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RESEARCH ARTICLE

# Spectral imaging technology of epithelial tissue based on two-photon excited fluorescence and second-harmonic generation

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**Abstract** The layer structures of the esophageal and oral tissues were investigated by using spectral imaging technology based on two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG). Because spectral imaging technology allows a simultaneous record of both the spectra and image, it is capable of identifying the layered structures of the epithelial tissues, including the keratinizing layer, epithelial cell layer and stromal layer in the molecular level, which are strongly correlated to tissue pathology. All this work indicates that this technique has the potential to provide more accurate and comprehensive information for the early pathological diagnosis of tissues with the stratified squamous epithelia.

**Keywords** spectral imaging, epithelial tissue, two-photon excited fluorescence (TPEF), second-harmonic generation (SHG)

## 1 Introduction

Most neoplasms occur in stratified squamous epithelia. According to the presentation of new cancer statistics from the American Cancer Society, the five-year survival rate of the esophagus and oral cavity are 15.6% and 58.8%, respectively [1]. The most important factor contributing to this fact is the lack of early non-invasive diagnosis of epithelial tissues. It is well-known that the epithelial tissues generally consist of the topmost keratinizing epithelial layer, epithelial cell layer and underlying stromal layer. In fact, during the forming of the epithelial carcinogenesis, the morphological structures of these three layers gradually develop some obvious alterations, such as increase in the keratinizing layer thickness, cellular nuclear enlargement in the epithelial cell layer and variation of collagen framework in the stromal layer [2–5]. The corresponding autofluorescence spectroscopy of different layers also shows apparent changes [6–9]. In biology and medicine, the spectroscopic measurement is combined with an optical microscope to generate images with spatially-resolved biochemical functional information, which is known as spectral imaging. However, there have been a limited number of studies on the combination of spectral imaging based on tissue intrinsic emission [10,11]. In this study, a spectral imaging technology based on two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) was presented to investigate the layer structure of epithelial tissues, including the esophageal and oral tissues. This method can simultaneously show the multiphoton images of tissue microstructure and corresponding spectra [12]. The experimental results demonstrated that this technology has a capability to identify the layered structures of the epithelial tissue including the keratinizing layer, epithelial cell layer and stromal layer in the molecular level, which are strongly correlated to the tissue pathology. All the studies indicate its potential on providing more accurate and comprehensive information for the early pathological diagnosis of the tissues with stratified squamous epithelia.

## 2 Materials and methods

The esophageal tissues of the experimental rabbits and oral mucosa tissues of the experimental mice were examined in this study. The fresh specimens were excised from the live experimental rabbits and mice that were provided by the Animal Center of Fujian Medical University. First, they were rinsed briefly with PBS solution (PH = 7.4) to remove the residual blood on their surfaces. Then the samples were sandwiched between the microscope slide

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and a piece of the cover glass with the keratinizing layer facing the objective. Moreover, to avoid dehydration or shrinkage during the whole imaging process, the specimens were sprinkled with PBS solution.

In this study, the spectral imaging process based on TPEF and SHG was performed on a Zeiss LSM 510 META laser scanning microscope equipped with a mode-locked femtosecond Ti:sapphire laser (110 fs, 76 MHz), tunable from 700 to 980 nm (Coherent Mira 900-F), which was used in the previous work [12]. The emission signal was collected by the META detector, which consists of a high-quality, reflective grating and an optimized 32-channel PMT array detector. The emission signal from biological samples was directed onto a wavelength-dispersive element, and then a stack of *x*-*y* images at a series of emission wavelength bands can be obtained. Thus, a spectral resolved image could be acquired by making all *x*-*y* images match the emission data with a pseudo-color that matches the wavelength range in the visible spectrum and over all the *x*-*y* images. The spectral imaging technology allowed a simultaneous record of both the spectra and image. All the 32 photomultipliers of the META detector covered a spectral width of approximately 340 nm ranging from 377 to 716 nm, and a single PMT covers a spectral range of 10.7 nm. A Plan-Apochromat 63× ($NA = 1.4$) oil immersion objective (Zeiss) was employed in the experiment. All the images were 512 × 512 pixels and had a 12 bit pixel depth. The spectral images were acquired at 2.56 μs per pixel. The average excitation power was controlled at lower than 35 mW at different imaging depths of the epithelial tissues. No photobleaching was observed in a series of spectral images at different depths within the epithelial tissue.

