Extracting diagnostic stromal organization features based on intrinsic two-photon excited fluorescence and second-harmonic generation signals

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Abstract. Intrinsic two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) signals are shown to differentiate between normal and neoplastic human esophageal stroma. It was found that TPEF and SHG signals from normal and neoplastic stroma exhibit different organization features, providing quantitative information about the biomorphology and biochemistry of tissue. By comparing normal with neoplastic stroma, there were significant differences in collagen-related changes, elastin-related changes, and alteration in proportions of matrix molecules, giving insight into the stromal changes associated with cancer progression and providing substantial potential to be applied *in vivo* to the clinical diagnosis of epithelial precancers and cancers. [DOI: 10.1117/1.3088029]

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A number of optical spectroscopic and imaging techniques have widely been used for the *in vivo*, real-time detection of epithelial precancers and cancers.[1] However, most studies focus only on the epithelium to analyze morphological, structural, and architectural changes that accompany development of epithelial precancers.[2] Recently, it has been recognized that stromal biology is also altered significantly with various pathological processes.[2] However, there is a lack of utilizing the alterations in the stroma as an intrinsic indicator of disease states, which can extract quantitative information about the biomorphology and biochemistry and can provide a new means to improve early detection of neoplastic changes.

Multiphoton microscopy has several advantages over traditional confocal microscopy, providing high-resolution images at increased imaging depths, minimal out-of-plane absorption, and inherent optical sectioning.[3] It is well suited for the observation of unstained samples based on intrinsic sources of nonlinear signals,[4] such as two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG). It also has been used for *in vivo* cancer imaging.[3]

Our primary goals in this study are to investigate the intrinsic TPEF and SHG signals in epithelial stroma and to extract biomorphologic and biochemical features for potential tissue diagnosis.

The multiphoton system used in this study has been described previously.[4] Briefly, it is an Axiovert 200 microscope (Zeiss LSM 510 META) equipped with a mode-locked femtosecond Ti:sapphire laser (Coherent Mira 900-F). The polarization of laser light is the linear polarization. To suppress TPEF signals from collagen, the excitation wavelength at 850 nm was used in this work.[4] An oil immersion objective ($\times 63$ and NA$=1.4$) was employed. The META detector was used to detect all signals in backscattered geometry. The average laser power at the sample was 5 mW. In this study, all images were 512×512 pixels. The images were obtained at 2.56 μs per pixel. In this work, considering that the surface of the sections is not absolutely flat and that the signals in the surface of sections is not absolutely maximal, we defined a depth of 0 μm, referring to the position where the multiphoton signals of reflection from the interface between the tissue and the glass coverslip reaches to maximum. All measurements were performed at depth of 0 μm. In this work, clinically normal and abnormal esophagus biopsy pairs from nine patients, were used. Each biopsy was divided into two parts. One part was immediately stored in a bottle of liquid nitrogen ($-196\,^{\circ}$C) and then embedded in optimal cutting temperature (OCT) medium and sectioned into 200-μm-thick transverse tissue slices (the stromal part is our region of interest) for multiphoton measurement. The second part was placed in buffered formalin for histopathology, showing that 9 samples were esophageal cancer at different clinical stages (3 T1N0M0, 1 T2N0M0, 3 T1N1M0, 2 T3N1M0) and 9 were normal esophagus.

To extract the biomorphologic and biochemical features of human esophageal stroma, multiphoton images of the stromal portion from each specimen were obtained. Figure 1 shows the representative multiphoton images of the human esophageal stroma from a biopsy pair. The SHG images are shown in the left column, the TPEF images are shown in the middle column, and the combined SHG/TPEF images are displayed in the right column. According to the previous studies,[3,4] the SHG signal results from collagen, whereas the TPEF images mainly arise from the elastin in the stroma. It is seen in Fig. 1 that several interesting results can be obtained. First, the collagen matrix of the normal stroma is a denser and more organized structure with long fibrils, whereas the collagen matrix of the neoplastic stroma exhibits a loss of fine structure and structural organization. Second, elastin in normal stroma displays the morphology of strings, while elastin in neoplastic is shorter and more fragmental, and it also has an irregular getting together tendency and an increased amount, like stromal elastosis.[5] Last, as can be seen in the combined SHG/TPEF figures, there is a significant alteration in the proportions between collagen and elastic fibers, consistent with which the

