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***In vivo* deep tissue fluorescence imaging of the murine small intestine and colon**

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ABSTRACT

Recently we described a novel technical approach with enhanced fluorescence detection capabilities in two-photon microscopy that achieves deep tissue imaging, while maintaining micron resolution. This technique was applied to *in vivo* imaging of murine small intestine and colon. Individuals with Inflammatory Bowel Disease (IBD), commonly presenting as Crohn's disease or Ulcerative Colitis, are at increased risk for developing colorectal cancer. We have developed a *Gia2* gene knock out mouse IBD model that develops colitis and colon cancer. The challenge is to study the disease in the whole animal, while maintaining high resolution imaging at millimeter depth. In the *Gia2*^{-/-} mice, we have been successful in imaging *Lgr5*-GFP positive stem cell reporters that are found in crypts of niche structures, as well as deeper structures, in the small intestine and colon at depths greater than 1mm. In parallel with these *in vivo* deep tissue imaging experiments, we have also pursued autofluorescence FLIM imaging of the colon and small intestine-at more shallow depths (roughly 160 μ m)- on commercial two photon microscopes with excellent structural correlation (in overlapping tissue regions) between the different technologies.

Keywords: fluorescence, deep-tissue imaging, microscopy, cancer, colon, small intestine

1. INTRODUCTION

Two-photon fluorescence microscopy [1] has been a fundamental tool to image biological tissues since its invention in 1990. High-resolution imaging of deep tissue layers is achieved by inducing two-photon fluorescence via the near-infrared light, which can penetrate deeper inside tissue samples [2-10]. Biological tissue is inherently a turbid medium, with optical properties characterized by multiple scattering and inhomogeneity of the refractive index. The excitation light that effectively reaches the focal area is attenuated by scattering so that the imaging depth is limited. Some tissue components, such as blood and melanin [12, 13], may present a prominent absorption at certain excitation wavelengths. However, the contribution of absorption and its effect on imaging depth is usually negligible for most biological tissues when compared with attenuation by multiple scattering [6,11].

To increase imaging depth in turbid media most of the research reported in the literature was focused on methods that allow the delivery of more excitation power to the focal area and consequently increase fluorescence intensity at depths at which fluorescence signals can still be detected. Imaging depth of 1mm was achieved in brain tissue with the aid of a regenerative amplifier to increase the excitation pulse power [2].

Another major problem associated with in-depth fluorescence imaging is the harvesting of fluorescence photons. While acquiring images of deep layers, the detected signal decays with depth due to the decrease in number of induced fluorescence photons, as well as to the attenuation of fluorescence by multiple scattering and possible absorption. Obviously the ability of the imaging system to collect fluorescence photons propagating in turbid sample will strongly affect the imaging depth, and thus deeper layers can be imaged with sensitive fluorescence detectors. Previously [14] we

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described the use of the detection method that allowed us to perform imaging in turbid samples with brain-like optical properties at depths up to 3 mm, while the maximum depth at which images could be acquired on the same samples by the state-of-the-art commercial two-photon microscope (Zeiss LSM 710) was limited to about 500 μ m.

We have modified our deep tissue imaging system, described below, to adapt it for the *in vivo* imaging of small animals. Using this system we were able to successfully obtain high resolution images in small intestines and colons of the $\text{G}\alpha 2^{-/-}$ mice. In the US, roughly half a million individuals suffer from IBD; a crippling, painful and debilitating disease. The cause of these diseases is unknown. We have developed a murine model of IBD to aid in the study of IBD and its transformation to malignancy. Specifically, the model is a $\text{G}\alpha 2$ gene knock out mouse that develops colitis and colon cancer, presumably through resistance to normal growth suppressors and apoptosis agents.

2. EXPERIMENTAL METHODS

2.1 Two-photon microscope system

Previously we described the principles of operation and characteristics of our two-photon microscope system that is capable of imaging inside turbid media to a few mm depth [14]. The system, described in this paper, is a modified upright version of the previous model that is more suitable for imaging live small animals. The experimental system diagram is shown in figure 1. A femtosecond Ti:Sapphire laser (Mai Tai, Spectra-Physics) equipped with a DeepSee attachment for group velocity dispersion (GVD) compensation was used for the two-photon excitation of the specimen. The excitation beam was directed to an acousto-optic modulator (AOM, AA Opto-Electronics MT 110-B50A1) to adjust the power level. Subsequently the beam was passed through an x-y-scanner (ISS) coupled to an Olympus BX illumination module equipped with a long working distance (7mm) Olympus LCPlanFl 20x/0.4 air objective. The focusing and specimen positioning was achieved by means of a motorized x-y-z stage. The two-photon fluorescence was induced by the IR beam focused inside the sample from its top side and collected by the detector (described below) from the opposite side of the sample. To prevent external light from reaching the sensitive detector, the components were enclosed in a light-sealed box made out of black cardboard. We have also used a commercial two-photon scanning microscope Zeiss LSM 710 to compare results obtained with our instrument.

