Two-photon autofluorescence spectroscopy and second-harmonic generation of epithelial tissue

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A spectroscopy system is developed for studying the two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) of epithelial tissue in backscattering geometry. Our findings show that TPEF signals from epithelial and underlying stromal layers exhibit different spectral characteristics, providing information on the biomorphology and biochemistry of tissue. The SHG signal serves as a sensitive indicator of collagen to separate the epithelial layer from underlying stroma. The polarization dependence of the SHG signal reveals a well-ordered orientation of collagen fibers in the stromal layer. The results demonstrate the potential of depth-resolved TPEF and SHG in determining the pathology of epithelial tissue. © 2005 Optical Society of America

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Autofluorescence spectroscopy has been a widely explored noninvasive technique for *in vivo* diagnosis of epithelial tissue, where most early cancers originate.[1] Recently we demonstrated that depth-resolved fluorescence spectroscopy based on a confocal technique can isolate the autofluorescence from different sublayers of epithelial tissue and provide more accurate information in assessing tissue pathology.[2,3] The promising alternatives for depth-resolved measurements of tissue are two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG), in which the quadratic power dependence of excitation results in optical sectioning without the use of a spatial filter.[4,5] Because TPEF and SHG involve different mechanisms, they can be used in tandem to provide complementary information on the biomorphology and biochemistry of tissue.[6] Our primary goals in this study are to investigate the endogenous TPEF and SHG signals in epithelial tissue and to extract the information for potential tissue diagnosis.

The spectroscopy system is similar to the confocal system used in previous work.[2] The excitation source was a tunable Ti:sapphire femtosecond laser. The average power was controlled at 40 mW for five excitation wavelengths: 710, 725, 750, 775, and 810 nm. A water immersion objective lens (×40 and NA=1.15) focused the excitation laser beam into the tissue sample and was also used to collect backscattered nonlinear optical signals from the sample. The signals were conducted to an imaging spectrometer via a 200 μm optical fiber and recorded by a cooled-CCD camera. Each recorded spectral signal represented the integration of TPEF and SHG over an area of 150 μm × 150 μm in a sampled tissue layer at a certain depth. The details of the depth-resolved measurement are described in Ref. 2. The biological samples used in this study were fresh oral, esophageal, and colonic tissue specimens from experimental rabbits. The TPEF–SHG signals were measured at five excitation wavelengths with an exposure time of 1 s. The histology and chemical analysis of the tissue samples were performed after the measurements.

Representative nonlinear optical signals measured from a fresh tissue specimen are shown in Fig. 1. The TPEF and SHG excited at 750 nm were obtained from a nonkeratinized oral tissue. Each TPEF spectrum is normalized to the fluorescence peak intensity, and the SHG signals are scaled down so that they can be displayed with the TPEF spectra. It was found that the TPEF from the tissue surface (0 μm) to a depth of 70 μm exhibit spectral characteristics different from the TPEF recorded from the underlying tissue layer of depth 100–200 μm. Strong SHG signals were observed only in the underlying tissue layer. The changes in the depth-resolved nonlinear optical signals indicate that the examined tissue has a two-layered structure. The interface of two tissue layers is at a depth of about 80–90 μm, where transitional TPEF spectral signals and small SHG are observed. To confirm the information extracted from the TPEF and SHG signals, the Masson-stained section of the examined tissue specimen is also presented in Fig. 1. As can be seen, the nonkeratinized oral tissue is covered with an ~80 μm thick epithelial layer. The underlying layer is the collagen-rich connective tissue. The information derived from the nonlinear optical signals is consistent with the histological analysis.

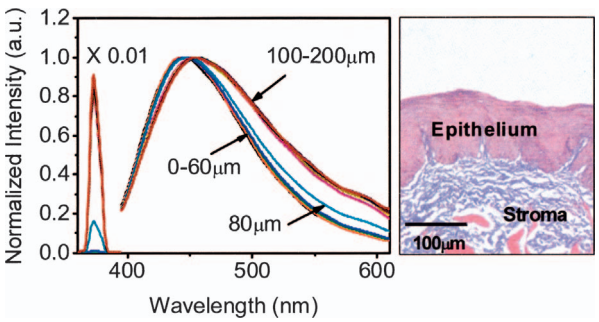

Fig. 1. Left, excited TPEF and SHG signals of nonkeratinized oral tissue; Right, Masson-stained section, coloring collagen blue.