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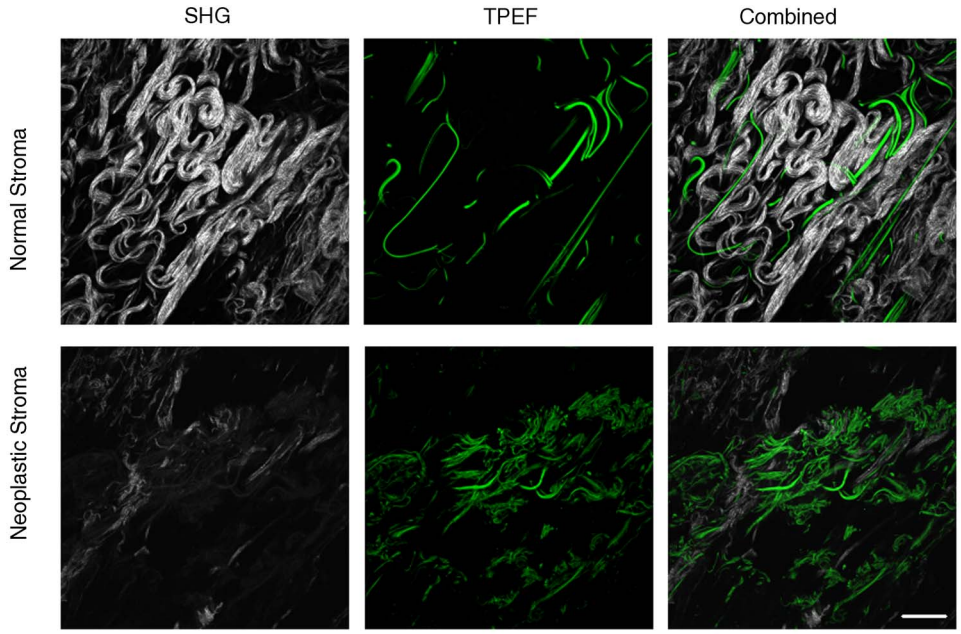

Fig. 1 From left to right: SHG, TPEF, and combined SHG (gray) and TPEF (green) images of the human esophageal stroma. Top to bottom: normal stroma and neoplastic stroma. The excitation wavelength λ_{ex} was 850 nm. Scale bar=20 μm. (Color online only.)

neoplastic stroma consists of abnormal proportions of matrix molecules.[2] In the following, we performed the quantitative analysis of these interesting results.

To quantitatively assess the collagen-related changes, we did two analyses. First, we performed gray-level co-occurrence matrices (GLCMs) texture analysis to SHG images. Based on gray-level statistical patterns between neighboring pixels, the GLCMs can provide texture features. In particular, by indicating the fibril and separation, the correlation feature, a measure of intensity correlation as a function of pixel distance, relates to collagen fibril structure. In detail, if the correlation falls off sharply with pixel distance, the collagen matrix presents distinct, linear fibrils; if it remains elevated as pixel distance is increased, the collagen matrix has less defined fibrillar structure.[6] In this work, we calculated the correlation for distances ranging from 1 to 60 pixels (0.4 μm to24.0 μm) in the horizontal direction of each optical section. (The vertical direction had similar results.) As can be seen in Fig. 2 (left), the normal stroma correlation fell off sharply with distance, indicating distinct, linear fibrils, whereas the neoplastic stroma correlation remained elevated as distance increased, suggesting less defined fibrillar structure. The correlation value at the distance of 30 pixels shows significant differences between normal and neoplastic stroma ($P<0.01$), shown in Fig. 2 (right). Second, we calculated the collagen area. In each SHG image, the ratio of the SHG pixels to the whole pixels is defined as the collagen area. In this study, the collagen area in normal stroma is 0.727 ± 0.056 ($n=27$ slices of 9 biopsies), and in neoplastic stroma, it is 0.213 ± 0.082 ($n=27$ slices of 9 biopsies), reflecting collagen loss in the neoplastic stroma. There were significant differences between the normal and neoplastic stroma ($P<0.01$). Thus, the neoplastic stroma with higher correlation and lower

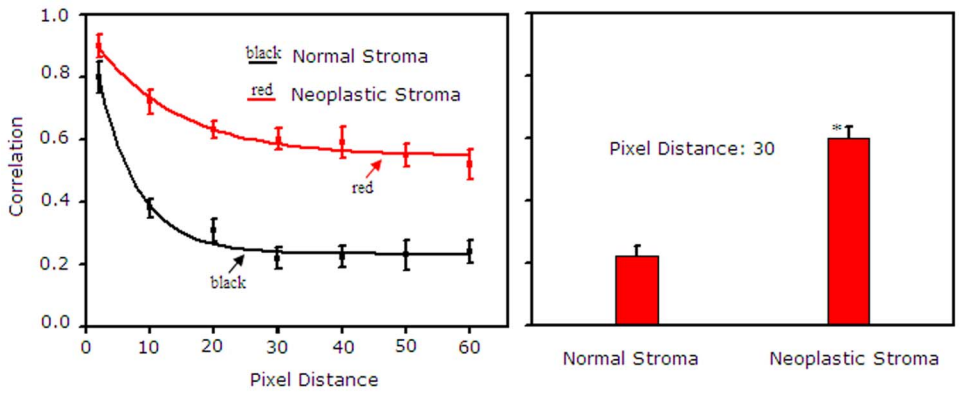

Fig. 2 Left: Quantitative collagen-related changes, showing that the neoplastic stroma displayed higher correlation with distance consistent with a loss of fine fibril structure. Right: correlation value with 30 pixels distance in normal versus neoplastic stroma. Error bars indicate calculated standard deviations. A Student *t*-test is performed against normal stroma for comparison. $^{*}P<0.01$. (Color online only.)

