Depth-resolved spectral imaging of rabbit oesophageal tissue based on two-photon excited fluorescence and second-harmonic generation

Jianxin Chen$^{1,3}$, Shuangmu Zhuo$^1$, Rong Chen$^1$, Xingshan Jiang$^1$, Shusen Xie$^1$ and Qilian Zou$^2$

$^1$ Key Laboratory of Optoelectronic Science and Technology for Medicine (Fujian Normal University), Ministry of Education, Fuzhou 350007, People’s Republic of China
$^2$ Division of Cell Biology and Genetics, Fujian Medical University, Fuzhou 350004, People’s Republic of China
E-mail: chenjianxin@fjnu.edu.cn

New Journal of Physics 9 (2007) 212
Received 14 November 2006
Published 4 July 2007
Online at http://www.njp.org/
doi:10.1088/1367-2630/9/7/212

Abstract. A novel depth-resolved spectral imaging based on two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) is developed for simultaneously investigating images and spectra at different depths within rabbit oesophageal tissues in backscattering geometry. Our results show that this method has a capability to identify the layered structures of oesophageal tissue including the keratinizing layer, epithelial cell layer and stromal layer, which are strongly correlated to tissue pathology. By integrating several system analysing tools, morphology and spectroscopy in different layers can be quantitatively obtained. Our findings demonstrate that this technique has the potential to provide more accurate and comprehensive information for the pathological diagnosis of tissues with stratified squamous epithelia.

$^3$ Author to whom any correspondence should be addressed.
1. Introduction

Most of neoplasms occur in stratified squamous epithelia (ACS: Cancer Facts and figures 2002, Georgakoudi et al 2002). Tissues with stratified squamous epithelia include the oesophagus, oral cavity, cervix and colon. Such tissues often consist of the topmost keratinizing layer, epithelial cell layer and underlying stromal layer, as shown in figure 1. During disease progression, the morphological structures of these layers present obvious alterations, such as an increase in the keratinizing layer thickness, cellular nuclear enlargement in the epithelial cell layer and variation of collagen framework in the stromal layer (Skala et al 2005, Sun et al 2004, Wilder-Smith et al 2004). The corresponding autofluorescence spectroscopy of different layers also shows apparent changes (Ramanujam et al 2000, Richards-Kortum et al 1996, Wu et al 2005). The keratin signals become strong, which can reflect the enhancement of keratinizing degree of the epithelial tissue. An increase of cellular NADH fluorescence and a decrease of cellular FAD fluorescence in the case of epithelial neoplasms indicate higher metabolic rate in precancerous epithelia. The structural alterations of collagen in the stromal layer result in the reduction of second-harmonic generation (SHG) signal. The variations of both the morphological structure and emission signal intensity of these layers are tightly related to the developing process of neoplasms. The detection of early alterations is important to improve diagnosis and effective therapy. With the advent of optical imaging technology such as reflectance confocal microscopy and optical coherence tomography, noninvasive tissue imaging with enhanced resolution in the clinical setting has become possible (Drexler et al 2001, Rajadhyaksha et al 1995). Recently, multiphoton microscopy has become a powerful tool for imaging unstained samples. The multimodal nonlinear optical imaging method based on two-photon excited fluorescence (TPEF) and SHG has been used to noninvasively evaluate and monitor the morphological structure and functional state of the layered tissues (Mertz 2004, Pons et al 2006, Zoumi et al 2002). However, the recently developed nonlinear spectral imaging technique has more attractive characteristics. The method has many advantages over those that only give the autofluorescence spectra or the morphological structure of tissues. It has great potential to provide comprehensive information.
Figure 1. Schematic drawing of a vertical section of stratified squamous epithelia, including the keratinizing layer, epithelial cell layer and stromal layer.

For the diagnosis and therapy of tissues, and it has been applied to the biochemical analysis of living mouse skin tissues at different depths using two prisms (Palero et al 2006). With the development of a miniaturized multiphoton apparatus (Bird et al 2003, Jung et al 2003), one can envision that this approach can be used for in vivo diagnosing diseases of the internal organs, such as the oesophagus. To the best of our knowledge, depth-resolved nonlinear spectral images of the oesophagus based on intrinsic signals have not been obtained. Especially, using grating optics to achieve depth-resolved nonlinear spectral images is not reported in previous studies.

