High-Contrast Multimodel Nonlinear Optical Imaging of Collagen and Elastin

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Abstract. Collagen and elastin, as the major components in the extracellular matrix (ECM), are intrinsic indicators of physiological and pathological states. Here, we have developed a high-contrast multimodel nonlinear optical imaging technique to imaging collagen and elastin by detecting simultaneously two photon-excited fluorescence (TPEF) from elastin and second-harmonic generation (SHG) from collagen. Our results show that this technique can obtain a high-contrast TPEF/SHG image in human dermis and permit direct visualization of collagen and elastin. It was shown that the technique can provide collagen and elastin structural information to determine collagen and elastin fibril orientation and distribution and acquire some morphometric properties. It was found that the in-depth TPEF/SHG imaging and 3-D reconstruction of TPEF/SHG images can extract more collagen and elastin structural and biochemical information. The study results suggest that the high-contrast multimodel nonlinear imaging provides a powerful tool to study ECM intrinsic components and has the potential to provide more important information for the diagnosis of tissue.

1. Introduction
Collagen and elastin are important structural proteins of extracellular matrix (ECM). Collagen plays an important structural role in skin, interstitial tissue and basal laminae. Modifications of the collagen fibrillar matrix structure are associated with various physiologic processes, such as wound healing, cornea diseases, osteoarthritis, liver fibrosis, and cancer [1-5]. Elastin is the protein responsible for the characteristic elastic properties of many tissues [6-8]. The elastic function complements collagen fibrils, which impart tensile strength. However, to understand the healthy and diseased tissues, a complete understanding of the arrangement and modification of the major structural proteins, such as collagen and elastin, is crucial. Thus, the visualization of the major structural proteins of the extracellular matrix (ECM), such as collagen and elastin, is of great value in gaining structural and diagnostic information.

So far, few techniques can achieve the selective visualization of collagen and elastin. Most techniques required the invasive removal of tissue samples, slicing by mechanical tools, embedding, fixation, and histological or immunohistochemical staining procedures [9, 10]. This fact has created the impossibility to detect collagen and elastin components within their natural environment and prompts to develop new techniques to observe collagen and elastin.
The last decade witnessed the emergence of multiphoton microscopy (MPM) as a powerful tool for the observation of unstained samples based on intrinsic sources of nonlinear signals [11, 12]. Since the high photon flux required for non-linear excitation limits the excitation volume, a confined and spot, multiphoton microscopy has stronger axial depth discrimination and less photodamage and photobleaching, allowing in vivo imaging application [13, 14]. The use of near infrared light significantly extends imaging depth because of reduction in tissue absorption and scattering. For multiphoton excitation, the excitation wavelength is much farther removed from the emission band, so it has high Signal-to-Background Ratio [13]. Furthermore, second-harmonic generation (SHG) can be used as a second-order nonlinear optical process imaging tool for examining intrinsic structural protein [15, 16]. SHG is a nonlinear coherent scattering process and requires laser interaction with a medium lacking a center of symmetry such as an interface, for example, a membrane, or the chiral structure collagen [1-3, 15, 16].

In this study, we demonstrated that the high-contrast multimodel nonlinear optical imaging technique to imaging collagen and elastin by the coregistration of TPEF and SHG in the human dermis. We also show that the technique has the capability of in-depth TPEF/SHG imaging and 3-D reconstruction. Finally, we qualitatively interpret the origin of the backward SHG signals from tissue.

2. Physics background
In general, the nonlinear polarization for a material is defined by:

$$P = X^{(1)}E^1 + X^{(2)}E^2 + X^{(3)}E^3 + ...$$

Where $P$ is the induced polarization, $X^{(n)}$ is the nth order nonlinear susceptibility, and $E$ is the electric field vector of the incident light; The first term describes normal absorption and reflection of light; the second term describes SHG, sum and difference frequency generation, hyper-Rayleigh; the third term covers both two and three photon absorption, third harmonic generation, and coherent anti-Stokes Raman Scattering. The processes of two-photon excited fluorescence (left) and second harmonic generation (right) are shown in Figure 1.

