Origins of Backward Second-Harmonic-Generation Emission in a Biological Sample Examined by Laser Scanning Microscopes

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Abstract: We characterize the backward second-harmonic-generation (SHG) emission in biological samples with a SHG microscope. Contributions from different mechanisms, including direct backward emissions, reflection and back-scattering of the forward emissions, are identified and clarified.
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Recently, second-harmonic-generation (SHG) is emerging as a powerful contrast mechanism in nonlinear optical microscopy. In contrast to laser-induced fluorescence, SHG is known to leave no energy deposition to the interacted matters due to the energy conservation characteristic, providing the optical noninvasive nature desirable for microscopy applications, especially for clinical imaging [1]. Due to its nonlinear nature, the generated SHG intensity depends on square of the incident light intensity. Similar to multiphoton induced fluorescence, this nonlinear dependency allows localized excitation and is ideal for intrinsic optical sectioning in laser scanning microscopy. It is important to utilize the intrinsic nonlinear harmonic generation for imaging purpose to replace unnecessary usage of invasive and toxic fluorophores in common multiphoton fluorescence microscopy. Moreover, since SHG is sensitive to local anisotropy, to the distribution of molecular hyperpolarizabilities, to phase-matching properties, to membrane potential [2], and to surface plasmon enhanced interactions [3], it can be used as a complementary imaging modality to the well-developed multiphoton fluorescence microscopy and third harmonic generation microscopy.

Because harmonic generation is a coherent process, the emitted SHG must satisfy phase matching constraints, producing highly directed radiation rather than isotropic emission. SHG directionality is dependent on the distribution and directionality of the induced dipoles within the focal volume. Due to momentum conservation, SHG emission tends to propagate along with incident laser light and thus forward collection is preferred. Thus most biological SHG microscopy adopts a forward collection geometry. However, for clinical applications with optically thick bio-tissues, it is essential to develop backward-collected SHG (B-SHG) microscopy. Though some experiments were arranged to collect backward SHG [1,4], the backward nonlinear signals were commonly designated as the backscattering from the forward-propagating signals, instead of direct backward emission. The comparison of forward-SHG (F-SHG) and B-SHG has been made very recently by Cox et al. [5] and Zipfel et al. [6]. The former study concluded that very little primary SHG was generated in backward direction from collagen fibrils due to their dense arrangement in tissue while the latter only provided a description of the relationship between the collagen fibril orientation and SHG intensity in both forward and backward directions.

In this talk, we will present the characterization the B-SHG as well as the F-SHG from biological samples, with a focus on collagen and muscle tissues which together constitute more than 50% of human body weight and both are known to exhibit strong SHG emission. Spectroscopic and power-scaling methods were adopted to certify the collected backward signal to be SHG. The difference of B-SHG/F-SHG is quantitatively compared in muscle and collagen tissues, and a surprisingly strong backward SHG emission is unraveled within collagen matrix for the first time. Contributions from different mechanisms, including direct backward emissions, reflection and back-scattering of the forward emissions, are identified and clarified.

We first simultaneously acquired F-SHG and B-SHG images in a 4-μm thick mouse leg muscle microtomic slice.

