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

Shuangmu Zhuo, Jianxin Chen,* Shusen Xie, Zhibin Hong, and Xingshan Jiang
Fujian Normal University,
Key Laboratory of OptoElectronic Science and Technology for Medicine of Ministry of Education,
Fujian Provincial Key Laboratory for Photonics Technology,
Institute of Laser and Optoelectronics Technology,
Fuzhou 350007 China

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. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3088029]

Keywords: multiphoton microscopy; human esophageal stroma; diagnosis.

Paper 08382LRR received Oct. 27, 2008; revised manuscript received Dec. 28, 2008; accepted for publication Jan. 15, 2009; published online Mar. 19, 2009.

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.³ 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 ab-
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 (\(0.4 \mu\text{m}\) to 24.0 \(\mu\text{m}\)) 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 collagen area presented.

---

**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 \(\lambda_{ex}\) was 850 nm. Scale bar=20 \(\mu\text{m}\). (Color online only.)

---

**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.)
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. 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. 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 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. Moreover, TPEF signals can feature the amount of elastin. 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.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (Grant Nos. 60508017, 60678054), the Natural Science Foundation of Fujian Province of China (2007J0007, C0720001), the Science and Technology Planning Key Program of Fujian Province (2008Y0037), and the Program for New Century Excellent Talents in University (NCET-07-0191). Zhibin Hong is a trainee from Northeastern University.

References