Depth-resolved fluorescence spectroscopy reveals layered structure of tissue

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ABSTRACT: A confocal fluorescence spectroscopy system is instrumented to study depth-resolved autofluorescence in biological tissue. The system provides the capability of optical sectioning with the maximal detection depth up to 120 μm in the examined tissue samples. It was found that the topmost keratinizing epithelial layer produces strong fluorescence similar to collagen. The fluorescence signal from epithelial tissue between the keratinizing layer and stroma can be well resolved. The study results show that depth-resolved fluorescence spectroscopy has the potential to provide more accurate information for the diagnosis of tissue pathology.

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OCIS codes: (170.6510) Spectroscopy, tissue diagnostics.

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1. Introduction

Most of cancers originate in epithelial tissue. During the development of epithelial precancers, not only epithelial cells undergo pathological transformations, but also the cross-talk between epithelia and connective tissues can be changed. Autofluorescence spectroscopy has been investigated as an effective and noninvasive tool for sensing the changes in morphology and biochemistry of tissue associated with cancer development [1-3]. A study based on fluorescence microscopy of fresh cervical tissue revealed an increase of NADH fluorescence and a reduction of FAD fluorescence in the area of dysplasia, which indicates higher metabolic rate in precancerous epithelia [4]. In addition, a significant decrease in stromal fluorescence observed in dysplasitc tissues suggests that the fluorescence of collagen and elastin in connective tissue under the epithelium may be also associated with the malignant change of epithelial tissue [4-6].

In general, autofluorescence measured by a conventional spectroscopy system is a mixture of fluorescence signals from epithelial and connective tissues. The fluorescence of collagen and elastin in deep structure create interference when extracting the epithelial fluorescence that is weak, but important for the characterization of tissue pathology. A depth-resolved fluorescence technique is desirable to separate the epithelial and stromal fluorescence. A few techniques have been explored recently to obtain the depth-resolved autofluorescence [7,8]. In this work, we use a confocal system to obtain high quality depth-resolved autofluorescence signal. We demonstrate that the information derived from depth-resolved autofluorescence can reveal the fine structure and biochemistry of epithelial tissue.

2. Materials and methods

The confocal system for measuring depth-resolved autofluorescence is similar to that reported in Ref. [9]. Briefly, two solid-state lasers with the wavelengths at 349 nm and 457 nm are used as excitation sources. The UV laser favors excitation of NADH, while the wavelength of blue laser is near the excitation peak of FAD. An objective lens ($\times$40 magnification, NA = 1.15) is used both for focusing the laser beam as well as collecting the autofluorescence from tissue sample. A 100 µm optical fiber acts as a confocal pinhole and conducts the fluorescence signal to an imaging spectrometer for spectral analysis. The confocal fluorescence spectra are recorded with a cooled-CCD camera. The axial and lateral resolutions of the system are about 3 µm and 1.8µm in reflection mode, respectively. A pair of galvo scanners scan the laser beam to create a 150$\times$150 µm sampling area in the focal plane. The scanning speed is about 5 f/s. The recorded depth-resolved spectral signal is the integration of the autofluorescence in the sampled tissue layer at certain depths. The purpose is to increase the signal-to-noise ratio (SNR) and average out the inhomogeneity of fluorophores distribution in the sampled tissue layer. The system sacrifices the imaging capability offered by conventional confocal microscope, but produces high quality depth-resolved fluorescence spectral signal and reduces phototoxicity of excitation sources to the tissue sample. In this work, the power of excitations at 349 nm and 457 nm on sample are set to about 12 µW and 50 µW, respectively. The exposure time of CCD is set to 1 s to record the autofluorescence signal from the sampled tissue layer at a certain depth.

The samples used in this study were fresh esophageal and oral tissues from ten experimental rabbits, and cervical tissues from three human subjects. All the samples were rinsed briefly with PBS solution to remove the residual blood on the surfaces before the measurements. To avoid possible degradation of tissue, the measurements were conducted within one hour after the tissue resection from animal and human subjects [10]. A cover glass was used to keep the tissue surface flat. The histology and chemical analysis of the tissue samples were performed after the fluorescence examinations.