![Energy state diagram. Second harmonic generation (SHG) involves virtual transitions in which no energy is absorbed by the specimen (right). In contrast, two-photon excited fluorescence (TPEF) involves the absorption of energy (real transitions) and excitation of molecules (left). The wavelength of SHG is half of the excitation wavelength, while TPEF has an emission wavelength more than half of the excitation wavelength.](image)

Two-photon excited fluorescence is a nonlinear process involving the absorption of two photons whose combined energy is sufficient to induce a molecular transition to an excited electronic state. In the process of two-photon excited fluorescence, a molecular makes a transition from its ground state ($s_0$) to a real excited state ($s_1$) by the simultaneous absorption of two laser photons and quickly transits to a metastable state ($s_2$), approximately $10^{-11}$s, and then emits one photon with higher frequency ($\omega_{TPEF}$ but less than $2\omega_{ex}$) while returning to the ground state ($s_1\rightarrow s_0$), about $10^{-9}$s. SHG, by contrast, is
a nonlinear coherent scattering that conserves energy, and therefore the SHG wavelength is always half the excitation wavelength. In this case, both the intermediate levels are virtual states. So, the spectral profiles of TPEF and SHG are very different. In TPEF, the relative energies and geometries of ground and first excited electronic states determine the wavelength and width of the emission spectrum. Thus the emission properties are dependent only on the molecular properties and are independent of the characteristics of the excitation laser. By contrast, the spectral characteristics of SHG are derived from the laser source: the bandwidth scales as $1/\sqrt{2}$ of the bandwidth of excitation laser. A 100-fs laser pulse has a full width half-maximum (FWHM) bandwidth of about 10nm, for example, and thus results in a SHG spectrum of about 7nm FWHM. Moreover, SHG can always be distinctly separated from other emission for the ability to shift SHG wavelength by tuning the excitation wavelength. Therefore, the two imaging modalities (TPEF and SHG) are easy to implement simultaneously and differ only in optical filter selection and detector placement.

3. Materials and Methods

All the experiments described in this paper were based on ex-vivo human skin. Human skin was obtained from Affiliated XieHe Hospital of Fujian Medical University. The specimens were stored at a bottle of 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 sandwiched between the microscope slide and a piece of the cover glass. To avoid dehydration or shrinkage during the whole imaging process, the specimen was sprinkled with PBS solution (PH 7.4). The dermal part of ex-vivo human skin is our region of interest.

In this study, the high-contrast multimodel nonlinear optical imaging was performed on a Zeiss LSM 510 META laser scanning microscopy equipped with a mode-locked femtosecond Ti: sapphire laser (110fs, 76MHz), tunable from 700nm to 980nm (Coherent Mira 900-F), as shown in Figure 2.

An Acousto-Optic Modulator (AOM) is used to control the laser intensity attenuation. A Plan-Apochromat 63× (N.A.=1.4) oil immersion objective (Zeiss) was employed for focusing the excitation beam and for collecting of the backward signals. We detected all signals in the backward direction. The signals were directed by a main dichroic beam splitter (MDFS) to the META detector. The META detector with 32-gated photon counting module is used to collect x-y images at a series of emission wavelengths, covering from 377nm to 716nm. Each photon counting module can cover a spectral range of 10.7nm. An IR beam block filters (Zeiss KP650), which is in front of META detector, was be used to ensure that excitation light was filtered out and only emission signals were recorded. The LSM 510 META laser scanning microscopy is controlled via a standard high-end Pentium PC and linked to the electronic control system via an ultrafast SCSi interface. This system has an especial multichannel imaging mode. The multichannel mode has eight independent-channels and
each channel can selectively be set to detect emission signals within the random range from 377 to 716 nm to achieve imaging. In this study, we selected two independent-channels from eight channels to achieve SHG (387-409nm) and TPEF (447-628nm) imaging of human dermis, respectively. The acquisition of a single 512x512 pixel image was generally achieved within a few seconds. All the images presented in this article are single optical sections. The average laser power at the specimen was maintained at <6mw and no photonbleaching was observed at this low power level.

4. Results and Discussions

4.1. High-contrast multimodel nonlinear optical imaging of human dermis

To demonstrate high-contrast multimodel nonlinear optical imaging, we have examined samples of human dermis. A representative high-contrast multimodel nonlinear optical image of human dermis acquired for an excitation wavelength $\lambda_{ex}=800\text{nm}$ is shown in Figure 3.