In this study, using a high sensitivity detector and grating optics, we present a novel depth-resolved spectral imaging based on TPEF and SHG to investigate the microstructure and biochemical components of rabbit oesophagus. This method was used to acquire real-colour RGB (red, green, blue) representations of the nonlinear spectral images from oesophageal tissue at various depths, and to identify the layered structure of oesophageal tissue including the topmost keratinizing layer, epithelial cell layer and underlying stromal layer. High-contrast, high-resolution images of tissue components with completely separated emission spectral ranges are obtained; and the keratinizing thickness, cellular nuclear area and diameter of elastic fibres are quantitatively analysed. Furthermore, for different excitation wavelengths, emission spectra from the cell layer and the stromal layer were recorded and compared.

2. Materials and methods

2.1. Preparation of rabbit oesophageal sample

The oesophageal tissues of six experimental rabbits were examined in this study. The fresh specimens were excised from living experimental rabbits, provided by the Animal Center of Fujian Medical University. Firstly, they were rinsed briefly with PBS solution (pH 7.4) to remove the residual blood on their surfaces. Then the oesophagus were opened longitudinally and sandwiched between the microscope slide and a piece of cover glass with the topmost keratinizing layer facing the objective. Moreover, to avoid dehydration or shrinkage during the whole imaging process, the specimen was sprinkled with PBS solution.

2.2. Experimental set-up

In this study, the depth-resolved spectral imaging was performed on a Zeiss LSM 510 META laser scanning microscope equipped with a mode-locked femtosecond Ti:sapphire laser (110 fs,
Figure 2. Experimental set-up for the depth-resolved spectral imaging of rabbit oesophageal tissue.

76 MHz), tunable from 700 to 980 nm (Coherent Mira 900-F), as shown in figure 2. The polarization direction of laser light is the horizontal polarization. An acousto-optic modulator (AOM) is used to control the laser intensity attenuation. The light enters a Zeiss Axiovert 200 microscope and is reflected into the objective through the main dichroic beam-splitter (MDBS). An optional HRZ (fast Z-scanning) 200 fine focusing stage (HRZ 200 stage, Carl Zeiss) is used to change the focus position, allowing for imaging at various depths. A Plan-Apochromat 63 \times (N.A. = 1.4) oil immersion objective (Zeiss) was employed in these experiments. The backward signal was collected by the META detector. The META detector consists of a high-quality, reflective grating and an optimized 32-channel photomultiplier tube (PMT) array detector. The emission signal from biological samples is directed on to a wavelength-dispersive element and a stack of \( x-y \) images at a series of emission wavelength bands can be obtained. Thus, by matching the emission data with a pseudo-colour that matches the wavelength range in the visible spectrum and overlaying all \( x-y \) images, a real-colour RGB nonlinear spectrally resolved image can be acquired. All 32 photomultipliers of the META detector cover 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. In this work, all images were 512 \times 512 pixels and had a 12-bit pixel depth. The acquisition of a single 512 \times 512 pixels spectral image was about 1.57 s. The excitation power was 10 mW on the specimen surface. At greater depths, a higher power value was employed by using automated power control of this system to obtain high contrast images. The highest power is approximately 25 mW. In our system, the diameter of the laser focal spot is approximately 15 \( \mu \)m. No photobleaching was observed in a series of spectral images at different depths within the oesophageal tissue, as shown in figures 3 and 4.

2.3. Analysing tools of the experimental system

There are many analysing tools in our experimental system. The stage tool can present the accurate location of imaging depth. The ROI (region of interest) tool can reveal the emission spectra of...
Figure 3. Representative real-colour spectral images and corresponding spectra at various depths. Scale bar is 50 µm.

a region of interest by plotting the mean intensity of all pixels within the ROI versus the centre wavelength of each emission band. In this work, we used this tool to identify intrinsic species spectra. Using the extracting channel tool, high contrast, high-resolution images of different tissue components with completely separated emission spectral ranges can be obtained. Specifically, we choose two channels to obtain the high contrast image of keratin and porphyrin derivatives.
Figure 4. High-contrast images of different microstructural components from various layers by using the extracting channel tool. Scale bar is 50 µm. (a) Depth: 0 µm keratinizing layer. First channel (green colour-coded): 382–628 nm, Second channel (red colour-coded): 628–714 nm. (b) Depth: 62.7 µm epithelium layer. One channel (green colour-coded): 425–607 nm. (c) Depth: 150 µm stomal layer. First channel (green colour-coded): 382–425 nm, Second channel (red colour-coded): 425–714 nm. (d) Depth: 83.6 µm stomal layer. First channel (green colour-coded): 382–425 nm, Second channel (red colour-coded): 425–714 nm.