During the analysis of the experimental data, many analysis tools were used in the experimental system. The stage tool can present the accurate location of the imaging depth; by using the region of interest (ROI) tool, the emission spectra of different epithelial tissue layers can be extracted from their spectral imaging; and by using the extracting channel tool, high contrast, high-resolution images of different tissue components with completely separated emission spectral ranges can be obtained from their spectral imaging. With the use of the morphometric tool, the average nuclear area of the cells in the epithelial cell layer and the average diameters of the elastin fibers in the stromal layer are determined.

## 3 Results and discussion

### 3.1 Spectral imaging of keratinizing layer in epithelial tissues

Figure 1 shows the multiphoton images and corresponding spectra extracted from the spectral imaging of the keratinizing layer in the esophageal and oral tissues. The excitation wavelength is set as $\lambda_{ex} = 810$ nm. As can be seen in Fig. 1(a), the keratinizing layer of esophageal tissue presents the morphological features with well defined keratinocytes, and this layer of oral tissues consist of some keratinocytes without nuclei (regular polygons), which is in excellent agreement with the previous histological observations [13,14] as shown in Fig. 1(b). Although the microstructure of these two samples has some apparent differences, it can be identified that they are the keratinizing epithelial layers. The emission spectra of this layer in the two samples have the same peaks at 511 nm as shown in Figs. 1(c) and 1(d). According to previous study [15], the 510 nm peak corresponds to the keratin emission spectra. In the keratinizing layer of the esophageal tissue, the emission spectrum also has a peak at 671 nm, which originated from the contribution of the porphyrin derivative [8]. The flourescence of the porphyrin derivatives is not observed from the oral tissue.

### 3.2 Spectral imaging of epithelial cell layer in epithelial tissues

With the increase of the imaging depth, the keratinocytes are gradually replaced by individual cells of the epithelium cell layer in both esophageal and oral tissues. Figure 2(a) shows the multiphoton image from the spectral imaging of the epithelium cell layer in the esophageal tissue at a depth of 62.7 μm for an excitation wavelength of $\lambda_{ex} = 810$ nm. Since the nuclei are less fluorescent, the cells can be identified by the presence of the dim nuclei. Using a morphometric tool, the cellular nucleus areas are outlined and measured. The average nuclear area is about 25.9 $\mu m^2$ in rabbit esophageal tissue. The previous investigations have proven that an excitation wavelength of $\lambda_{ex} = 730$ nm is preferred for imaging cellular structures [16,17]. To obtain more clear cellular microstructures of the epithelial layer, an excitation wavelength of $\lambda_{ex} = 730$ nm is selected to image the cell layer of the oral sample. Figure 2(b) gives the multiphoton images of the cell layer in the oral sample at a depth of 20 μm. It can be seen that the microstructure of the epithelia squamous cells can be more easily identified through the fluorescent cytoplasm and dark non-fluorescent nuclei without any artifact. The cytoplasm is filled with fluorescent granules stemming from the intrinsic mitochondrial NADH. Because of the absence of a suitable fluorophore at $\lambda_{ex} = 730$ nm, the nuclei surrounded by the fluorescent granules remain non-fluorescent and are dark as shown in the figures. The cells connect with each other through cellular processes, and the average nuclear area is about 28.1 $\mu m^2$ in mouse oral tissue.

In the epithelial cell layer of the esophageal tissue, the emission spectrum has three apparent peaks at 475, 511, and 535 nm for an excitation wavelength of $\lambda_{ex} = 810$ nm. In previous studies, it has been suggested

