Combined depth- and time-resolved autofluorescence spectroscopy of epithelial tissue

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A time-resolved confocal fluorescence spectroscopy system is built to measure the fine structure and localized biochemistry of epithelial tissue. It is found that the autofluorescence excited at 405 nm is sensitive to the cellular metabolism and can be used to sense the metabolic status of epithelial tissue. The decay of autofluorescence excited at 405 nm can be accurately fitted with a dual-exponential function consisting of short lifetime (0.4–0.6 ns) and long lifetime (3–4 ns) components. The ratio of the amplitudes of the two components provides information on the fine structure of epithelial tissue. We demonstrate that the combined depth- and time-resolved measurements with single excitation can potentially provide accurate information for the diagnosis of tissue pathology. © 2006 Optical Society of America

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Most human cancers arise from the epithelium, a cell-rich superficial tissue, because of frequent exposure to various forms of physical and chemical damage that cause the development of cancer. Autofluorescence spectroscopy has been a widely explored technique for *in vivo* and noninvasive diagnosis of epithelial precancers.[1] Recent studies show that the depth-resolved fluorescence technique based on the confocal measurement provides accurate information on the fine structure and metabolic status of epithelial tissue and can potentially improve the accuracy of fluorescence detection of epithelial precancers.[2,3] In the depth-resolved measurements of keratinized epithelial tissue, the keratin, nicotinamide adenine dinucleotide (NADH), and collagen fluorescence spectral signals excited by a 355 nm UV laser produced the contrast to differentiate the topmost keratinized epithelial layer, the underlying normal epithelium, and the stroma. The redox ratio of the NADH signal excited by the UV laser over flavin adenine dinucleotide (FAD) signals induced by a 457 nm blue laser were used for the assessment of metabolic status of the epithelium.

To accurately assess the metabolic status of the epithelium, it is critical to separate the fluorescence signal of nonkeratinized epithelium from the signals of the topmost keratinized layer and the underlying stromal layer because most of the squamous epithelial tissues were found to keratinize.[2,3] Though it could be achieved using the depth-resolved spectroscopy system with UV and blue excitations, the measurement procedure became more complicated than a more desirable system with a single excitation. Note that the time decay of fluorescence provides additional information on the characteristics of a specific fluorophore and its microenvironment.[4] The Monte Carlo simulation showed that the temporal characteristics of tissue fluorescence were determined by the fluorescence time decay of each sublayer of tissue.[5] In this Letter we demonstrate that a time-resolved confocal fluorescence spectroscopy system with single excitation can provide a depth-resolved assessment of a layered structure and the metabolic status of epithelial tissue. Specifically, the excitation is selected at the wavelength where the intensity of NADH and FAD fluorescence are comparable for a good estimation of the redox ratio, and the temporal characteristics of the major fluorophores are used to produce the contrast to identify different tissue layers.

The fluorescence spectroscopy system used in this study is similar to the confocal system described in Refs. 2 and 3. The excitation source is the second-harmonic generation of a pulsed Ti:sapphire laser with the pulse duration from 150 to 200 fs and tunable range from 355 to 435 nm. A water immersion objective lens ($40\times$ and NA=1.15) focuses the excitation beam into the sample and collects the backscattered fluorescence signals. The excitation and fluorescence are separated by a dichroic mirror. A 100 μm optical fiber is used as a pinhole to collect the confocal fluorescence and conducts the signal to the detection system. In the spectral analysis measurement, the confocal fluorescence is analyzed by an imaging spectrometer and recorded by a cooled-CCD camera. In the time-resolved measurement, the decay of confocal fluorescence signals at a series of wavelength bands are analyzed using a time-correlated single photon counter (TCSPC). The spectral and temporal measurements are performed sequentially. The measured signals are the averaged fluorescence signals over a sampling area of $150\,\mu\text{m}\times150\,\mu\text{m}$. In this work, the excitation power in the biological samples was controlled below 100 μW, and the acquisition time was set to 1 s for the measurement at a certain depth.

First, we investigated the fluorescence characteristics of epithelial tissues to find an excitation wavelength at which the intensity of NADH and FAD fluorescence in the epithelium are comparable. The representative fluorescence spectra measured from a squamous epithelial tissue are shown in Fig. 1. The detailed procedures to measure the depth-resolved fluorescence and correlate the fluorescence signals to tissue histology are described in Ref. 2. As can be seen, the contrast in spectral signals between differ-

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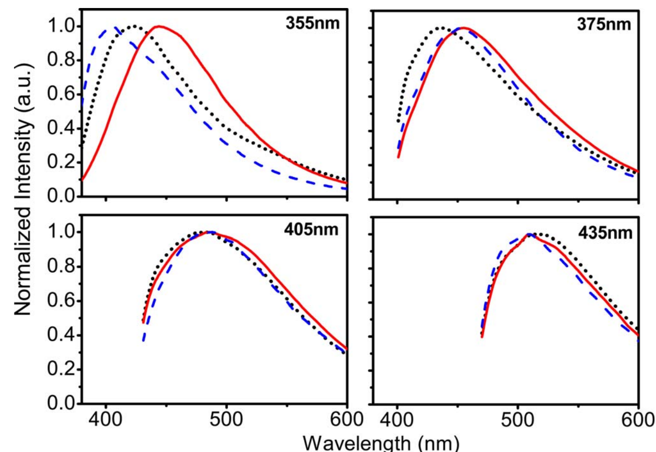

Fig. 1. (Color online) Depth-resolved fluorescence spectra measured from rabbit esophageal tissue at different excitation wavelengths. Dotted curves, keratinized epithelial layer with depths of 0–30 μm; solid curves, nonkeratinized epithelial layer with depths of 40–120 μm; dashed curves, stromal layer with depths of 140–200 μm.

