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Quantitative structural markers of colorectal dysplasia in a cross sectional study of ex vivo murine tissue using label-free multiphoton microscopy

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Abstract

Two-photon excitation of label-free tissue is of increasing interest, as advances have been made in endoscopic clinical application of multiphoton microscopy, such as second harmonic generation (SHG) scanning endoscopy used to monitor cervical collagen in mice¹. We used C57BL mice as a model to investigate the progression of gastrointestinal structures, specifically glandular area and circularity. We used multiphoton microscopy to image ex-vivo label-free murine colon, focusing on the collagen structure changes over time, in mice ranging from 10 to 20 weeks of age. Series of images were acquired within the colonic and intestinal tissue at depth intervals of 20 microns from muscularis to the epithelium, up to a maximum depth of 180 microns.

The imaging system comprised a two-photon laser tuned to 800nm wavelength excitation, and the SHG emission was filtered with a 400/40 bandpass filter before reaching the photomultiplier tube. Images were acquired at 15 frames per second, for 200 to 300 cumulative frames, with a field of view of 261μm by 261μm, and 40mW at sample. Image series were compared to histopathology H&E slides taken from adjacent locations. Quantitative metrics for determining differences between murine glandular structures were applied, specifically glandular area and circularity.

Keywords

colorectal cancer; multiphoton; histopathology; label-free; image analysis; mouse model

1. INTRODUCTION

Colorectal cancer is the second leading cause of cancer deaths in the United States, despite high 5-year survival rates when diagnosed early². Traditionally, progression of colorectal dysplasia has been investigated using histopathology. This has allowed for the understanding of how most colorectal dysplasia begins in the epithelial layer, and increases in risk to the patient when it extends beyond that layer and into the submucosa, muscularis, and serosa^{3,4}. While histopathology is considered the standard, current research has made advances in different imaging modalities that would allow for faster results, preferably in situ. Imaging modalities such as high-resolution microendoscopy⁵, two-photon excitation fluorescence⁶, second-harmonic generation (SHG)⁷, fluorescence lifetime imaging⁷, and optical coherence

tomography⁸ are all examples of optical imaging which allow for real-time data extraction, and have used to further understanding of colorectal dysphasia, and potentially serve as tools for aiding in screening, such as guiding the clinician in choosing regions for biopsies.

Label-free multiphoton imaging has been used to study different diseases, such as inflammatory bowel disease⁹, breast cancer¹⁰, and lung carcinoma¹¹. Its ability to provide image data from bulk tissue, often 150um or more below the tissue surface, makes this imaging modality ideal for in situ study of epithelial dysplasia, for example, endoscopic clinical application of multiphoton microscopy to monitor cervical collagen in mice¹. Using label-free multiphoton imaging, specifically second harmonic generation (SHG), we investigated possible quantitative metrics for determining differences between wildtype and heterozygous (6J-APCmin) colorectal murine tissue.

Colorectal cancer progression causes structural changes in the epithelial tissue, normally leading to irregularities in gland and crypt shapes and patterns^{3, 5, 12}. While qualitative differences are important for clinicians who might implement an optical imaging modality, quantitative difference are a complementary tool of interest for reducing the burden of training as well as inter-observer variability. Quantification of image data, specifically glandular area and circularity, using label-free multiphoton imaging, can provide in situ data that could be relevant in the future design of imaging modalities for early detection and treatment. In this manuscript we qualitatively and quantitatively investigate glandular area and circularity of murine colonic tissue.

2. METHODS

2.1 Multiphoton microscopy system

The multiphoton microscopy system (Fig. 1) used comprises a Mai Tai Ti:Sapphire laser (Spectra-Physics, USA), half wave plate (10RP52-2, Newport, USA) mounted in a motorized rotation mount (PRM1Z8, Thorlabs, USA), polarizing beam splitter, beam dump, 50/50 beam splitter, Si Avalanche Photodetector (APD120A2, Thorlabs, USA), two mirrors, a Galvo-Resonant scan head and tube lens kit with four-channel acquisition (MPM-SCAN4, Thorlabs, USA), 40x water immersion objective (0.8 NA, Nikon, USA), and motorized platform for movement in the x and y axes (Z806, Thorlabs, USA) as well as z axis (ZFS06, Thorlabs, USA). Four photomultiplier (PMT) tubes acquired backscattered signal through the following filters: Channel A, designed for SHG, had a bandpass filter of 400nm center wavelength and 40nm bandwidth, Channel B had a bandpass filter of 466nm center wavelength and 40nm bandwidth, Channel C had a bandpass filter of 525nm center wavelength and 45nm bandwidth, and Channel D had a bandpass filter of 607nm center wavelength and 70nm bandwidth.