With such a thin slice, the effect of backscattering should be negligible. In muscle fibrils, the B-SHG intensity was about 5% of F-SHG, close to the reflectivity of the underneath interfaces. Negligible direct backward emitted SHG from muscle was produced due to destructive interference of the coherently emitted SHG in the backward direction. However, the B-SHG can be 2 ~ 4 times stronger than F-SHG in a single collagen fibril, too large to be simply contributed by interface reflection or backscattering of the F-SHG. To simplify the condition, collagen fibrils fabricated on a cover glass were used to characterize the B-SHG from a single fibril. Figure 1 A-C shows an example AFM, B-SHG, and F-SHG images, acquired from the same fabricated collagen fibril matrix. Fig. 1 *D* gives the polarization dependency of both F-SHG and B-SHG of a single collagen fibril, which are integrated within the selected area (shown in Fig. 1, *B* and *C*). The observed polarization behavior indicates a cylindrical fibril structure symmetry. Both the F-SHG and B-SHG exhibit broadened maxima with local minima in the center. For the detailed study on the relation between SHG intensity and collagen fibril thickness, the half-wave plate was then fixed at 30-deg (the laser polarization is shown as arrows in Fig. 2, *B* and *C*) with relatively strong B-SHG and F-SHG emissions. The SHG power variation along the selected fibril is shown in Fig. 2 *E*. The power variation is found to be induced by the local collagen fibril structure and arrangement, which can be revealed with a nanometer resolution in the AFM image. Detailed polarization behavior on individual artificial collagen fibrils can then be analyzed with the help of the elegant connection between polarization and the coherence effect (not shown here), which in turn was found to determine the B-SHG/F-SHG intensity ratio.

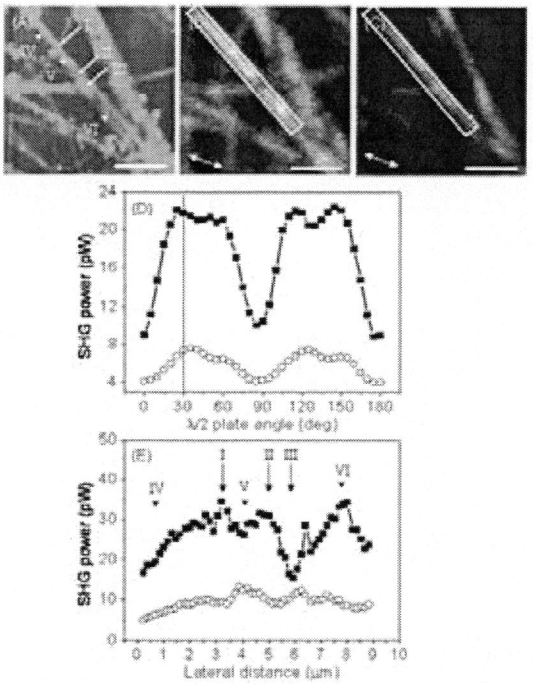

Fig.1 (A) AFM, (B) B-SHG, and (C) F-SHG images of an artificial collagen fibril sample on a cover glass. Scale bar: 3-μm. (D) is the polarization dependency of the selected rectangular area for B-SHG (solid rectangles) and F-SHG (hollow circles). No polarizer before the PMT is used. (E) is the integrated linegraph of the rectangular area for B-SHG (solid rectangles) and F-SHG (hollow circles), showing the SHG power variation along the selected fibril. These are acquired as the halfwave plate rotated at 30-deg (laser polarization is shown as double-sided arrows in (B) and (C)).

Fig. 2, *A* and *B*, show the scanning B-SHG and F-SHG images taken from a 4-μm microtomic slice around a dermis-muscle junction from a laboratory mouse leg. The muscle fibers exhibit strong F-SHG while only weak B-SHG, mainly from reflection of slice/glass interfaces, can be observed. It can be found that although the B-SHG and F-SHG images around collagen tissue part are qualitatively similar, they cannot match exactly with each other. Detailed fibril thickness and interaction can only be revealed through careful comparison between B-SHG and F-SHG intensity as well as polarization dependency. For example, the similarity of polarization dependency of B-SHG and F-SHG in area I (Fig. 2 *C*) suggests that there is only a single fibril or parallel-aligned fibrils within the selected region. From the power ratio between B-SHG maximum and F-SHG maximum, we can expect a ~ 300-nm fibril diameter in this area (refer to the point III in Fig. 1). However the coherent interference of two crossing collagen fibril results in different polarization dependency of B-SHG and F-SHG in areas II and III (Fig. 2 *E, F*).

