Microstructure imaging of human rectal mucosa using multiphoton microscopy

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Abstract. Multiphoton microscopy (MPM) has high resolution and sensitivity. In this study, MPM was used to image microstructure of human rectal mucosa. The morphology and distribution of the main components in mucosa layer, absorptive cells and goblet cells in the epithelium, abundant intestinal glands in the lamina propria and smooth muscle fibers in the muscularis mucosa were clearly monitored. The variations of these components were tightly relevant to the pathology in gastrointestinal system, especially early rectal cancer. The obtained images will be helpful for the diagnosis of early colorectal cancer.

1. Introduction

It is well known that the rectal carcinoma has the high mortality and is the second most common gastrointestinal malignancy. With the western patterns of diet and physical inactivity, the incidence rate of colorectal cancer is increasing. The greatest increase in the incidence of colorectal cancer is in Asia[1]. Patients with stage I colorectal cancer have a 5-year survival of ≥90%, whereas patients with stage II, III, IV colorectal cancer have a 5-year survival of 82.5%, 59.5%, 8.1% respectively[2]. So, reducing the mortality of colorectal cancer relies on early detection. Unfortunately, the routine endoscopy is difficult to identify the early colorectal cancers, especially flat adenomas and carcinomas in situ, mainly due to the absence of typical morphological structures. Therefore, much more progress
has been made towards developing new optical image-systems that improve the predictability of dysplastic and malignant colorectal lesions, such as photodynamic diagnosis[3-5], fluorescence imaging systems[6-7], and laser-induced spectroscopy[8-9].

Multiphoton microscopy (MPM) based on two-photon excited fluorescence and second harmonic generation has the ability to obtain the images of tissues at the cellular and subcellular level[10-12]. It has some advantages in imaging unstained tissue samples and has been used to evaluate and monitor morphological structure and functional state of epithelial tissues[10-11,13]. MPM has the potential to become a powerful tool for noninvasively early diagnosis of cancers in colorectal system[14]. The variations of mucosa and submucosa layers are important to early diagnoses of rectal cancer. So, in this study, the microstructures of human rectal mucosa are investigated using multiphoton microscopy. The obtained images will be helpful for the diagnosis of early colorectal cancers.

2. Materials and methods
In this study, the ex-vivo human rectum tissues were examined. The fresh specimens were provided by Fujian Provincial Tumor Hospital, originated from five patients aged 40-60 years old. Every patient had his consent to participate in the study. The samples were cut into 5 μm thickness sections by the freezing microtome and stored in a low temperature refrigerator (-86°C) before they were used. Each section was surely comprised of a complete transverse cross-section of the mucosa layers and sandwiched between the microscope slide and cover glass. To avoid dehydration or shrinkage during the whole imaging process, the specimen was sprinkled with PBS solution (PH 7.4). In this study, we are chiefly interested in the rectal mucosa layer.

The multiphoton microscopic imaging system used in this study has been described in previous publications[9,15]. It was mainly an Axiovert 200 microscope (Zeiss LSM 510 META) equipped with a mode-locked femtosecond Ti: sapphire laser (Coherent Mira 900-F), tunable from 700 nm to 980 nm (110 fs, 76 MHz). A Plan-Apochromat 63× (NA=1.4) oil immersion objective (Zeiss) was employed for focusing the excitation beam and for collecting the backward signals. The multichannel mode can achieve TPEF and SHG images, respectively, which separated by the dichroic mirror in the detection path. In this study, the excitation wavelength of 800 nm was used. So, one channel was corresponding to the wavelength range of 387 - 409 nm to show microstructure of tissue component from SHG signals (red color-coded), whereas another channel covered the wavelength range from 430 to 708 nm in order to present the morphology of tissue component from TPEF signals (green color-coded ). An optional HRZ 200 fine-focusing stage (HRZ 200 stage, Carl Zeiss) was used to obtain a large-area image of rectum tissue and record the focus position.

3. Results and discussions
Figure 1 shows a large-area TPEF/SHG image (877.5 ×585.0μm) of human rectal mucosa. As can be seen from figure 1a, a little collagen (↓ white arrow) with mesh morphology of lamina propria in mucosa layer can be imaged alone by one channel (red color-coded). The thin red band surrounds individual glands (showed in figure 1c), which likely represents the basement membrane and portions of myofibroblastic sheath[14]. Similarly, in figure 1b the absorptive cells, goblet cells, and a large number of intestinal glands can be isolated from collagen using another channel (green color-coded).
The content and distribution of absorptive cells, goblet cells, intestinal glands and some scattered smooth muscle bundles (↓ white arrow) between the intestine glands, smooth muscle fibres in the muscularis mucosae can be clearly visible in figure 1b. Overlaying two channels yields a high-contrast TPEF/SHG image of mucosa, as shown in figure 1c, indicates that a little collagen located between two intestinal glands. Imaging results of normal rectal mucosa are in excellent agreement with histology as showed in reference 16.