2.2 Animal preparation

C57BL mice were bred and sacrificed in accordance with the University of Arkansas Institutional Animal Care and Use Committee (IACUC # 15009). In summary, standard triad breeding was used, and after weaning and gender separation, mice were sacrificed at different ages - 10, 12, 14, 16, and 20 weeks. A midline laparotomy incision was made, and

the gastrointestinal tract cut as close to the rectum as possible. Freshly resected tissue for imaging was sectioned into 1cm pieces, no further than 6cm from the rectal end, and each piece was longitudinally sectioned, then cut in half, with half the tissue placed in 10% formalin for histopathology and the other half placed epithelium down on a petri dish, then moistened with a drop of 1x phosphate buffered saline (PBS) and covered with a No.1 coverslip.

2.3 Image acquisition

Images were acquired at 755nm and 800nm two-photon excitation, and collected in the B and A channels, respectively. Image acquisition settings ranged between 200 and 300 cumulative frames at 15 frame per second (fps) acquisition, at 90/100 PMT gain, with a 261um by 261um field of view. Most images were acquired at 512×512 pixels, within 2 hours of excision. For most regions, images were acquired in 20um steps, up to 180um in depth.

2.4 Quantitative image metrics

Glandular area and circularity were extracted from SHG images after drawing the perimeters manually in ImageJ (Fig. 2). Since depth stacks of images were acquired, many of which were not representative of the collagen structure supporting glandular crypts, the quantified SHG images were chosen for having clear, complete, outlines of glandular structure. The perimeter of at least five glands per image were manually drawn in ImageJ, and measured and averaged for glandular area and circularity.

3. RESULTS

3.1 Qualitative review of SHG images

Our SHG images support current understanding of collagen structures in colorectal tissue¹². The image series, taken at depths of 20um increments, show the collagen fibers in orthogonal directions (Fig. 3a, b), followed by the relatively round collagen structures that surround the glandular crypts (Fig. 3c). Fig. 3a shows collagen structures in the longitudinal muscle portion of the muscularis (horizontal orientation), while Fig. 3b shows collagen structures in the circular muscle portion of the muscularis (vertical orientation). These two layers of orthogonally oriented collagen structures appear in the outer layers of every mouse colon imaged. Fig. 3a and 3b are at 20um and 70um depths respectively, with Fig. 3c, which is the glandular crypt region (mucosa), at a depth of 140um. Normal epithelial tissue exhibits homogenous distribution of relatively round glands, seemingly clustered in smaller groups. The edges are smooth and the glands are homogenous in size. The combination of images from 755nm excitation and 800nm excitation (Fig. 3d) show collagen structures (blue) surrounding glandular structures (green).

3.2 Quantitative image metrics

Quantitative measurements of glandular area and circularity in SHG images of 6 different murine colorectal epithelium show the viability of quantitative metrics for distinguishing differences in normal and dysplastic tissue. Figure 4 shows a comparison between (a) normal and (b) slightly abnormal colonic epithelium. The edges are not smooth, and

quantification of the glands in this region (Fig. 5, age 10*) are compared against normal glandular area and circularity in mice of 10, 12, and 14 weeks of age. Preliminary quantification show that glandular area and circularity remains relatively stable relative to the age of the mouse, but that small changes in collagen structure (such as Fig. 4b) are quantifiable.

4. DISCUSSION

Qualitatively, the collagen structures in the SHG images agree with previous research and traditional histopathology. Glandular outlines are smooth and homogeneously spaced. Quantitatively, glandular area and circularity show promise in measuring the homogeneity in our cross-sectional study, and preliminary quantification of a slightly abnormal region shows that these quantitative metrics are capable of distinguishing slight difference between qualitatively different regions.