in the keratin layer: one channel shows the output of keratin that falls between 382 and 628 nm, whereas another channel shows the output of porphyrin derivatives covering the range from 628 to 714 nm. And one channel is used to image cells in the epithelial cell layer (425–607 nm) in order to eliminate the interference of background signal and SHG signal of the extracellular matrix, collagen. Similarly, the distributions of collagen (382–425 nm) and elastin (425–607 nm) in the stromal layer are clearly presented by using two channels. By the use of the morphometric tool, the average nuclear area of cells in the epithelial cell layer and the average diameters of elastin fibres in the stromal layer are determined.
3. Results and discussion

3.1. Depth-resolved spectral imaging of oesophageal tissue

To investigate the layered structure of oesophageal tissues, a series of spectral images are obtained at different depths within the tissue. Figures 3(a)–(c) show real-colour RGB representations of the typical nonlinear spectral images from fresh oesophageal tissue at various depths from 0 to 150 µm for excitation wavelength $\lambda_{\text{ex}} = 810\,\text{nm}$. The accurate positions of imaging depths were obtained by using the stage tool. As can be seen, there are three kinds of completely different real-colour RGB representations. Specifically, from the morphological features with well defined keratinocytes in the surface (0 µm), one can identify that it is the keratinizing epithelial layer of oesophageal tissue. With the increasing of imaging depths, keratinocytes are gradually replaced by individual cells of the epithelium cell layer, as shown in the real-colour spectral image at 62.7 µm deep. Above 85.0 µm deep, the whole spectral images have two main colour structures: blue and purple. The blue-fluorescing rope-like structures are elastin of the stromal layer and the purple colour refers to the second-harmonic signal generated by collagen of the stromal layer. Moreover, using the accurate measurement of the stage tool and according to the characteristics of spectral images at various depths, we found that the transition zone of the keratinizing layer and epithelium cell layer is at the depth range of $35.43 \pm 2.75\,\mu\text{m}$ and the interface of the epithelium layer and stromal layer is at a depth of about $75.73 \pm 4.01\,\mu\text{m}$. In other words, the keratin thickness is about $28.23 \pm 1.26\,\mu\text{m}$ and the full epithelial cell layer thickness is approx. $38.53 \pm 1.00\,\mu\text{m}$. It is obvious that the depth-resolved spectral imaging has a capability to identify the layered structure of oesophageal tissue including the keratinizing layer, epithelial cell layer and stromal layer, which are tightly associated with the pathology of tissues.

3.2. Depth-resolved emission spectra of oesophageal tissue

The spectra from various depth optical planes were revealed by using the ROI tool, as shown in figures 3(d)–(f). The presented spectra have been corrected for the wavelength dependent instrument response and dark noise spectra are subtracted from the acquired sample spectra. Each spectrum is normalized to the maximal peak intensity. In the keratinizing layer, the emission spectrum has two distinct peaks at 511 and 671 nm. According to previous studies (Pena et al 2005, Ramanujam et al 2000), they originated from keratin and porphyrin derivatives. In the epithelial cell layer, the red fluorescence peak at 671 nm vanishes while there are three strong and apparent peaks at 475, 511 and 535 nm. It has been suggested in previous studies that the blue fluorescence peak at 475 nm and the yellow fluorescence peak at 535 nm are contributions of cellular NADH and FAD, which are the enzymes carrying information on cellular metabolism (Drezek et al 2001, Georgakoudi et al 2002, Palero et al 2006). According to the fluorescence peaks at 511 nm of keratin in the keratinizing layer and elastin in the stromal layer (as seen in the following analysis), we believe that the strong fluorescence peak at 511 nm in the epithelium cell layer is responsible for the contribution of cellular structural protein. In addition, a very small peak at 405 nm originated from the SHG signal of the extracellular matrix (collagen). It was reported in previous work that the SHG signal serves as a sensitive indicator of collagen to separate the epithelial layer from underlying stroma (Wu et al 2005). However, our findings show that small SHG fail to discriminate the epithelial layer from underlying stroma. So, in addition to emission spectra, signal imaging is necessary for discriminating the epithelial layer from underlying stroma. In the stromal layer, the SHG signal of collagen at 405 nm and the
TPEF signal of elastin at 511 nm are observed. Moreover, there are two emission signals at 475 and 535 nm peak, which correspond to NADH and FAD in the fibroblasts (as shown in the spectra at 150 µm deep), respectively. These findings suggest that spectral analysis of various depths can provide depth-resolved information on keratin, NADH, FAD, elastin and collagen, the important biomarkers of precancer development in oesophageal tissue.