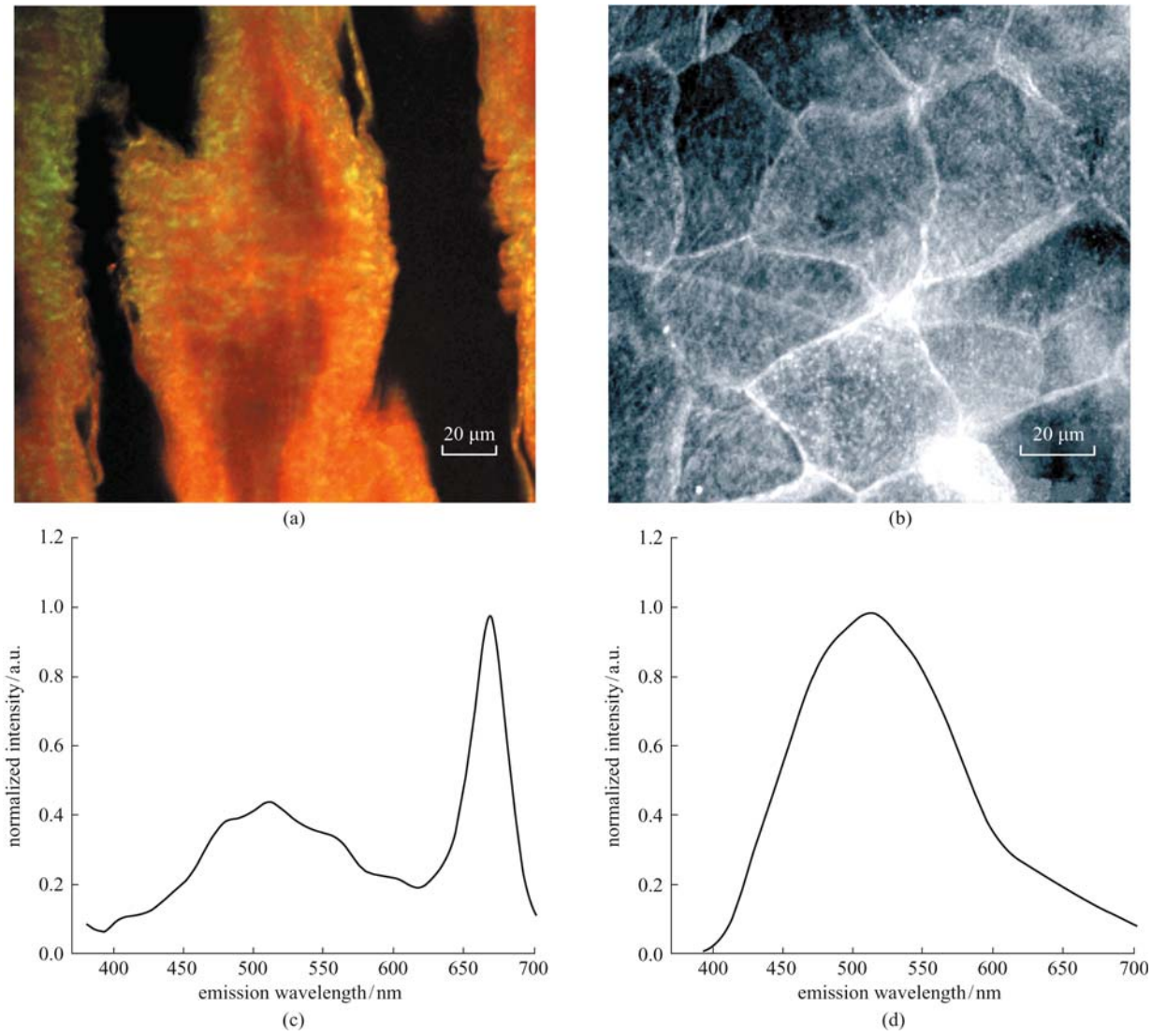


**Fig. 1** Multiphoton images and corresponding spectra extracted from spectral imaging of keratinizing layer in esophageal and oral tissues. (a) Multiphoton image of keratinizing layer in esophageal tissue; (b) multiphoton image of keratinizing layer in oral tissue; (c) corresponding spectrum of keratinizing layer in esophageal tissue; (d) corresponding spectrum of keratinizing layer in oral tissue

that the fluorescence peak at 475 and 535 nm are the contributions of the cellular NADH and FAD, which are the enzymes carrying information of the cellular metabolism [2,8,11]. According to the fluorescence peak of the keratin in the keratinizing layer and the elastin of the stromal layer (shown in the following analysis) at 511 nm, it can be inferred that the strong fluorescence peak at 511 nm of the epithelium cell layer is responsible for the contribution of the cellular structural protein. In addition, a small peak at 405 nm may have originated from the SHG signal of DNA in the chromatin, because it has a typical large molecular anisotropy and is second-order nonlinear [18]. The spectral characteristics of the epithelial cell layer in the oral tissue are the same with that in the esophageal tissue, so the emission spectrum of the epithelial cell layer in the oral tissue is not presented.