3. Results and discussion

Typical depth-resolved fluorescence spectra measured from the rabbit esophageal and oral tissues are shown in Fig. 1. Every spectrum shown in the figure is normalized to its peak

intensity and the inlet of each figure provides the peak intensity of raw fluorescence signals at different depths. The histology of corresponding samples are also displayed as references. The reflection confocal signal of excitation laser from the interface between the cover glass and tissue sample was used to identify the tissue surface. The depth of 0 µm refers to the tissue surface where reflection confocal signal reaches to the maximum.

Figure 1(a) shows the depth-resolved fluorescence signals of rabbit esophageal tissue excited at 349 nm. As can be seen, the fluorescence of tissue layer from the surface to the depth of 10 µm is peaked at 400 nm and similar to those of collagen and elastin [1-3]. The fluorescence spectra measured from the tissue at depths from 40-120 µm are peaked at 450 nm. They are corresponding to NADH fluorescence [11]. A transition zone where the fluorescence peak shifts from 400 nm to 450 nm can be clearly observed at the depth range of 20-30 µm. The red fluorescence peak at 680 nm observed at all the depths except surface may be the contribution of porphyrin derivatives [1-3]. Overall, the information derived from the depth-resolved spectra indicate that the examined epithelial tissue is layered and the dominant fluorophores in the top layer is different from that in the epithelial tissue beneath. The H&E stained section of the corresponding tissue examined by fluorescence measurement is shown in Fig. 1(c). The topmost layer of thickness about 20 µm is the keratinizing epithelium that has different cellular morphology and biochemistry from the epithelium beneath. The histological analysis confirmed that the information derived from the depth-resolved fluorescence excited at 349 nm correctly revealed the layered epithelial structure.

The depth-resolved fluorescence spectra excited at 457 nm are shown in Fig. 1(b). The measurement site was the same as that using 349 nm excitation. It was found that the signals from the epithelial tissue under the keratinizing layer are peaked at 535 nm and corresponding to FAD fluorescence [11]. However, the signal of keratinizing epithelium also appears similarly in spectral line-shape as FAD fluorescence. It means that fluorescence emission of the dominant fluorophore in keratinizing layer is similar to that of FAD under the excitation at 457 nm. The fine structure of epithelium can not be clearly distinguished. This indicates that the selection of excitation wavelength is important to generate good contrast between the tissues with different morphology and biochemistry. A strong fluorescence peak at 680 nm excited by 457 nm laser further confirms the existence of porphyrin derivatives in the esophageal epithelial tissue because the maximum excitation wavelength of porphyrin is in the range from 400-450 nm [1-3].

The depth-resolved fluorescence spectra measured from rabbit oral tissues are shown in Fig. 1(d-i). It was found that the thickness of keratinizing layer in oral tissue samples varied greatly from 0 to a few tens microns. The fluorescence of porphyrin derivatives was not observed from the oral tissue sample. The results shown in the figure were obtained from two extreme cases: a sample with highly keratinizing epithelium and a sample with non-keratinizing epithelium. Fig. 1(d) displays the normalized depth-resolved fluorescence excited at 349 nm from one of the oral tissue samples. The variation of spectral characteristics as a function of depth is similar to that observed from esophageal tissue shown in Fig. 1(a), which indicates that the sample has two-layered epithelium. The thickness of keratinizing layer derived from the fluorescence measurement is consistent with corresponding histology shown in Fig. 1(f). It should be noted that a shoulder on the left side appears in the fluorescence signal from the epithelial tissue under the keratinizing layer. It may be contributed from the residual fluorescence of the keratinizing layer. This demonstrates that the strong non-confocal signals from the topmost layer can be suppressed, but can not be completely rejected by the confocal technique.