3.3. Selective visualization of microstructural components at different layers

The microstructural alterations of different components in biological tissues are tightly related to the multi-step process of tissue carcinogenesis. Comparing with the normal tissue, the keratinizing layer of the dysplastic lesion becomes thick and less regular. In the epithelial cell layer, the dysplastic cells show nuclear enlargement, crowding and irregular distribution. Collagen in the stromal layer undergoes architectural changes (Ramanujam et al 2000, Skala et al 2005). So, enhanced understanding of early microstructural alterations will improve diagnosis and development of effective therapeutic methods. Using the real-colour RGB representations of depth-resolved spectral images coupled with several analysing tools in our system, we can easily present visualization of the major components in the three layered structure of oesophageal tissue and quantitative analysis of microstructures. According to the spectral shape of the topmost keratinizing layer, it is possible to see that the emission spectral range (382–628 nm) of keratin is well separated from that of porphyrin derivatives (628–714 nm). Thus, using the extracting channel tool, we obtained the clearly relative distributions of keratin and porphyrin derivatives in the keratinizing layer, as shown in figure 4(a). One channel shows the output of keratin between 382 and 628 nm, whereas another channel shows the output of porphyrin derivatives from 628 to 714 nm. The green colour-coded image is keratin and red colour-coded image is porphyrin derivatives. It is noted that distributions of keratin and porphyrin derivatives are not uniform. Similarly, the distributions of epithelial cells (425–607 nm) and underlying collagen (382–425 nm) and elastin (425–607 nm) of the stromal layer are presented in figures 4(b) and 4(c). In figure 4(b), the cells can be identified by the presence of dim nuclei since the nuclei are less fluorescent. And the microstructure of cells becomes more distinct. By the use of the morphometric tool, the cellular nuclear areas are outlined and measured. The average nuclear area is 21.05 ± 3.80 μm² as shown in figure 4(b). In figures 4(c) and (d), collagen with the mesh morphology is distinguished from elastin fibres with the morphology of thick ropes. To the best of our knowledge, we show, for the first time, the high-contrast, high-resolution images of collagen and elastin fibres in the stromal layer of rabbit oesophageal tissue. Related changes in the distribution of collagen and elastic fibres in the dermis have been demonstrated to be an indicator for evaluating skin aging or other pathological processes involving variations of the extracellular matrix (Koehler et al 2006, Lin et al 2005). So, the microstructural changes of collagen and elastin in the stromal layer may possibly be used to assess pathological processes in oesophageal tissues. Using the morphometric tool, the average diameters of elastin fibres are about 1.43 ± 0.04 μm and 1.33 ± 0.11 μm at depths of 150.0 and 83.6 μm, respectively, which indicates that the diameter of elastin fibres in the stromal layer has no apparent variation with increasing detecting depth. These results demonstrate that the real-colour RGB representations of depth-resolved spectral imaging coupled with several analysing tools can provide high-contrast, high-resolution visualization of microstructural components of various layers and selectively study the tissue components such as keratin, porphyrin derivatives, cells, elastin and collagen.
3.4. Optimal excitation wavelengths