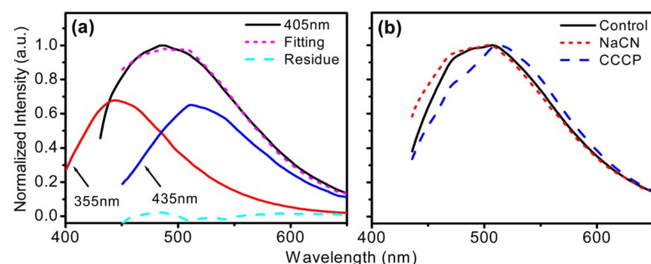

Fig. 2. (Color online) (a) Epithelial fluorescence excited at 405 nm fitted with the fluorescence spectra excited at 355 and 435 nm. (b) Variations of autofluorescence of SiHa cells excited at 405 nm when treated with NaCN and CCCP.

ent tissue layers decreases with the increase of excitation wavelength because of the redshift of collagen and keratin fluorescence spectra.[2,6] Studying the nonkeratinized epithelial signals that are free of interferences from keratin and collagen, we found that the fluorescence excited at 375 and 405 nm could be well fitted using the fluorescence excited at 355 and 435 nm. The fluorescence excited at 355 nm is dominated by the NADH signal, while the signal excited at 435 nm is dominated by FAD fluorescence.[7,8] In particular, the fitting result in Fig. 2(a) shows that with 405 mm excitation the NADH and FAD fluorescence reaches a balanced level, indicating that the ratio of the fluorescence in the wavelength band dominated by the NADH signal to the fluorescence in the band dominated by FAD signal could produce a good estimation of redox ratio for the assessment of epithelial metabolism.

To demonstrate that the fluorescence excited at 405 nm is sensitive to the cellular metabolism, the fluorescence was measured from the monolayered cell culture (SiHa cell line) before and after being treated with the mitochondria inhibitor NaCN and uncoupler carbonyl cyanide 3-chloro-phenylhdrazene (CCCP).[7] Typical results are shown in Fig. 2(b). We calculated the ratio of the signal in a blue band of wavelength range from 440 to 480 nm to the signal in a yellow band from 530 to 570 nm. It was found that the ratio increased ~15% when cells were treated with NaCN, and decreased ~23% when treated with CCCP. The results are consistent with the studies of cell autofluorescence using single- and two-photon excitations.[7,9] The redox ratio can be well estimated by the autofluorescence excited at 405 nm. Therefore the following time-resolved study was focused on the autofluorescence excited at 405 nm.

To understand the temporal characteristics of the major endogenous fluorophores in epithelial tissue, the time-decay fluorescence of free NADH, free FAD, collagen I, and keratin (N8129, F6625, C7661, K0253, Sigma-Aldrich, St. Louis, Missouri) were measured using the TCSPC detector. The exponential functions were used to quantify the fluorescence decay process. As shown in Table 1, the time decay of NADH and FAD fluorescence are well fitted with a single exponential function, and the time decay of collagen and keratin fluorescence are well fitted with a dual-exponential function model, $A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$. The measured lifetimes of NADH, FAD, and collagen fluorescence are consistent with the published literature,[10,11] while keratin fluorescence exhibits the characteristics of time decay similar to collagen.

To demonstrate that the temporal characteristics of the major fluorophores in epithelial tissue can produce the contrast to identify different tissue layers, the depth- and time-resolved autofluorescence in blue and yellow bands were measured from fresh esophageal and oral tissue samples of experimental rabbits. The bandpass filters were inserted in the fluorescence channels of the confocal setup to select the blue and yellow windows for temporal measurements. As the representative results, the time-decay fluorescence measured from an oral tissue without keratinized epithelium and an esophageal tissue with keratinized epithelium are shown in Figs. 3(a) and 3(b), respectively. The corresponding Masson-stained histologies are shown in Figs. 3(c) and 3(d), respectively. As can be seen, the time decay of nonkeratinized epithelial fluorescence is obviously different from those of keratinized epithelial and stromal fluorescence. The time decays of the fluorescence in blue and yellow bands measured at the same tissue site have similar temporal characteristics. It was found that the fluorescence measured from oral and esophageal tissue samples exhibits almost the same temporal characteristics. The dual-exponential function model produced the best fitting to the decay of the fluorescence from different tissue layers. The coefficients of determination, r^2, in all the fittings are