![High-contrast multimodel nonlinear optical imaging of human dermis](image)

As can be seen from Figure 3 (a), the collagen mesh of dermis can be imaged alone by one channel (387-409nm, green color-coded). Similarly, in the Figure 3 (b) the elastin and fibroblasts components of dermis can be isolated from collagen using another channel (447-628nm, red color-coded). Overlaying two channels yields a high-contrast TPEF/SHG image of human dermis, where structural details of the sample can be readily distinguished, as shown in Figure 3 (c). The image (green color-coded) shows a fine mesh morphology which correspond to collagen and probes the distribution and orientation of collagen. Collagen is the predominant structural protein in most biological tissues, as well as an efficient source of SHG [1-3, 15, 16-19]. Modifications of the collagen fibrillar matrix structure are associated with various physiologic processes, such as wound healing, aging, diabetes, and cancer. Therefore, SHG is very promising as a sensitive probe in tissue morphology and physiology studies. The image (red color-coded) displays the morphology of thick ropes that correspond to elastin fibers with diameters of~1.96$\mu$m. Elastin that coil and recoils like a spring within the elastic fibers of connective tissue and accounts for the elasticity of structures such the skin, blood vessels, heart, lungs, intestines, tendons, and ligaments. It should be noted that the image contrast is significantly improved in the present study as compared with the previous study [8]. Since two channels are independent and can possess different system parameters as shown in Table 1, they can acquire images with the optimal system parameters which can enhance the image contrast. The method is effective in improving the image (SHG and TPEF) contrast.
Table 1. System parameters of two independent-channels.

| Channels | Detector Gain | Amplifier Offset | Amplifier Gain | Laser Power(mw) |
|----------|--------------|------------------|----------------|----------------|
| One(SHG) | 759          | 0                | 1              | 6.0            |
| Two(TPEF)| 742          | -0.17            | 1              | 2.6            |

4.2. In-depth high-contrast multimodel nonlinear optical imaging and reconstruct 3-D TPEF/SHG images
To illustrate this technique has capability to generate high-contrast structural images deep inside highly scattering tissues, high-contrast multimodel nonlinear optical images were obtained at various depths, z, into the human dermis ranging from 0 to 40μm. Figure 4 (a)-(e) shows channel-mode nonlinear optical images from human dermis at depths of 0, 10, 20, 30, 40μm, for. As represented in Figure 3, detailed structural information can be deduced from the images throughout the examined depth range. The signal progressively degrades with increasing depth, but the images still bear high-contrast. This technique also allows us to reconstruct high-contrast 3-D TPEF/SHG images by reconstructing the overall image from each channel and overlaying two channels. This was done by imaging a 60μm section of dermis in 0.5μm increments at an excitation wavelength through each channel. 3-D images were then reconstructed for these channels. Channel-mode nonlinear optical image of human dermis 3-D reconstruction were shown in the Figure 5, which provides more collagen and elastin structural information.

Figure 4. High-contrast multimodel nonlinear optical image of human dermis at depths of 0, 10, 20, 30, and 40μm (P_{SHG}=6.09mw, P_{TPEF}=2.61mw, \lambda_{ex}=800nm).

Figure 5. 3-D reconstruction of TPEF/SHG images from human dermis, obtained from a stack of 120 images acquired every 0.5μm at the same region as Figure 4 (P_{SHG}=6.09mw, P_{TPEF}=2.61mw).

5. Conclusion
We have coupled a mode-locked femtosecond Ti: sapphire laser to a Zeiss LSM 510 META laser scanning microscopy to develop a multimodel nonlinear optical imaging technique and employ it for the imaging of collagen and elastin. The experimental results demonstrate that the technique can permit high-contrast visualization of collagen and elastin. Our findings indicate that in-depth TPEF/SHG imaging and 3-D reconstruction of TPEF/SHG images can provide more collagen and elastin structural and biochemical information.
A major advantage of the multimodel nonlinear optical imaging technique is that it relies on nonlinear physical processes, namely TPEF of elastin and SHG in collagen molecules, providing the unique possibility to image selectively the extracellular components without any staining and slicing procedures. Another major advantage is that the technique can provide unique contrast enhancement to the previous study in TPEF/SHG image by using two-independent channels with the optimal system parameters.

The technique has therefore the potential to become valuable tools for studying ECM intrinsic components and to diagnose a variety of diseases including ECM related dermatological disorders.

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