![Figure 1](image.png)

**Figure 1.** A large area SHG/TPEF image of human rectum mucosa: (a) SHG image of collagen (↓ white arrow); (b) TPEF image of epithelial cells in the epithelium, absorptive cells, goblet cells, crypts of Lieberkuhn in the lamina propria, and some scattered smooth muscle bundles (↓ white arrow) between the intestine glands, smooth muscle fibers in the muscularis mucosae; (c) overlay of the SHG/TPEF image. Scale bar is 50 μm.

In order to clearly present the microstructure of mucosa layer, figure 2 shows the epithelium image of the mucosa, white box 1 in figure 1c. The imaging field of view is 140.0 × 213.1 μm. As can be seen, the absorptive cells (↓ white arrow ①) and goblet cells (↓ white arrow ②) in figure 2b can be distinctly identified and show the fluorescent cytoplasmatic granules of mitochondria. The non-fluorescent nuclei displayed darkly on the optical image and surrounded by the fluorescent mitochondria, and the clear cellular boundary.
Figure 2. The microstructure of the mucous epithelium in figure 1c (white box 1), showing SHG image of collagen in figure 2a, the absorptive cells (↓white arrow ①) and goblet cells (↓white arrow ②) in figure 2b,. Scale bar is 20 μm

Figure 3. SHG/TPEF image of the lamina propria of human rectum, regions of interest (ROI) in figure 1c (white box 2): (a) SHG image of collagen (↓white arrow); (b) TPEF images of absorptive cells (↓white arrow ①), goblet cells (↓white arrow ②), scattered smooth muscle bundles (↓white arrow ③) in figure 3b; (c) SHG/TPEF image was obtained by overlaying two channels. Scale bar is 20 μm
Displayed in Figure 3 are the TPEF/SHG images of the lamina propria of human rectal mucosa. Imaging field of view is 296.5 × 192.5 μm. Figure 3a shows a little collagen (↓ white arrow) with mesh morphology of lamina propria in mucosa layer surrounding the intestine glands can be imaged alone by one channel (red color-coded) just like in figure 1a. Similarly, figure 3b reveals the two-photon excited fluorescence images (green color-coded) of absorptive cells (↓ white arrow ①), goblet cells (↓ white arrow ②) in the intestine glands, scattered smooth muscle bundles (↓ white arrow ③) between the intestine glands. The high-contrast TPEF/SHG image is shown in figure 3c.

![Figure 3](image)

**Figure 3.** The TPEF/SHG images of the lamina propria of human rectal mucosa. Imaging field of view is 296.5 × 192.5 μm. Figure 3a shows a little collagen (↓ white arrow) with mesh morphology of lamina propria in mucosa layer surrounding the intestine glands can be imaged alone by one channel (red color-coded) just like in figure 1a. Similarly, figure 3b reveals the two-photon excited fluorescence images (green color-coded) of absorptive cells (↓ white arrow ①), goblet cells (↓ white arrow ②) in the intestine glands, scattered smooth muscle bundles (↓ white arrow ③) between the intestine glands. The high-contrast TPEF/SHG image is shown in figure 3c.

The morphology of smooth muscle fibres in the muscularis mucosae is also displayed in figure 4 (imaging field of view was 119.4 × 151.4 μm). SHG signals (red color-coded) of fibers are of inner circular (↓ white arrow ①) and outer longitudinal (↓ white arrow ②), as shown in the figure 4a. Similarly, figure 4b also presents the two-photon excited fluorescence images (green color-coded) of an inner circular and outer longitudinal layer of smooth muscle in the corresponding location. The high-contrast TPEF/SHG image is shown in figure 4c.

The morphology and function of the intestinal glands and cells in rectum mucosa may alter with disease progression of early rectum cancers [17]. The thickness of mucosa [18] and the morphology of smooth muscle fibres in the muscularis mucosae also changed when cancer occurs. So, the microscopic imaging of rectal mucosa layer may further develop a new method for histological evaluation in the early diagnosis of rectum diseases.

4. Conclusions
In conclusion, we demonstrate that multiphoton microscopic imaging technique has the potential to present the microstructures of human rectal mucosa. And the imaging results of normal rectal mucosa are in excellent agreement with histology. These imply that the multiphoton microscopic imaging
technique has the potential to non-invasively diagnose early rectal cancer.

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