Table 1. Fluorescence Decay of Chemicals

	τ_1 (ns)	τ_2 (ns)	A_1/A_2	r^2
NADH	0.44	–	–	0.999
FAD	2.94	–	–	0.998
Collagen	0.61	3.32	0.57	0.997
Keratin	0.61	4.80	1.77	0.997

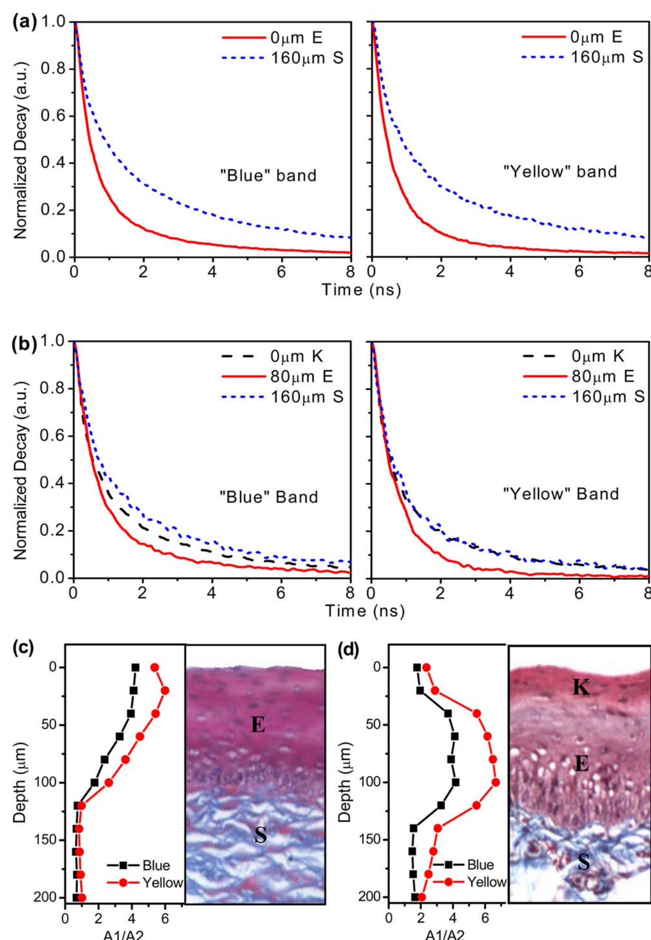

Fig. 3. (Color online) (a), (c) Time-resolved fluorescence of a nonkeratinized oral tissue and the corresponding histology. (b), (d) Time-resolved fluorescence of a keratinized esophageal tissue and the corresponding histology. K, keratinized layer; E, epithelial layer; S, stromal layer.

Table 2. Fluorescence Decay of Different Tissue Layers[a]

		τ_1 (ns)	τ_2 (ns)	A_1/A_2
Blue band	K	0.45±0.02	3.38±0.20	1.61±0.16
	E	0.49±0.04	3.28±0.27	3.31±0.72
	S	0.51±0.05	3.76±0.34	1.78±0.38
Yellow band	K	0.50±0.04	3.32±0.24	2.86±0.42
	E	0.56±0.07	3.08±0.34	5.76±1.79
	S	0.57±0.06	4.03±0.51	2.49±0.88

[a]K, keratinized layer; E, epithelial layer, S, stromal layer.

over 0.995. The quantitative analyses of the fluorescence time decay based on the measurements from the oral and esophageal tissue samples of six experimental rabbits are summarized together in Table 2.

The fluorescence decay measured in different layers is characterized with the similar short lifetime (0.4–0.6 ns) and long lifetime (3–4 ns). However, the amplitudes of the short lifetime and long lifetime components vary significantly from layer to layer. The ratio of the short lifetime term to long lifetime term, A_1/A_2, provides the contrast to differentiate the epithelial layer from the keratinized layer and stromal layer with high statistical significance ($p \leqslant 0.001$). The A_1/A_2 values measured from an oral tissue without a keratinized layer and an esophageal tissue with a keratinized layer are displayed together with their corresponding histology in Figs. 3(c) and 3(d). It is demonstrated that the A_1/A_2 value provides the information on the fine structure of tissue accurately correlated to the histology.

Comparing the data in Tables 1 and 2, it is noted that the fluorescence decay in the keratinized epithelial and stomal layers is similar to that measured from the pure keratin and collagen samples. This is consistent with the results in a previous study that keratin and collagen are the major fluorophores in keratinized epithelial and stromal layers, respectively.[2,3] However, the fluorescence time decay of nonkeratinized epithelial tissue in the blue band, dominated by the NADH signal, and in the yellow band, dominated by FAD signal, are significantly different from those of free NADH and FAD fluorescence. The decay in both bands consists of a short lifetime term with large amplitude and a long lifetime term with relatively small amplitude. There may be two physical reasons causing the differences. It is known that the protein-bound NADH generally has a much longer fluorescence lifetime (~2 ns) than free NADH, and protein-bound FAD has shorter lifetime (<1 ns).[10,12] Because of the cross talk between the NADH and FAD signals in the blue and yellow bands, the short lifetime term in both bands should be determined by the free NADH and protein-bound FAD, and the long lifetime term should be determined by the free FAD and protein-bound NADH.

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