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It is known that the autofluorescence of nonkeratinized epithelium is determined mainly by the signals of NADH and FAD, the enzymes carrying information of cellular metabolism.[2,3] The normalized epithelial TPEF spectra excited at five wavelengths are shown in Fig. 2a for comparison. The corresponding single-photon epithelial fluorescence spectra excited at 355 and 405 nm are also presented in the figure for comparison. As can be seen, the spectral signals excited at 710, 725, and 750 nm are similar to the single-photon fluorescence excited at 349 and 355 nm, indicating that NADH, with a fluorescence peak at 450 nm, is the major fluorophore at these excitation wavelengths, and the single- and two-photon NADH fluorescences involve the same excitation mechanism. With a further increase of the excitation wavelength to 810 nm, the spectral peak shifts to 470–475 nm, and the spectral bandwidth is broadened, showing that the contribution of the FAD signal, peaking at 530 nm, becomes significant.[7] It was reported in previous work that the ratio of NADH over FAD fluorescence, a parameter related to the tissue redox ratio, has a strong correlation with tissue pathology.[3] Thus the epithelial TPEF excited at 810 nm, a combination of NADH and FAD fluorescence, can be used for evaluating the redox ratio and monitoring the metabolic state in epithelium.

The TPEF spectral signals recorded from the underlying stromal layer are shown in Fig. 2b. The spectral characteristics of the TPEF in stroma exhibit obvious differences from those of epithelial signals shown in Fig. 2a. To understand the signals in the stromal layer, the TPEF of pure collagen (Sigma-Aldrich Corporation, St. Louis) at all the excitation wavelengths was studied, and the results are shown in Fig. 2c. Strong SHGs are obtained from both the stromal layer and pure collagen. The spectral characteristics of the stromal TPEF signal are almost identical to those of collagen. It is confirmed that collagen is the source of SHG and TPEF in the stromal layer, which is consistent with the study of single-photon excited fluorescence.[2] In addition, the comparison between single- and two-photon excited fluorescence in stroma and pure collagen samples show that the TPEF signal of collagen exhibits different spectral characteristics from the corresponding single-photon excited fluorescence. The corresponding single-photon excited stromal fluorescence and collagen fluorescence spectra are shown in Figs. 2b and 2c for comparison. For instance, the collagen fluorescences excited at 355 and 405 nm peak at 405 and 475 nm, respectively.[2] However, the TPEF peaks with excitations of 710 and 810 nm are at 446 and 488 nm, respectively. This suggests that the single- and two-photon fluorescence of collagen may involve different excitation mechanisms.

The representative TPEF–SHG spectra measured from esophageal tissue with keratinizing epithelium and colon tissue with 750 nm excitation are shown in Fig. 3. The previous work based on single-photon excitation showed that keratin fluorescence has spectral characteristics similar to collagen.[2,3] The fluorescence measured in the keratinizing epithelium is a

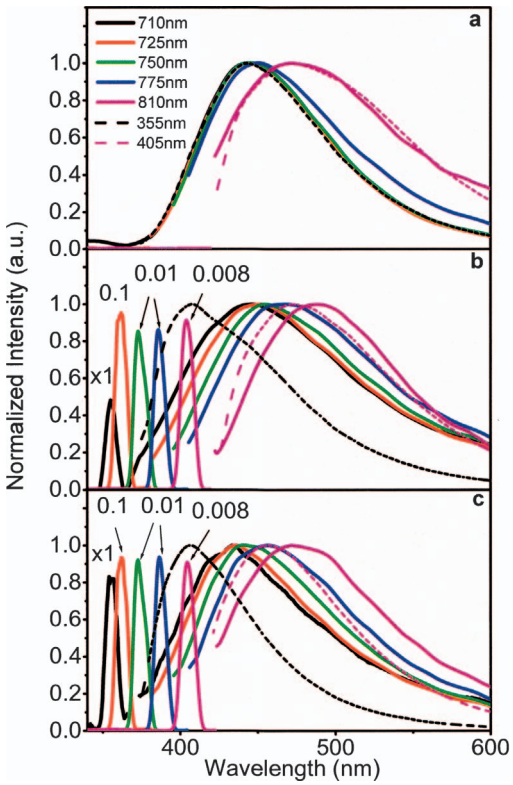

Fig. 2. Normalized two- and single-photon excited fluorescence spectral signals. a, Epithelial layer; b, stromal layer; c, pure collagen.