In order to characterize the back scattering effect in a thick tissue, the amount of backscattering light was compared with an incident light at the SHG wavelength on samples with different thickness. Even though our study indicated that the backscattering is not the dominant backward emission source for collagen fibrils, it is however the dominant back emission mechanism for SHG generated in muscle fibers, due to the destructive interference of the direct backward emitted SHG from the optically thick muscle fiber. It is worth to notice that although the overall backscattering of collagen tissue is not very strong (~ 5%), the strong reflection of individual collagen fibril can severely deform the focus, resulting in weaker collection efficiency deep inside the sample.

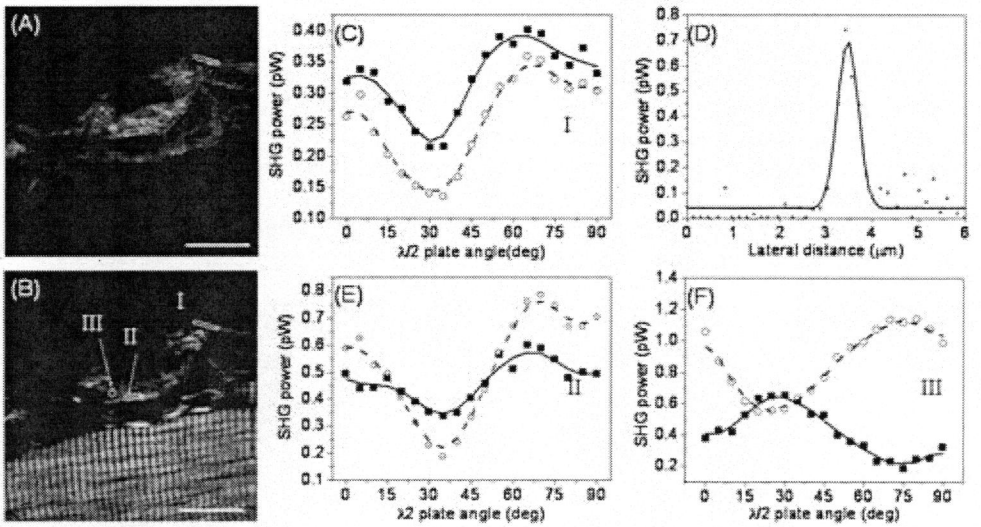

Fig. 2. (A) and (B) are scanning B-SHG and F-SHG images of a 4-μm slice from a mouse leg. Scale bar: 20-μm. (C), (E), and (F) are the polarization dependencies of B-SHG (solid rectangles) and F-SHG (hollow circles) at the selected areas I, II, and III, respectively. Solid and dashed lines are smoothing curve for B-SHG and F-SHG, respectively. (D) is a cross-sectional linegraph of area I with a Gaussian fitting curve. The FWHM is ~ 550-nm.

In summary, we studied the physical origin of the backward SHG emission in biological samples with a SHG microscope. The B-SHG in muscle is mainly contributed by the backscattering of F-SHG from underlying tissues, and the estimated collectable B-SHG from the muscle tissue in a thick sample is on the order of a few femtowatt in a normal experimental condition, much less than our current system sensitivity. For collagen tissues, on the other hand, we proposed that the bipolar emission from each collagen fibril is asymmetric with backward emission preferred. We have demonstrated, for the first time, that with a single collagen fibril of 100 ~ 200-nm diameter, B-SHG can be 2 ~ 4 times stronger than F-SHG. We also demonstrate that tight relationship among the ratio of B-SHG/F-SHG, local fibril arrangement such as fibril crossing, and laser polarization arrangement. It is explicitly shown for the first time that SHG emission power is extremely sensitive to collagen fibril diameter and may be used as a noninvasive tool for collagen fibrillogenesis study and for potential early disease diagnosis. This work is sponsored by National Health Research Institute of Taiwan through NHRI-EX93-9201EI and NTU Center for Genome Medicine.

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