Label-free multiphoton imaging has various advantages which make it desirable in the study of colorectal dysplasia. These include imaging of bulk tissue, forgoing the need for stains, and capability for depth sectioning. When combined with quantitative image metrics, multiphoton imaging provides a range of structural markers for detecting dysplasia. Future work would include in vivo imaging, which would provide additional data such as metabolic differences between normal and dysplastic tissue. This data could then serve to guide future designs for optical screening and diagnosing imaging modalities.

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REFERENCES

- [1]. Zhang Y, Akins ML, Murari K, Xi J, Li MJ, Luby-Phelps K, Mahendroo M, Li X. A compact fiberoptic SHG scanning endomicroscope and its application to visualize cervical remodeling during pregnancy. *Proc.Natl.Acad.Sci.U.S.A.* 2012; 109(32):12878–12883. [PubMed: 22826263]
- [2]. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA: a cancer journal for clinicians.* 2015; 65(1):5–29. [PubMed: 25559415]
- [3]. Bujanda L, Cosme A, Gil I, Arenas-Mirave JJ. Malignant colorectal polyps. *World J.Gastroenterol.* 2010; 16(25):3103–3111. [PubMed: 20593495]
- [4]. Bujanda L, Cosme A, Gil I, Arenas-Mirave JJ. Malignant colorectal polyps. *World J.Gastroenterol.* 2010; 16(25):3103–3111. [PubMed: 20593495]
- [5]. Parikh ND, Perl D, Lee MH, Shah B, Young Y, Chang SS, Shukla R, Polydorides AD, Moshier E, Godbold J. In vivo diagnostic accuracy of high-resolution microendoscopy in differentiating neoplastic from nonneoplastic colorectal polyps: a prospective study. *AmJ.Gastroenterol.* 2014; 109(1):68–75.
- [6]. Rogart JN, Nagata J, Loeser CS, Roorda RD, Aslanian H, Robert ME, Zipfel WR, Nathanson MH. Multiphoton Imaging Can Be Used for Microscopic Examination of Intact Human Gastrointestinal Mucosa Ex Vivo. *Clinical Gastroenterology and Hepatology.* 2008; 6(1):95–101. [PubMed: 18065276]
- [7]. Adur J, Pelegati VB, Bianchi M, de Thomaz AA, Baratti MO, Carvalho HF, Casco VH, Cesar CL. Multimodal nonlinear optical microscopy used to discriminate human colon cancer. *SPIE BiOS.* 2013:85881J–85881J-8.

- [8]. McNally JB, Kirkpatrick ND, Hariri LP, Tumlinson AR, Besselsen DG, Gerner EW, Utzinger U, Barton JK. Task-based imaging of colon cancer in the Apc Min/ mouse model. *Appl.Opt.* 2006; 45(13):3049–3062. [PubMed: 16639453]
- [9]. Schürmann S, Foersch S, Atreya R, Neumann H, Friedrich O, Neurath MF, Waldner MJ. Label-free imaging of inflammatory bowel disease using multiphoton microscopy. *Gastroenterology.* 2013; 145(3):514–516. [PubMed: 23850960]
- [10]. Wu, X.; Chen, G.; Lu, J.; Zhu, W.; Qiu, J.; Chen, J.; Xie, S.; Zhuo, S.; Yan, J. Label-free detection of breast masses using multiphoton microscopy. 2013.
- [11]. Wang C, Li F, Wu R, Hovhannisyan VA, Lin W, Lin S, Dong C. Differentiation of normal and cancerous lung tissues by multiphoton imaging. *J.Biomed.Opt.* 2009; 14(4):044034–044034-4. [PubMed: 19725745]
- [12]. Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. *Journal of gastrointestinal oncology.* 2012; 3(3):153. [PubMed: 22943008]

Figure 1.
Two-photon microscopy schematic.

Figure 2.
Example of manually drawn glandular crypt for quantitative data measurement. Scale bar is 100 μ m.

Figure 3.

Label-free two-photon images of murine colon tissue. (a) Outer layer of muscularis, 800nm excitation. (b) Inner layer of muscularis, 800nm excitation. (c) Glandular crypts, 800nm excitation. (d) Colored combination of 800nm excitation (red), and 755nm excitation (green). Scale bar is 100um.

Figure 4.
Murine colon. (a) Normal region. (b) Slightly abnormal region.

Figure 5.

Bar plots of average measured glandular (a) area and (b) circularity. Age 10* refers to image region shown in Fig. 4b. Error bars show standard deviation.