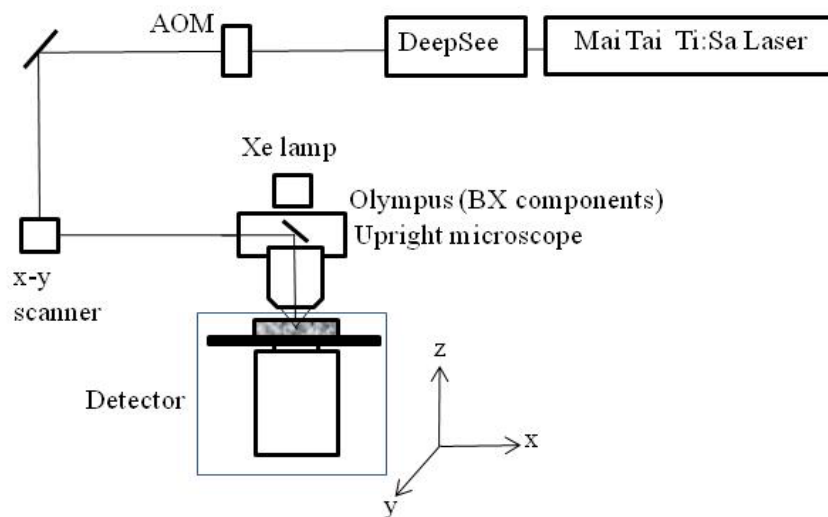


Figure 1. Schematic diagram of the deep tissue imaging system

The detector is the essential feature of the described experimental setup. The detector and the principle of its operation are shown schematically in figure 2a. The detector is comprised of a head-on photomultiplier tube with a 25 mm diameter photocathode (Hamamatsu PMT R-1104) operating in photon-counting mode. The PMT is coupled to a glass

cylinder with mirror coated walls. The cylinder acts as a light-guide by directing fluorescence photons to the PMT photocathode. The optical shutter, connected to the glass cylinder, is made of two 25 x 3mm optical filters (Schott BG-39) that transmit fluorescence, but block the access of the excitation light to the PMT. A ~0.2 mm gap between the filters allows the insertion of a 0.1mm thin aluminum plate to close the shutter. All the detector components are connected with index matching compounds as well as the gap between filters, which is filled with an index matching liquid to minimize losses of fluorescence photons due to reflection at the boundaries. For the same purpose, during measurements the detector was placed directly in contact with the surface of the samples.

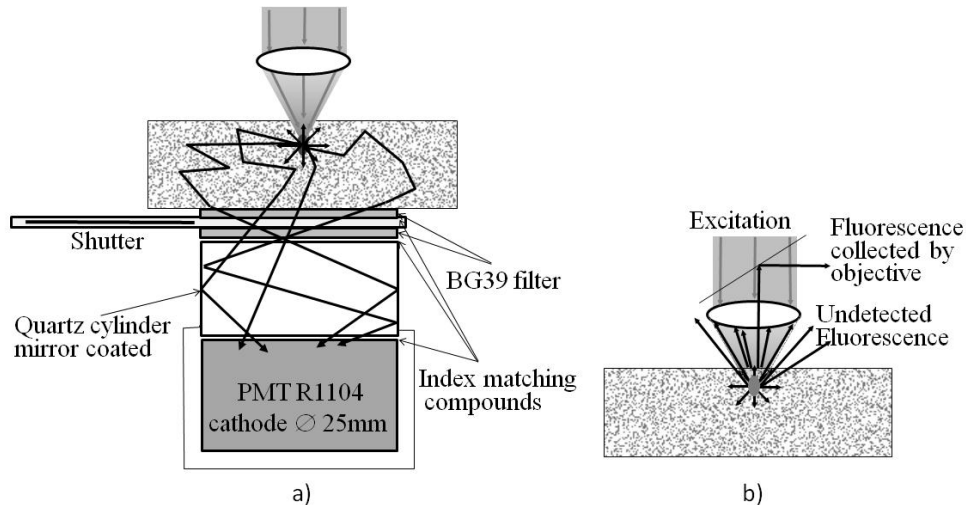


Figure 2. Fluorescence detection methods: developed at the LFD (a) and used in conventional 2-photon microscopy (b)