### 3.3 Spectral imaging of stromal layer in epithelial tissues

Above the 83.6 μm depth of the esophageal tissue, the whole multiphoton images have two main structures: rope-like and mesh morphological, as shown in Fig. 3(a).

It is known that the collagen and elastic fiber are the main structural components in the stromal layer. From the observed morphological structures, it can be deduced that the rope-like structure and the mesh morphological structure are the elastic fiber and collagen of the stromal layer, respectively. In Fig. 3(c), the corresponding emission spectrum presents two apparent peaks. One is the two-photon excited fluorescent signal of elastin at 511 nm peak. The other peak locates at 405 nm, which is half of the excited wavelength. Its full width at half

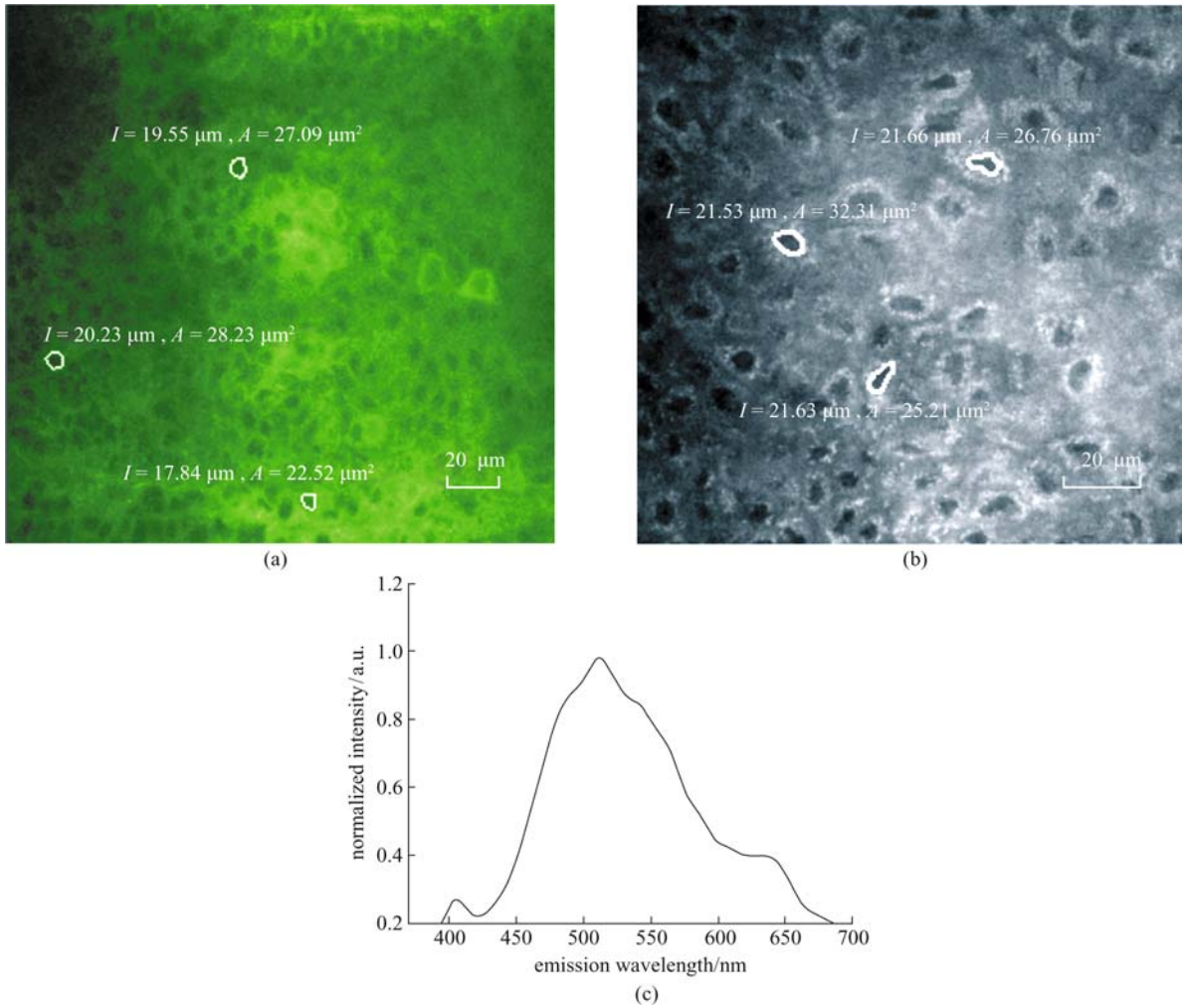


**Fig. 2** Multiphoton images and corresponding spectra from spectral imaging of epithelium cell layer in esophageal and oral tissues. (a) Multiphoton image of cell layer in esophageal tissue; (b) multiphoton image of cell layer in oral tissue; (c) corresponding spectrum of cell layer in esophageal and oral tissues

maximum bandwidth is approximately 10 nm, which is $1/\sqrt{2}$ of the excitation light. In addition, the dependence of the spectral intensity on the excitation power presents a quadratic relationship. Similarly, as the excitation wavelength varies, the site of the spectrum peak also changes and is precisely at half the wavelength of the excitation wavelength. This is the characteristic of the nonlinear optical effect called SHG. From the observed mesh morphological structure, it can be deduced that the SHG signal of collagen is responsible for it. The same result can also be obtained in the oral tissue as shown in Fig. 3(b). In Figs. 3(a) and 3(b), the average diameters of the elastin fibers are about 1.33 and 0.95 μm, respectively.

### 3.4 Layer structure of epithelial tissues based on spectral imaging technology

From the above experimental results, three kinds of completely different microstructures in the keratinizing layer, the epithelial cell layer and the stromal layer can be extracted from their spectral images based on TPEF and SHG. According to the characteristics of microstructures at various depths, one can obtain that the transition zone of the keratinizing layer and epithelium cell layer is at the depth range of 30–40 μm, and the interface of the epithelium layer and stromal layer is at a depth of about 70–80 μm in the esophageal tissue by the accurate measurement of the stage function. In other words, the keratin thickness is about 30 μm and the full epithelial cell layer thickness is approximately 40 μm. It is also shown that the multiphoton image from spectral imaging not only has a capability to identify the layered structure of the esophageal tissue including the keratinizing layer, the epithelial cell layer and the stromal layer, but also can quantitatively give the keratinizing layer and epithelial layer thickness, which are tightly associated with the pathology of tissues. Meanwhile, according to the spectral shape of the different layers in epithelial tissues, it is known that the emission spectral peak (511 nm) of the keratinizing layer is different from that of NADH and FAD in the epithelial cell layer