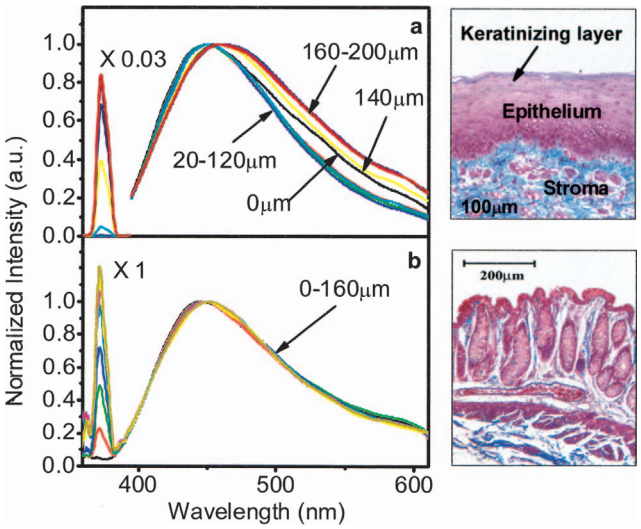

Fig. 3. a, Left, TPEF and SHG signals of esophageal tissue; right, Masson-stained section. b, Left, TPEF and SHG signals of colonic tissue; right, Masson-stained section.

mixture of NADH and keratin signals. As is shown in Fig. 3a, the TPEF measured in the normal epithelial layer and underlying stromal layer are dominated by NADH and collagen signals, respectively. The TPEF signals measured from the topmost keratinizing layer show a combined spectral signal of epithelial and stromal fluorescence. However, SHG was not recorded from keratinizing epithelium and pure keratin. The TPEF spectra in colon mucosa at different depths shown in Fig. 3b are almost identical and peaked at 450 nm, indicating the dominance of

NADH fluorescence. Weak SHG signals are observed at depths ranging from 30 to 160 μm. The Masson-stained histology reveals a small amount of collagen connecting the columnar epithelial cells and glandular cells in the colon mucosa. This demonstrates that SHG is a very sensitive indicator of collagen.

The SHG intensity is not only a function of the collagen content in tissue but is also modulated by the collagen organization. Therefore the polarization-dependent SHG provides information on the organization degrees of collagen fibers in tissue, the additional information of tissue structure.[8] We investigated the collagen SHG signals as a function of the excitation polarization at 800 nm. The polarization-dependent SHG was recorded from the stromal layers of rabbit oral and esophageal tissue samples and the mucosa of rabbit colon tissue over scanning areas from 15 μm$\times$15 μm to 150 μm $\times$150 μm to prevent photodamage of tissue. The setup for the study is the same as that reported in Ref. 9. The polarization-dependent SHG signals measured from oral tissue sample are shown in Fig. 4 as a representative result. As can be seen, the SHG signals are not sensitive to the scanning area, indicating that the organization degree of collagen does not change from the minimal to the maximal scanning areas investigated. To quantify the polarization dependence of SHG, the polarization modulation, δ, is defined as the ratio of the difference between the maximal and the minimal SHG over the minimal SHG. It was found that the δ-values in oral stroma, esophageal stroma, and colon mucosa are 1.34 ± 0.16, 0.84 ± 0.14, and 0.49 ± 0.17, respectively. The results suggest that the collagen fibers in the stromal layer of oral tissue are highly organized and that the orientation of collagen in colon mucosa is almost random.

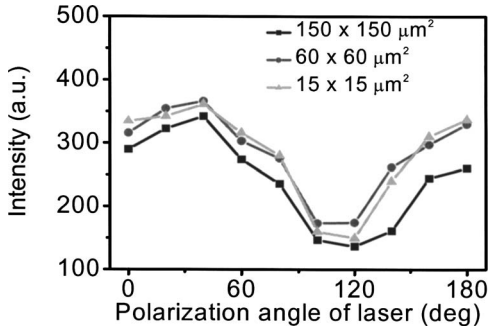

Fig. 4. Polarization-dependent SHG signals recorded from oral stroma.

In conclusion, we demonstrate that two-photon excited fluorescence spectroscopy and second-harmonic generation provide depth-resolved information on NADH, FAD, and collagen, the important biomarkers of precancer development in epithelium. Though the fluorescence spectral signals could be modulated by the wavelength-dependent scattering and absorption in tissue, the effects are negligible in the range of detection depth (0–200 μm) because of the optical sectioning capability and confocal measurement provided by our two-photon fluorescence system. The study shows the potential of the nonlinear optical method in noninvasive diagnosis of tissue pathology.

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