The depth-resolved fluorescence spectra shown in Fig. 1(g) display a different pattern of spectral characteristics as a function of depth. The signals measured from the surface to the tissue layer at the depths about 80 µm are peaked at 450 nm. They are corresponding to NADH fluorescence. With further increase of the sampling depth the fluorescence peak shifts to 400 nm and the collagen-like signal becomes dominant. It suggests that the connective tissue under epithelial tissue had been sampled. Comparing the results with the histology analysis shown in Fig. 1(i), it is found again that the depth-resolved fluorescence correctly

distinguished the epithelial tissue from connective tissue and produced accurate information of tissue structure. Furthermore, the results demonstrate that maximal sampling depth in the examined tissue is at least 120 μm.

The depth-resolved fluorescence of the same oral tissue samples excited at 457nm are shown in Figs. 1(e) and (h). With the increase of sampling depth no obvious spectral shift was observed as that in the fluorescence excited at 349 nm. The fluorescence emissions from the dominant fluorophores in keratinizing layer, normal epithelial tissue and connective tissue do not produce sufficient contrast to resolve the layered structure of tissue. This is consistent with the depth-resolved fluorescence of esophageal tissue with excitation at 457 nm.
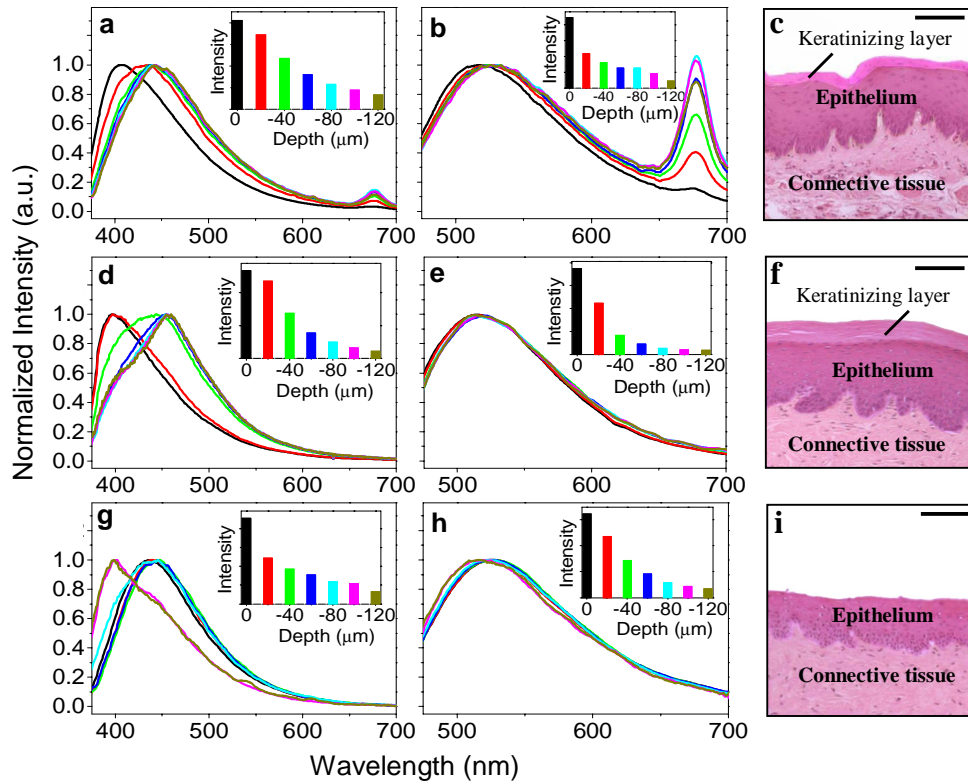

Fig. 1. Normalized depth-resolved fluorescence spectra excited at 349 nm and 457 nm, respectively. The inlet of each figure shows the raw fluorescence intensity as a function of depth. a, b & c: Fluorescence of esophageal tissue and the corresponding histology. d, e & f: Fluorescence of highly keratinizing oral tissue and the corresponding histology. g, h & i: Fluorescence of non-keratinizing oral tissue and the corresponding histology. Scale bar: 100μm.