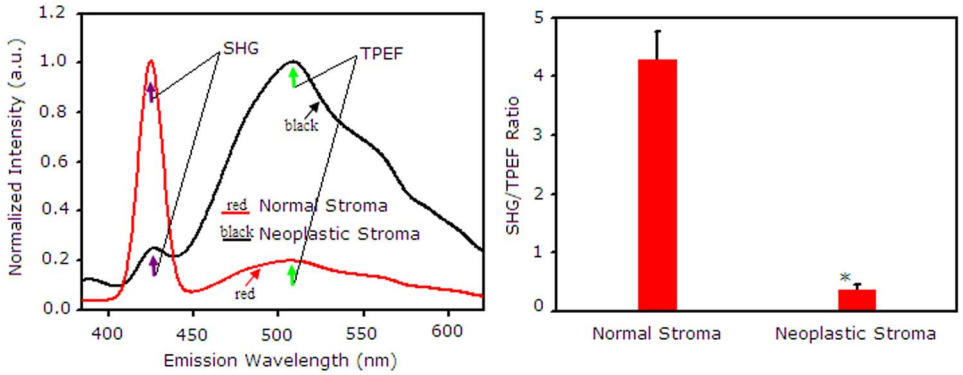

Fig. 3 Left: Emission spectra obtained from normal versus neoplastic stroma for $\lambda_{ex}=850$ nm. Right: Ratio of SHG/TPEF in normal versus neoplastic stroma. Error bars indicate calculated standard deviations. A Student *t*-test is performed against normal stroma for comparison. $^{*}P<0.0001$. (Color online only.)

collagen area suggest loss of the fine fibril structure and the amount of collagen with cancer progression, and the SHG signals may be used to quantitatively discriminate between normal and neoplastic stroma.

To quantitatively characterize elastin-related changes, two analyses were performed. First, we determined the spacing between elastic fibers within normal and neoplastic stroma. The approach for determining the spacing between elastic fibers has been described in our previous studies.[4] In this work, the spacing between elastic fibers in normal stroma is 14.13 ± 4.12 μm ($n=27$ slices of 9 biopsies) and in neoplastic stroma is 1.72 ± 0.57 μm ($n=27$ slices of 9 biopsies), indicating that the elastic fibers have a getting together tendency. Second, similar to the measurement of collagen, we also obtained the elastin area. In this study, the elastin area in normal stroma is 0.089 ± 0.012 ($n=27$ slices of 9 biopsies), whereas in neoplastic stroma, it is 0.188 ± 0.073 ($n=27$ slices of 9 biopsies), suggesting an increased amount of elastin. It was found that two values, the spacing between elastic fibers and the elastin area, are significantly different in normal and neoplastic stroma ($P<0.01$). It is, therefore, reasonable to propose that elastin-related changes are an ancillary feature in the early detection of neoplastic changes.

To better characterize the alteration in proportions of matrix molecules, the emission spectra from normal and neoplastic stroma were obtained using identical conditions. The obtained spectra have been corrected. Typical emission spectra are shown in Fig. 3 (left) at depth of 0 μm. As can be seen, there are two peaks at about 425 and 500 nm, resulting from collagen SHG and elastin TPEF, respectively.[3,4] Obviously, in normal stroma, the SHG accounts for most of the signals, reflecting the fact that the collagen is intact and is the major extracellular matrix protein. In neoplastic stroma, the SHG signals are greatly diminished, and TPEF signals increase, demonstrating the fact that the collagen structures responsible for SHG are disrupted and that the increased elastin enhances TPEF signals. In this work, SHG images showed a random distribution of fiber orientations and a wide variety of interfiber spacings in human esophageal stroma; therefore, the average SHG signal can reflect the total collagen content.[3] Moreover, TPEF signals can feature the amount of elastin.[4] So to quantitatively analyze the alteration in the proportions of matrix molecules, we performed an analysis of the ratio of SHG signal and TPEF signal. As can be seen in Fig. 3 (right), the ratio in normal stroma is 4.37 ± 0.61 ($n=27$ slices of 9 biopsies), whereas in neoplastic stroma, it is 0.32 ± 0.12 ($n=27$ slices of 9 biopsies). It is obvious that there were significant differences between normal and neoplastic stroma ($P<0.0001$). These differences in the molecular constituents of normal versus neoplastic stroma predict a possible difference in cancer progression.

In conclusion, this work demonstrates the potential of intrinsic TPEF and SHG signals to extract quantitative biomorphologic and biochemical features on collagen-related changes, elastin-related changes, and alteration in proportions of matrix molecules, the important hallmarks of cancer progression in stroma. With additional development, the intrinsic TPEF and SHG signals in epithelial stroma may offer a new means to improve early detection of neoplastic changes.

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