As shown in figure 2a, the two-photon induced fluorescence is multiply-scattered inside the turbid sample and is harvested by the wide area detector (25 mm in diameter). Due to multiple scattering, photons initially headed out of the detector have some probability to be redirected and enter the detector. For the same reason, in the multiple scattering media, where photons constantly change direction, light losses due to total internal reflection at the sample/detector boundary are reduced and fluorescence photons can be captured by the detector from any angle of incidence. In theory multiple scattering could even improve fluorescence detection, because in multiple scattering media the intensity of the scattered photons decays as the inverse of the distance from the excitation light focus [3], which is slower than the inverse of the squared distance decay in clear media. We have proven that the described detection method is very efficient in harvesting photons. Indeed, no microscope objective is capable of collecting photons from such a wide area. For comparison, figure 2b shows a conventional two-photon microscope diagram where fluorescence photons can be collected by a microscope objective only from a relatively small area and at a narrow angle.

The manufacturing of the detector is very simple, it practically does not require any optics, and it should be also effective for clear media samples.

2.2 Specimen preparation

All procedures followed were reviewed and approved by the Irvine University Institutional Animal Care and Use Committee. For *in vivo* imaging, 4-6 week old Lgr5+GFP mice were fasted for 24 hours prior to being anesthetized with 0.4 cc of ketamine-xylazine given IP, and immobilized (with clear tape) on their side on a glass slide. A 1 cm vertical incision was made 0.5 cm to the left of midline of the abdomen and a loop of small bowel exteriorized. The loop was placed on the surface of the glass slide, with a minimal volume of PBS to prevent desiccation of the serosal surface. The prepared animal was placed on the stage of the multiphoton microscope and imaged. At the end of the imaging session, the animal was euthanized with an overdose of ketamine.

For *ex-vivo* tissue imaging, the mouse is euthanized with an overdose of ketamine. Then the animal is dissected, and the selected freshly excised GI tissues are dissected out and imaged. Tissue was bathed in saline prior to imaging, and all imaging occurred within four hours of euthanasia.

3. RESULTS AND DISCUSSION

In Figure 3 and Figure 4, deep two-photon microscopy is used to visualize the morphology of the normal colon and small intestine tissue. We used transgenic mice that express GFP in the Lgr5 positive stem cells at the base of the small intestine and colon crypts [15]. Stem cells located at the crypts base are the ones responsible for generating the intestinal cancer [16].

The deep two-photon imaging is performed with an excitation wavelength of 880nm through the external muscularis mucosa up to a depth of 420 μm . We can distinguish different structures at different depths of the colon (Fig.3) and small intestine (Fig.4) tissue.

Starting from the surface of the colon tube we imaged first the muscularis externa, muscularis mucosa, and the submucosa [17]. In these layers, at 122.5 μm and 133 μm depth, we distinguish the collagen walls of a branching blood vessel. Deeper, at depth 175 μm and 195 μm , the beginning of the epithelium structure is characterized by the bases of the circular crypts, that contain the Lgr5+GFP stem cells and that are surrounded by collagen fibers. Single Lgr5+GFP stem cells are distinguishable (Fig. 3 217 μm depth) within the circular crypt with their typical alternated pattern [15]. Below 217 μm depth, the granular level of the colon is visible with circular crypts and the lamina propria that contains the capillary network [17].

