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Abstract. Most colorectal cancers arise from dysplastic lesions, such as adenomatous polyps, and these lesions are difficult to be detected by the current endoscopic screening approaches. Here, we present the use of an intrinsic second-harmonic generation (SHG) signal as a novel means to differentiate between normal and dysplastic human colonic tissues. We find that the SHG signal can quantitatively identify collagen change associated with colonic dysplasia that is indiscernible by conventional pathologic techniques. By comparing normal with dysplastic mucosa, there were significant differences in collagen density and collagen fiber direction, providing substantial potential to become quantitative intrinsic biomarkers for *in vivo* clinical diagnosis of colonic dysplasia. © 2011 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.3659715]

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Colorectal cancer (CRC) is a major cause of cancer-related deaths in the world.¹ It is commonly believed that adenomatous polyps, or adenomas, appear to be major precursors to CRC, and the morbidity rate from CRC could be significantly reduced if these dysplastic lesions were detected.^{2,3} However, it is challenging that the current endoscopic screening methods with white light detect these dysplastic lesions.^{4,5} Thus, the development of new approaches to identify such lesions is of great medical significance.

Recently, the remodeling of the stroma has been implicated in colonic dysplasia,^{6,7} and these alterations may provide a better biomarker of these lesions than currently available screening methods. The stroma is composed primarily of type-I collagen, which is known to induce a second harmonic generation (SHG) signal.^{8,9} SHG microscopy, in recent years, has emerged to be a promising technique for the qualitative and quantitative

characterization of stromal biology with advantages of being label-free, inherent three-dimension resolution, near-IR excitation for superior optical penetration, lower photodamage, and capable of providing quantitative information.^{8–11} Therefore, it has been recognized as promising approaches for cancer diagnostics.^{9,12–14} To date, we have found no previous studies that quantitatively investigated the remodeling of the stroma in dysplastic colonic mucosa, and this is what motivated us to do this work. In this study, we used SHG microscopy to analyze the collagen change of normal and dysplastic colonic mucosa.

SHG microscopy was achieved using a nonlinear optical system which has been described previously.¹⁴ In brief, SHG images were acquired using a commercial laser scanning microscopic imaging system (Zeiss LSM 510 META, Jena, Germany) coupled to a femtosecond Ti:sapphire laser (Coherent Mira 900-F) operating at 800 nm. The polarization direction of the laser light is the horizontal polarization. An oil immersion objective ($\times 63$ and $NA = 1.4$) was employed for focusing the excitation beam into tissue samples (average power less than 15 mW) and was also used to collect the backscattered intrinsic SHG signals. The images were obtained at 2.56 μs per pixel. A fine focusing stage (HRZ 200 stage, Carl Zeiss) was used to change the focus position for recording various optical sections.

A total of nine fresh *ex vivo* colon tissue samples were obtained immediately after resection from patients undergoing a colectomy for familial adenomatous polyposis. Prior to study participation, all patients signed an informed consent, and this study was approved by the Institutional Review Board of Fujian Medical University. Colonic adenomas are dysplastic lesions. Typical adenomas are polypoid structures and the adenomas are surrounded by normal tissue. In this study, the surrounding normal regions and the polyp regions were imaged. After SHG imaging, the tissue specimens were fixed in 10% formalin and prepared for pathologic examination using standard protocols. In this work, all data were presented as a mean value plus-or-minus its standard deviation, and analyzed using Student's *t*-test. Differences were considered to be statistically significant when the *P*-values were less than 0.05.

Shown in Fig. 1 are the representative *en face* SHG images from normal and dysplastic colonic mucosa. As can be seen from Fig. 1, the stroma can be identified because the epithelium consists mainly of columnar epithelial cells and goblet cells that are not effective in generating SHG signals and the stroma is composed primarily of type-I collagen that is capable of emitting strong SHG signals. Morphologically, normal and dysplastic colonic mucosa can be easily discriminated. In a normal case, the collagen displays a denser matrix, and the collagen fibers are almost parallel to the interface of epithelium and stroma. In dysplasia, a looser collagen matrix is observed, and the collagen fibers are located at an angle to the interface of epithelium and stroma. It is worth pointing out that these features are not visible in standard hematoxylin and eosin-stained sections, as presented in Fig. 2.

In the following, to further quantify these features, two quantitative analyses were performed. First, the depth-dependent decay (DDD) of the SHG signal was analyzed to determine the collagen density. In short, a series of SHG images were obtained at intervals of 1 μm up to a final depth of 30 μm ;

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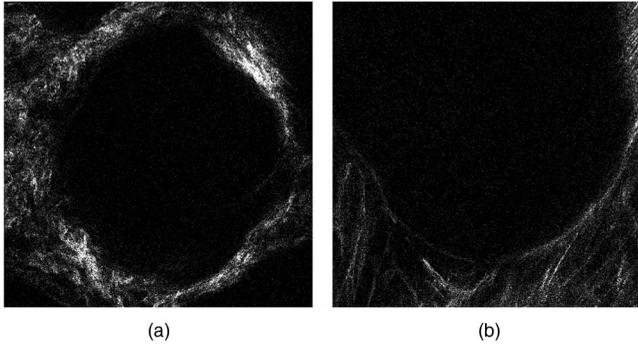


Fig. 1 Representative SHG images from (a) normal and (b) dysplastic colonic tissues. The size of the images is $146 \times 146 \mu\text{m}^2$.