It was found that fluorescence from the keratinizing tissue layer presents the spectral line-shape similar to collagen and elastin. To identify the major fluorophore in the keratinizing layer, Masson stain and Verhoff-Van Geison stain methods were used to map the distribution of collagen and elastin in examined tissue samples. The Masson stain colors collagen in green and muscle in red; and Verhoff-Van Geison stain colors elastin in black and collagen in red [12]. Typical Masson and Verhoff-Van Geison stained sections are shown in Fig. 2. The tissue sections are from the same rabbit oral tissue samples as those with histology shown in Fig. 1(f) and (I). As can be seen, collagen was not found in the keratinizing epithelial layer, while it is abundant in connective tissue under the epithelial layer. No elastin was found in the keratinizing epithelial layer and connective tissue. It is, therefore, concluded that the fluorescence in keratinizing epithelium is contributed from neither collagen nor elastin. In addition, the Masson stained section of the sample with non-keratinizing epithelium

confirmed that the fluorescence with peak at 400 nm from deep layer shown in Fig. 1(g) were indeed contributed from the collagen in connective tissue.
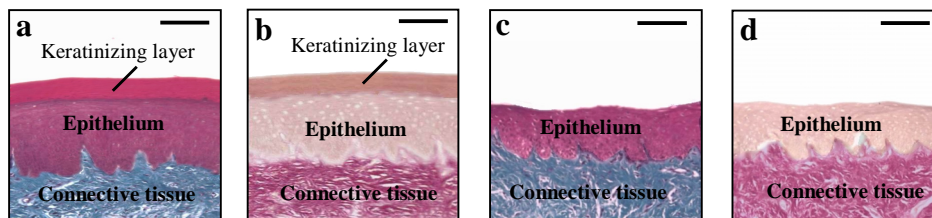

Fig. 2. Masson and Verhoff-Van Geison stained sections. a & b: highly keratinizing rabbit oral tissue. c & d: non-keratinizing rabbit oral tissue. Scale bar: 100µm.

It is known that the keratin content in keratinizing tissue is high. We investigated the fluorescence of keratin and compare its spectral characteristics with collagen and elastin, though keratin was not considered as one of the major endogenous fluorophores [1-3]. The fluorescence spectra of pure collagen, elastin and keratin excited at 349 nm and 457 nm are shown in Fig. 3. All the pure chemicals were obtained from Sigma-Aldrich Corp., St. Louis. It is an interesting finding that keratin produces strong fluorescence with spectral characteristics very close to collagen. The fluorescence of pure collagen and keratin excited at 457 nm are slightly blue-shifted comparing with the results from tissue. It may be caused by the difference in the interaction between the fluorophores and their microenvironment.
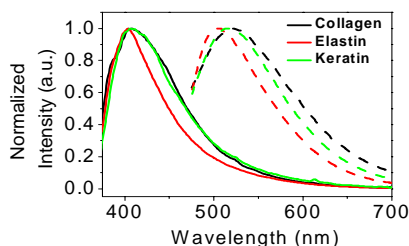

Fig. 3. Peak normalized fluorescence spectra of collagen, elastin and keratin excited at 349 nm (solid lines) and 457 nm (dash lines).