3.4.1. Optimal excitation wavelength for the estimation of cellular metabolism. It is well known that the autofluorescence of the epithelial cell layer is determined by NADH and FAD, the enzymes carrying information on cellular metabolism (Palero et al 2006, Skala et al 2005, Wu et al 2005, Zipfel et al 2003). It was reported in previous work that the ratio of NADH over FAD fluorescence, a parameter related to cellular metabolism, has strong correlation with tissue pathology. To obtain optimal excitation wavelength for the estimation of cellular metabolism, the normalized epithelial cell layer spectra for a series of excitation wavelengths ranging from 730 to 910 nm with increments of 10 nm were obtained for comparison. Figures 5(a)–(c) show the representative emission spectra at the same imaging depth of the epithelial cell layer and stromal layer for $\lambda_{\text{ex}} = 750, 810$ and 850 nm. As can be seen, at $\lambda_{\text{ex}} = 750$, TPEF of cellular NADH and structural protein that correspond to two peaks at 475 and 511 nm, respectively, are the main signal sources for imaging the epithelial cell layer. At this excitation wavelength, excitation efficiency of FAD fluorescence at 535 nm peak is very low. When using 850 nm excitation, FAD fluorescence can be efficiently excited whereas the 475 nm peak of NADH can not be identified. Only at 810 nm laser excitation, the NADH and FAD autofluorescence almost reach a balanced level. Because measuring with two excitations is complicated, 810 nm excitation wavelength, which produces the mitochondrial signal with NADH and FAD autofluorescence at comparable levels, is an optimal wavelength for the estimation of cellular metabolism.

3.4.2. Optimal excitation wavelength for monitoring the change of collagen SHG signal over elastin TPEF. According to previous studies, collagen SHG signal over elastin autofluorescence can be used to evaluate tissue diseases (Koehler et al 2006, Lin et al 2005). To better monitor the change of collagen SHG over elastin autofluorescence signal, the effect of excitation wavelength was investigated. Similarly, the normalized stromal layer spectra were acquired for excitation wavelengths from 730 to 910 nm with increments of 10 nm. Figures 5 (d)–(f) show the representative emission spectra at the same imaging depth of the stromal layer for $\lambda_{\text{ex}} = 750, 810$ and 850 nm. As can be seen, 850 nm excitation light is most suitable for imaging elastin, which is less affected by collagen (Zoumi et al 2002), cellular NADH and FAD autofluorescence. So, to acquire high contrast images of collagen and elastin and better monitor the change of collagen SHG over elastin TPEF signal in the stromal layer, 850 nm is an optimal excitation wavelength.

4. Conclusion

We have demonstrated a novel depth-resolved spectral imaging method based on TPEF and SHG to study the layered structures of rabbit oesophageal tissue. The real-colour RGB representation of the spectral images at different tissue depths can offer a simple method of discriminating the layered structure of rabbit oesophageal tissue, including the keratinizing layer, epithelial cell layer and stromal layer. Coupled with several system analysing tools, the method allows selective visualization of microstructural components and analysis of intrinsic spectra in various layers, quantitatively providing some important depth-resolved information on the biomorphology and biochemistry of the oesophagus. Our results also suggest that 810 nm is an optimal excitation wavelength for the estimation of epithelial cellular metabolism, while 850 nm excitation light
Figure 5. The emission spectra obtained from the epithelial cell layer (a)–(c) and the stromal layer (d)–(f) for $\lambda_{\text{ex}} = 750$, 810 and 850 nm.
is most suitable for acquiring high contrast images of collagen and elastin and monitoring the change of collagen SHG over elastin TPEF in the stromal layer.

These results imply that this method has the potential to provide more accurate and comprehensive information for the pathological diagnosis of tissues with stratified squamous epithelia. Moreover, this method does not require tissue biopsy, sectioning, or staining, and in the future could be applied in vivo in a clinical setting with multiphoton endoscopy (Bird et al 2003, Jung et al 2003). In the future, our work will focus on the application of this technique to systematically investigating human tissue with stratified squamous epithelia including normal and abnormal tissues.

Acknowledgments

The project was supported by the National Natural Science Foundation of China (no. 60508017, 60678054), the Scientific Program of the Educational Hall of Fujian Province (no. JA05215), the Natural Science Foundation of Fujian Province of China and Program for New Century Excellent Talents in Fujian Province University (2007).