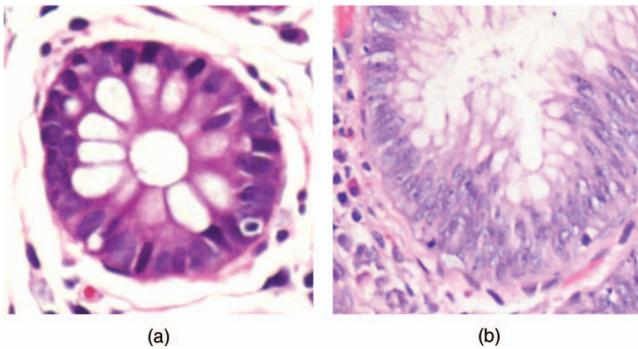


Fig. 2 Typical histological images (magnification, 40x), from (a) normal and (b) dysplastic colonic tissues.

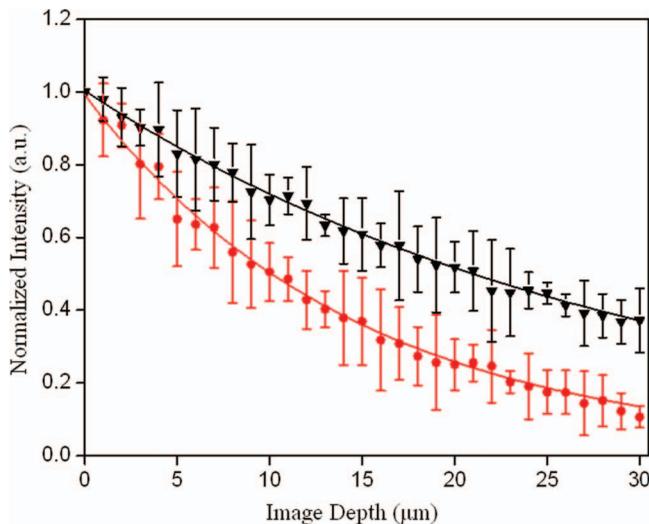


Fig. 3 Typical plot of the exponential fits from normal (circles), and dysplastic (triangles) colonic tissues; intensity at depth $0 \mu\text{m}$ has been normalized to unity in each case.

Table 1 Quantitative variables with dysplasia.

	DDD constant	Collagen fiber angle (deg)
Normal case	0.068 ± 0.012	3 ± 2
Dysplasia	0.031 ± 0.015	51 ± 7

the average SHG intensity of selected regions at each depth was then calculated and plotted as a function of depth. Decrease in SHG signal intensity with increasing depth can be approximated using a first-order model: $I = Ae^{-kx} + C$, where I is the SHG intensity, x is the imaging depth, A is a pre-exponential scaling factor, C is an arbitrary constant that adjusts the lowest signal intensity to zero, and k is the DDD constant.¹⁵ Figure 3 shows a typical plot of the exponential fits from normal and dysplastic colonic tissues. Recently, Dunn et al. found that although scattering plays a role in DDD, absorption is the main factor responsible for DDD in turbid media at least to a depth of focus of $412 \mu\text{m}$. Thus, although other factors, such as direction, distribution, and size of collagen fibers, may contribute to changes in the DDD value, it is a reasonable measure of collagen density.¹⁶ Moreover, the collagen fiber angle relative to the tangent of the interface of epithelium and stroma was measured every $5 \mu\text{m}$ using IMAGEJ software (National Institutes of Health), as reported previously.¹⁷ As shown in Table 1, the dysplastic colonic mucosa has a lower DDD constant and a bigger collagen fiber angle ($p < 0.05$), indicating loss of the collagen density and the collagen fibers located at an angle to the interface of epithelium and stroma in dysplasia, consistent with the above-mentioned observations. Therefore, these differences in the collagen density and the collagen fiber direction may be used to quantitatively discriminate between normal and dysplastic colonic mucosa, and serve as quantitative intrinsic biomarkers for quantifying the diagnosis of colonic dysplasia.

In conclusion, this study demonstrates the potential of intrinsic SHG imaging to provide biochemical and morphological biomarkers, including the collagen density and the collagen fiber direction, which can be used to discriminate between normal and dysplastic colonic mucosa. With the advent of the SHG-based endoscopy,¹⁸ we expect that the analysis of collagen change using intrinsic SHG imaging may become an important noninvasive methodology that provides quantitative information about the collagen change for diagnosing colonic dysplasia.

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