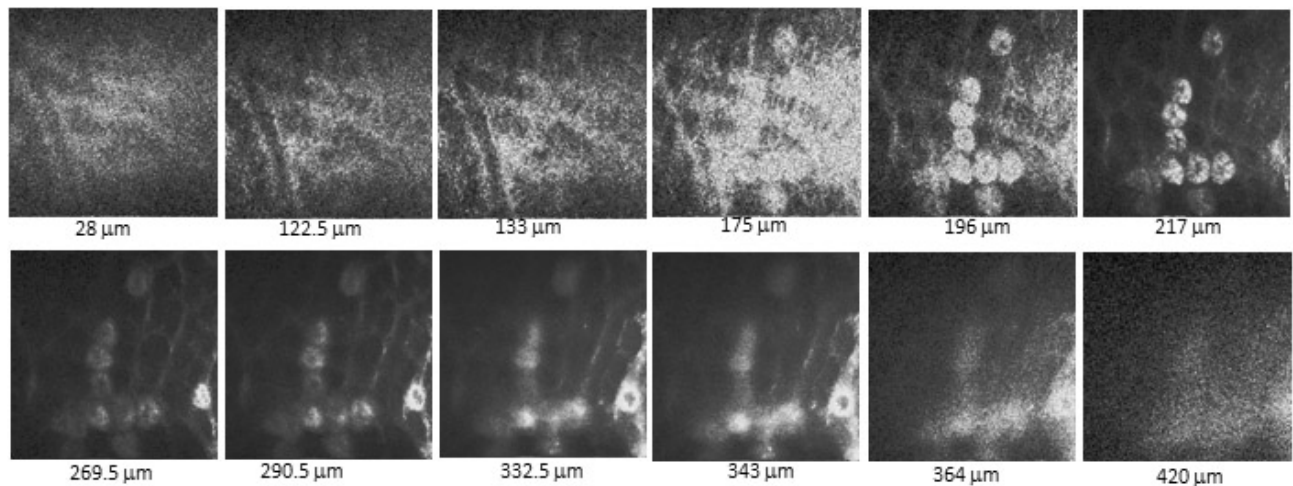


Figure 3. Images of colon at various depths; field of view 400x400 μm

The three-dimensional structure of the small intestine tissue (Figure 4) is similar to the colon with some differences [17]. Starting from the surface of the small intestine exteriorized loop we first imaged the smooth muscle layer, characterized by smooth muscle cells (Fig.4 depth 10 μm and 20 μm). A layer of collagen fibers is located at the base of the Lgr5-GFP stem cells positive crypts (arrows in Fig.4 depth 30 μm). Single Lgr5+GFP stem cells are visible (arrows in Fig. 4 depth 70 μm) within the circular crypt with their typical alternated pattern [15].

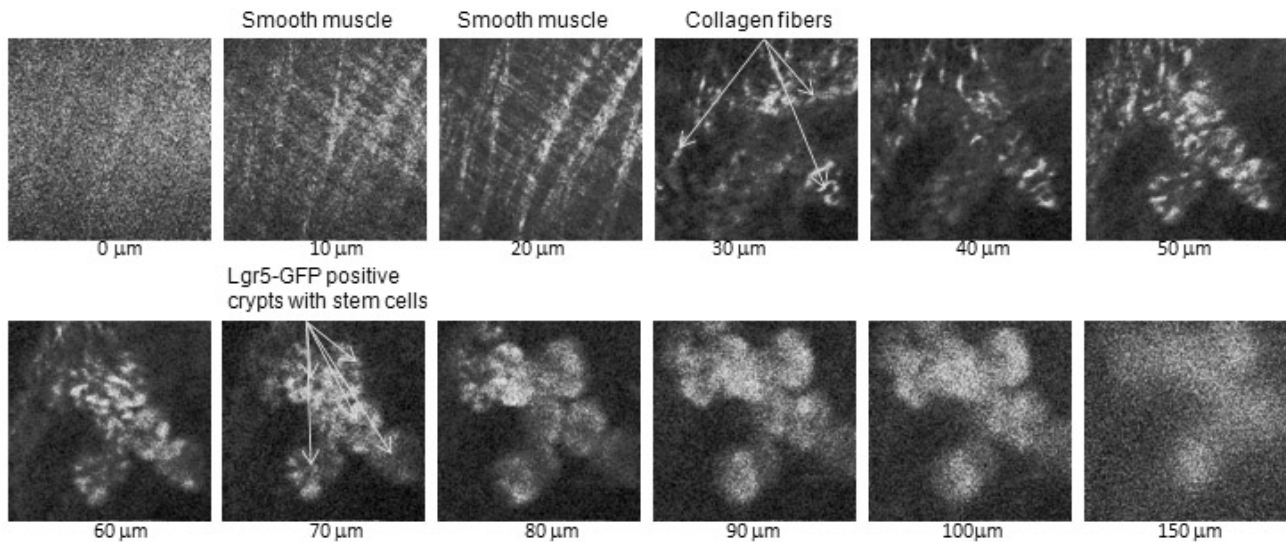


Figure 4. Images of small intestine at various depths; field of view 200x200 μm

4. CONCLUSIONS

Here we performed *in vivo* deep two-photon microscopy on an exteriorized loop of small intestine and freshly excised colon tube. We used a transgenic mice line that expresses GFP in the Lgr5 positive stem cells.

