomorphology and biochemistry information on collagen, elastin, NADH, melanin, and porphyrins, the important biomarkers of human skin. It was also found that two-photon excited spectra of *ex vivo* human skin endogenous species depend on the excitation wavelength. The spectral data arising from the two-photon excited autofluorescence and SHG signal of *ex vivo* human skin may have practical interest for disease diagnosis such as distinguishing between malignant and normal tissues.

This work was supported by the National Natural Science Foundation of China (No. 60508017), the Program for New Century Excellent Talents in Fujian Province University, the Natural Science Foundation of Fujian Province of China (No. A0510015), the Innovation Foundation for Young Technological Talents of Fujian Province (No. 2003J027), and the Scientific Program of the Educational Department of Fujian Province (No. JA05215). J. Chen's e-mail address is chenjianxin@fjnu.edu.cn.

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