The depth-resolved fluorescence spectroscopy of layered epithelium revealed strong keratin fluorescence from the keratinizing layer. Thus, spectral characteristics of the bulk autofluorescence measured by conventional method may be strongly affected by the variation of thickness of keratinizing layer, because the bulk fluorescence is a mixture of the fluorescence from different tissue layers. This may introduce serious challenge to extract NADH/FAD and collagen signals from bulk fluorescence. Figure 4 displays the bulk fluorescence of rabbit esophageal and oral tissue samples with the excitation at 349 nm. To measure the bulk fluorescence, the objective lens of confocal system was replaced with a lens of long focal length to create a 2 mm in diameter illumination/collection area on tissue sample. The bulk fluorescence measurement was performed on each tissue sample following the depth-resolved confocal measurements. As shown in the figure, the spectral line-shape of bulk fluorescence varies significantly from sample to sample even when the samples were obtained in the same organ sites from different animal subjects. On the contrary, the depth-resolved fluorescence spectroscopy technique can effectively suppress the strong keratin fluorescence and extract the epithelial fluorescence signal under the keratinizing layer. The influence of the optical properties (scattering and absorption) in connective tissue on fluorescence spectral line-shape is also eliminated. Moreover, the confocal system equipped with two calibrated excitation sources can be used to measure the redox ratio that is directly related to cellular metabolism of epithelial tissue [4].
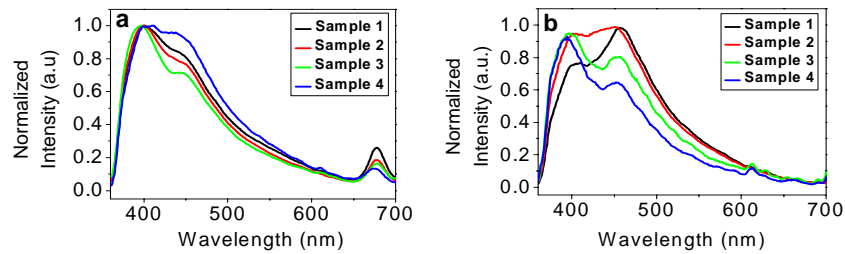
Fig. 4. Peak normalized bulk autofluorescence spectra. a: Rabbit esophageal tissues, b. Rabbit oral tissues.

A feasibility study of the depth-resolved fluorescence spectroscopy of human tissue was launched recently to investigate potential of the technique in clinical application. The very preliminary results are shown in Fig. 5. The spectra were measured from three fresh human cervical squamous tissue samples with confirmed pathology. The samples were obtained from three subjects undergoing the loop electrosurgical excision process (LEEP). The results demonstrate that high quality depth-resolved fluorescence can be obtained from human tissue. Very strong keratin-like fluorescence from the topmost layer provides the evidence of the keratinization of epithelial layer. The variations of spectral characteristics as the function depth among three samples suggest that thickness of keratinizing layer varies greatly from sample to sample. This is consistent with the results obtained from the animal model.
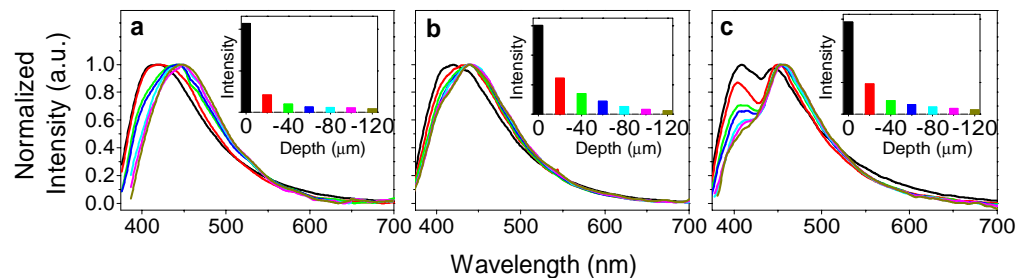

Fig. 5. Peak normalized depth-resolved fluorescence spectra excited at 349 nm from normal cervical squamous tissue (a) and the tissues with HPV infection (b, c). The inlet of each figure shows the raw fluorescence intensity as a function of depth.

4. Conclusion

In conclusion, we used a confocal system to measure the depth-resolved fluorescence of biological tissue. The experimental results demonstrate that the system can separate the fluorescence spectral signals from different tissue layers. The information derived from the depth-resolved autofluorescence was generally consistent with the histology of corresponding tissue specimen. It was found that keratin in the keratinized epithelium produces strong collagen-like fluorescence. The depth-resolved fluorescence method can effectively reduce the interference from the keratin fluorescence and extract useful diagnostic information, such as NADH and FAD fluorescence in epithelial tissue. The results of preliminary study on human cervical tissue shows the clinical potential of the depth-resolved fluorescence technique. Our future work will focus on the systematic investigation of depth-resolved spectroscopy of human tissues in various organ sites. In particular, the correlation of NADH and FAD fluorescence in epithelial tissue with the tissue pathology will be investigated. Furthermore, a system with miniaturized objective lens and scanners will be instrumented to study the diagnosis of diseased tissue with depth-resolved fluorescence spectroscopy at clinical level.