References

ACS 2002 Cancer Facts and Figures 2002 (New York: American Cancer Society)
Bird D and Gu M 2003 Two-photon fluorescence endoscopy with a micro-optic scanning head Opt. Lett. 28 1552–4
Drezek R, Brookner C, Pavlova I, Boiko I, Malpica A, Lotan R, Follen M and Richards-Kortum R 2001 Autofluorescence microscopy of fresh cervical-tissue sections reveals alterations in tissue biochemistry with dysplasia Photochem. Photobiol. 73 636–41
Drexler A W, Morgen U, Ghanta R K, Kartner F X, Schuman J S and Fujimoto J G 2001 Ultrahigh-resolution ophthalmic optical coherence tomography Nat. Med. 7 502–7
Georgakoudi I et al 2002 NAD(P)H and collagen as in vivo quantitative fluorescent biomarkers of epithelial precancerous changes Cancer Res. 62 682–7
Jung J C and Schnitzer M J Multiphoton endoscopy 2003 Opt. Lett. 28 902–4
Koehler M J, Koenig K, Elsner P, Buckle R and Kaatz M 2006 In vivo assessment of human skin aging by multiphoton laser scanning tomography Opt. Lett. 31 2879–81
Laiho L H, Pelet S, Hancewicz T M, Kaplan P D and So P T C 2005 Two-photon 3D mapping of ex vivo human skin endogenous fluorescence species based on fluorescence emission spectra J. Biomed. Opt. 10 024016–10
Lin S J, Wu R J, Tan H Y, Lo W, Lin W C, Young T H, Hsu C J, Chen J S, Jee S H and Dong C Y 2005 Evaluating cutaneous photaging by use of multiphoton fluorescence and second-harmonic generation microscopy Opt. Lett. 31 2275–7
Mertz J 2004 Nonlinear microscopy: new techniques and applications Curr. Opin. Neurobiol. 14 610–6
Palero J, Bruijn H, Heuvel A, Sterenberg H and Gerritsen H 2006 In vivo nonlinear spectral imaging in mouse skin Opt. Express 14 4395–402
Pena A M, Strupler M, Boulesteix T and Schanne-Klein M C 2005 Spectroscopic analysis of keratin endogenous signal for skin multiphoton microscopy Opt. Express 13 6268–74
Pons T and Mertz J 2006 Membrane potential detection with second-harmonic generation and two-photon excited fluorescence: a theoretical comparison Opt. Commun. 258 203–9
Ramanujam N 2000 Fluorescence spectroscopy of neoplastic and non-neoplastic tissues Neoplasia 2 89–117
Richards-Kortum R and Sevick-Muraca E 1996 Quantitative optical spectroscopy for tissue diagnosis Annu. Rev. Phys. Chem. 47 555–606

New Journal of Physics 9 (2007) 212 (http://www.njp.org/)
Rajadhyaksha M, Grossman M, Esterowitz D, Webb R H and Anderson R R 1995 *In vivo* confocal scanning laser microscopy of human skin: melanin provides strong contrast *J. Invest. Dermatol.* **104** 946–52

Skala M C, Squirrell J M, Vrotsos K M, Eickhoff J C, Gendron-Fitzpatrick A, Eliceiri K W and Ramanujam N 2005 Multiphoton microscopy of endogenous fluorescence differentiates normal, precancerous, and cancerous squamous epithelial tissues *Cancer Res.* **65** 1180–6

Sun J, Shilagard T, Bell B, Motamedi M and Vargas G 2004 *In vivo* multimodal nonlinear optical imaging of mucosal tissue *Opt. Express.* **12** 2478–6

Wilder-Smith P, Osann K, Hanna N, Abbadi N El, Brenner M, Messadi D and Krasieva T 2004 *In vivo* multiphoton fluorescence imaging: a novel approach to oral malignancy *Lasers Surg. Med.* **35** 96–103

Wu Y and Qu J Y 2005 Two-photon autofluorescence spectroscopy and second-harmonic generation of epithelial tissue *Opt. Lett.* **30** 3045–7

Zoumi A, Yeh A and Tromberg B J 2002 Imaging cells and extracellular matrix *in vivo* by using second-harmonic generation and two-photon excited fluorescence *Proc. Natl Acad. Sci. USA* **99** 11014–9

Zipfel W R, Williams R M and Webb W W 2003 Nonlinear magic: multiphoton microscopy in the biosciences *Nat. Biotechnol.* **21** 1369–77