We were able to image up to 420 μm depth and to discriminate and identify different important structures of the colon and small intestine tissue: blood vessels in the muscularis mucosa, smooth muscle layer, collagen fibers, lamina propria, circular crypts in the granular layer, and single Lgr5+GFP stem cells, located at the crypt base with their typical alternated pattern.

Performing *in vivo* deep tissue imaging on an exteriorized loop of small intestine allows us to monitor the spatial and temporal dynamics of Lgr5+GFP stem cells, intrinsic contrast and collagen organization in wild type and mutant mice that develop inflammation (*Gia2*^{-/-} mice) and cancer (APC^{min}).

This type of microscopy is very suitable to monitor the early stages and the progression of cancer in a living animal model with single cell resolution.

5. ACKNOWLEDGEMENTS

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REFERENCES

- [1] Denk, W., Strickler, J. and Webb, W., "Two-photon laser scanning fluorescence microscopy," *Science* 248(4951), 73–76 (1990).
- [2] Theer, P., Hasan, M. T. and Denk, W., "Two-photon imaging to a depth of 1000 micron in living brains by use of a Ti:Al₂O₃ regenerative amplifier," *Opt. Lett.* 28(12), 1022-1024 (2003).
- [3] Theer, P. and Denk, W., "On the fundamental imaging-depth limit in two-photon microscopy," *J. Opt. Soc. Am. A* 23(12), 3139-3149 (2006).
- [4] O'Malley, D., "Chapter 5: Imaging in depth: controversies and opportunities," *Methods Cell Biol.* 89, 95-128 (2008).

- [5] Gu, M., Gan, X., Kisteman, A. and Xu, M. G., "Comparison of penetration depth between two-photon excitation and single-photon excitation in imaging through turbid tissue media," *Appl. Phys. Lett.* 77(10), 1551-1553 (2000).
- [6] Deng, X. and Gu, M., "Penetration depth of single-, two-, and three-photon fluorescence microscopic imaging through human cortex structures: Monte Carlo simulation," *Appl. Opt.* 42(16), 3321-3329 (2003).
- [7] Gerritsen, H. C. and De Grauw, C. J., "Imaging of optically thick specimen using two-photon excitation microscopy," *Microsc. Res. Tech.* 47(3), 206-209 (1999).
- [8] Oheim, M., Beaurepaire, E., Chaigneau, E., Mertz, J., Charpak, S., Neurosci, J., "Two-photon microscopy in brain tissue: parameters influencing the imaging depth," *Methods* 11(1), 29-37 (2001).
- [9] Leray, A., Odin, C., Le, Y., "Out-of-focus fluorescence collection in two-photon microscopy of scattering media," *Opt. Commun.* 281, 6139-6144 (2008).
- [10] Botcherby, E. J., Juskaitis, R., Wilson, T., "Scanning two photon fluorescence microscopy with extended depth of field," *Opt. Commun.* 268, 253-260 (2006).
- [11] Yaroslavsky, A. N., Schulze, P. C., Yaroslavsky, I. V., Schober, R., Ulrich F., Schwarzmaier, H-J., "Optical properties in selected native and coagulated human brain tissue *in vitro* in the visible and near infrared spectral range," *Phys. Med. Biol.* 47, 2059-2073 (2002).
- [12] Masters, B. R., So, P.T.C., Buehler, C., Barry, N., Sutin, J. D., Mantulin, W. W., Gratton, E., "Mitigating thermal mechanical damage potential during two-photon dermal imaging," *J. Biomed. Opt.* 9(6), 1265-1270 (2004).
- [13] Cheong, W., Prahl, S. A., Welch, A. J., "A review of optical properties of biological tissue," *IEEE J. Quantum Electron.* 26(12), 2166-2185 (1990).
- [14] Crosignani, V., Dvornikov, A. S., Gratton, E., "Enhancement of imaging depth in turbid media using a wide area detector," *J. Biophotonics* 4(9), 592-599 (2011).
- [15] Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters. P.J., Clevers, H., "Identification of stem cells in small intestine and colon by marker gene *Lgr5*," *Nature* 449(7165): 1003-7 (2007).
- [16] Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., Clevers, H., "Crypt stem cells as the cells-of-origin of intestinal cancer," *Nature* 457(7229): 608-11 (2009).
- [17] Ross, M. H., Pawlina, W., "Histology: a text and atlas: with correlated cell and molecular biology," BaltimoreMD: Lippincott Williams & Wilkins, xvii (906 p.) (2006).