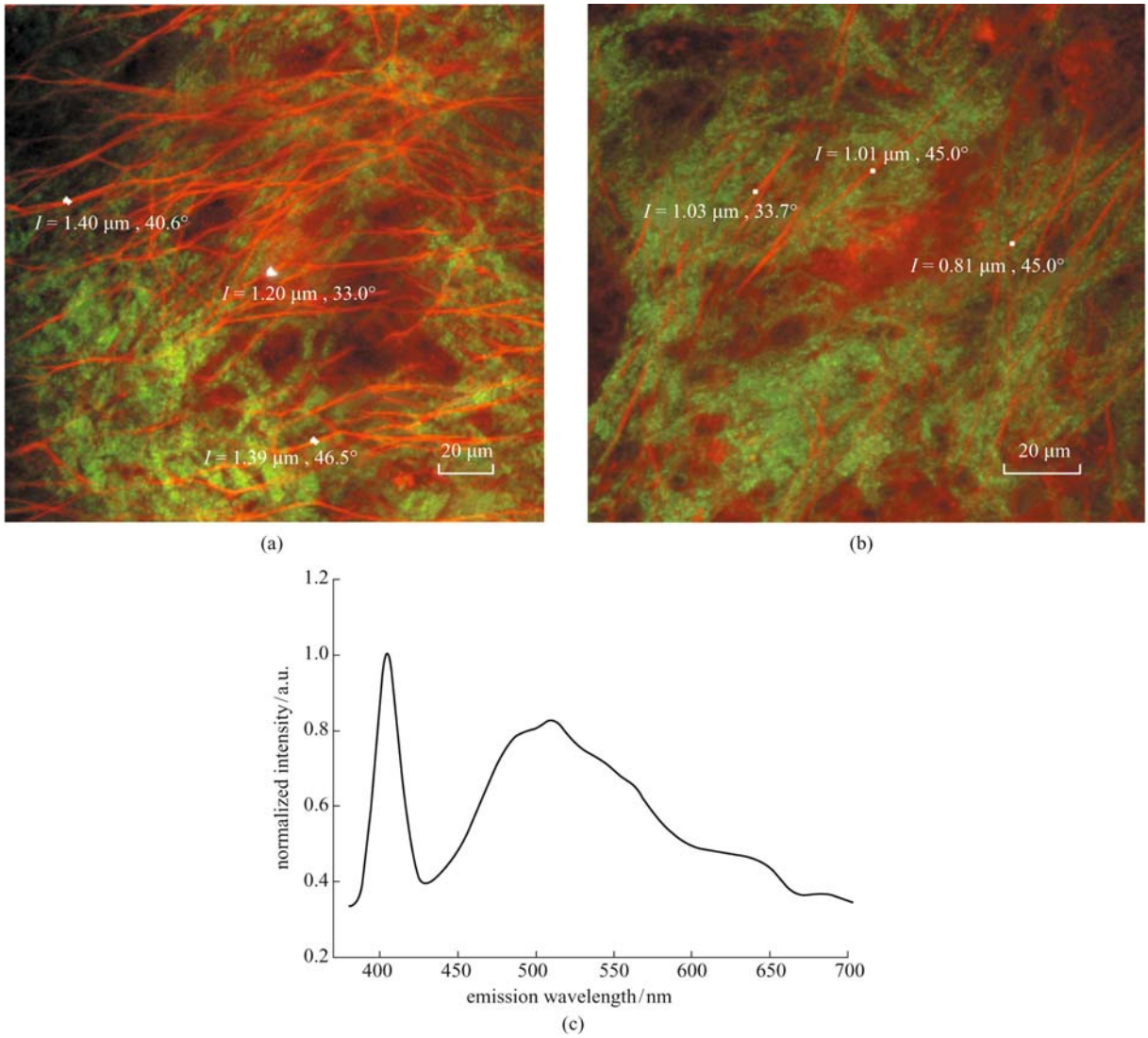


**Fig. 3** Multiphoton images and corresponding spectra from spectral imaging of stromal layer in esophageal and oral tissues. (a) Multiphoton image of stromal layer in esophageal tissue; (b) multiphoton image of stromal layer in oral tissue; (c) corresponding spectrum of stromal layer in esophageal and oral tissues

(475 and 535 nm). The emission spectral peaks of NADH and FAD in the epithelial cell layer can be distinguished from that of the underlying collagen (405 nm) and elastin (511 nm) in the stromal layer. All these findings suggest that spectral analysis of various depths not only can provide spectral information on keratin, NADH, FAD, elastin, and collagen (the important biomarkers of precancer development in esophageal tissue), but also indicate that one can identify the layer structure of the epithelial tissue according to the spectral characteristics of different layers extracted from their spectral imaging.

## 4 Conclusion

The morphological structures and spectral characteristics of epithelial tissues will develop obvious alterations during the formation of epithelial carcinogenesis. The spectral imaging technology can not only present three kinds of completely different microstructures in the keratinizing layer, the epithelial cell layer and the stromal layer, but can also show different emission spectral peaks of these three layers. These experimental results indicate that the spectral imaging has a capability to identify the layered structure of the epithelial tissue, which are tightly associated with the pathology of tissues. The spectral imaging technology is based on TPEF and SHG of the intrinsic components in epithelial tissues, which means that this method does not require any tissue biopsy, sectioning or staining. Thus, it will be of clinical practical interest for the pathological diagnosis of the tissues with stratified squamous epithelia, and could be applied in vivo in a clinical setting with a multiphoton endoscope. Our future work will focus on

the application of this technique to systematically investigate human tissue with stratified squamous epithelia including normal and abnormal tissues.

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