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RESEARCH ARTICLE

Fluorescence microendoscopy imaging based on GRIN lenses with one- and two-photon excitation modes

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Abstract With the rapid development of life sciences, there is an increasing demand for intravital fluorescence imaging of small animals. However, large dimensions and limited working distances of objective lenses in traditional fluorescence microscopes have limited their imaging applications mostly to superficial tissues. To overcome these disadvantages, researchers have developed the graded-index (GRIN) probes with small diameters for imaging internal organs of small animals in a minimally invasive fashion. However, dynamic imaging based on GRIN lens has not been studied extensively. Here, this paper presented a fluorescence endoscopic imaging system based on GRIN lenses using one-photon and two-photon excitation. GRIN lenses with 1.15 mm diameter and 7.65 mm length were used in the system. The images were acquired by a compact laser scanning imaging system with a resonant galvo-mirror system to scan the laser beam and a photomultiplier tube (PMT) to detect fluorescence signals. Experimental results showed that this system using two-photon excitation could implement dynamic fluorescence microendoscopic imaging and monitor the movement of blood flow beneath the skin in anesthetized mice while producing images with higher contrast and signal to noise ratio (SNR) than those using one photon excitation. It would be a useful tool for studying biological processes of small animals or plants *in vivo*.

Keywords one-photon excitation, two-photon excitation, graded-index (GRIN) lens, fluorescence micorendoscopy, dynamic imaging

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1 Introduction

In recent decades, there has been a remarkable growth in the use of fluorescence in biologic applications [1–20]. Fluorescence microscopy [1] is considered to be a primary tool in biochemistry, biomedicine and biophysics. Since Denk et al. perceived the two-photon microscopy in the laboratory of Watt W. Webb at Cornell University in 1990 [2], fluorescence microscopy imaging has been extensively used in physiology, neurobiology, embryology and tissue engineering [3]. As such, intravital fluorescence microscopic imaging has been broadly explored in different modalities and implementation settings. Readers can refer literatures listed herein on several topics, such as one-photon excited fluorescence microscopy *in vivo* [4,5], two-photon excited fluorescence microscopy and second harmonic generation imaging [6–9], fiber optic confocal fluorescence microscopy imaging [10], two-photon fluorescence lifetime microscopy imaging [18–20], and so on. Confocal and multiphoton fluorescence microscopy, combining with fluorescent biomarkers, have stood out and become powerful imaging tools in biomedical research if animal models are involved. Due to the optical sectioning capability of these techniques, depth-resolved imaging can be performed in highly scattering media such as biological tissues which are difficult to image using traditional wide-field imaging techniques. However, even with these techniques, the imaging depth in biological tissues is limited to a few hundred micrometers due to the low penetration depth of visible and near-infrared wavelengths. Therefore, *in vivo* optical imaging of biological tissue is mostly limited to superficial layers of skin and hollow organs. To overcome the depth limitation, microendoscopy is a promising approach for internal organ imaging *in vivo*. It combines conventional microscopy and miniature

endoscopy, using a narrow-diameter optical probe that provides minimally invasive access to internal organs which is difficult to perform with conventional instruments. To overcome these difficulties, several research groups have proposed endoscopy solutions that are based on two-photon excitation and graded-index (GRIN) lens delivery [7,10–17].

A GRIN lens is a non-conventional lens that typically has a shape of cylinder available in various sizes. The refractive index of the lens varies parabolically along the radius of the lens, containing a maximum number in the optical axis of the lens (lens center) and gradually decreases toward the outer edges. Due to this refractive index gradient, a light strikes on the front surface of the lens and follows a sinusoidal path along the lens rod. A GRIN lens can conveniently couple light into an optical fiber due to its cylindrical shape and therefore has been widely used in telecommunication devices. In the past 20 years, the applications of GRIN lens become more and more popular in different areas of optical microscopy [7,10–17]. As a cost-effective alternative to the direct miniaturization of conventional microscope objectives, GRIN lenses attracted lots of attentions. For example, in 2004, Jung et al. [7] built a microendoscope based on compound micro-lenses, and achieved high-resolution fluorescence imaging in mammalian brains. In 2008, Li and Yu [11] reported that they achieved intravital deep kidney imaging using a GRIN lens. In 2008, Kim et al. [13] developed a multicolor multimodal imaging system with cellular resolution in live mice using a GRIN lens. In 2011, Kyrish et al. [16] reported initial imaging with second-harmonic generation signal in collagen fibers and two-photon excited fluorescence of Convallaria rhizome with an ultra-slim plastic endomicroscope objective. In 2011, Pillai et al. [10] reported a GRIN lens-based microendoscope, that was suitable for accessing deep tissues within the body using confocal fluorescence imaging. In 2012, Huland et al. [14] proposed a multiphoton endoscopic system which could perform in vivo imaging of unstained tissues using long GRIN lens. All these demonstrations have shown that GRIN lens two-photon microendoscopy has great potentials in clinical applications. However, dynamic imaging based on GRIN lens has not been studied extensively. In this paper, we presented a one-photon and two-photon excited fluorescence microendoscopic imaging system using GRIN lenses of 1.15 mm diameter and 7.65 mm length. A resonant galvo-mirror system was used to accomplish quick 2D scanning of the laser beam, and a photomultiplier tube (PMT) was used to acquire fluorescence signals. The system was used to perform dynamic fluorescence microendoscopy imaging to study the blood flow in an anesthetized mouse.

2 Experimental setup

The experimental setup of the system is shown in Fig. 1. For one-photon excited fluorescence microendoscopic imaging, the light source is a semiconductor laser whose wavelength was 486 nm, which is close to excitation wavelength of 488 nm recommended for the GRIN lens. For two-photon excitation, the light source was replaced by a mode-locked Ti:Sapphire femtosecond laser (Spectral-Physics, Mai Tai DeepSee) which provides 100 fs pulses at 80 MHz repetition rate. The ultrafast laser has a tuning range from 690 to 1000 nm and output power of 3 W. We tuned the laser to 820 nm to acquire all the two-photon excited fluorescence images shown in this report. The laser scanning was performed by a resonant galvo-mirror system. We designed the microendoscopy system to contain a field of view (FOV) of 70 μm × 70 μm using a 10 × objective (UPlanFl 10×, 0.3NA, Olympus) and a GRIN lens with 4.65 × magnifications. The detail parameters are listed in the right of Fig. 1. To obtain optimal coupling efficiency, the proximal end of the GRIN lens was placed approximately at the focal plane of the objective. A PMT

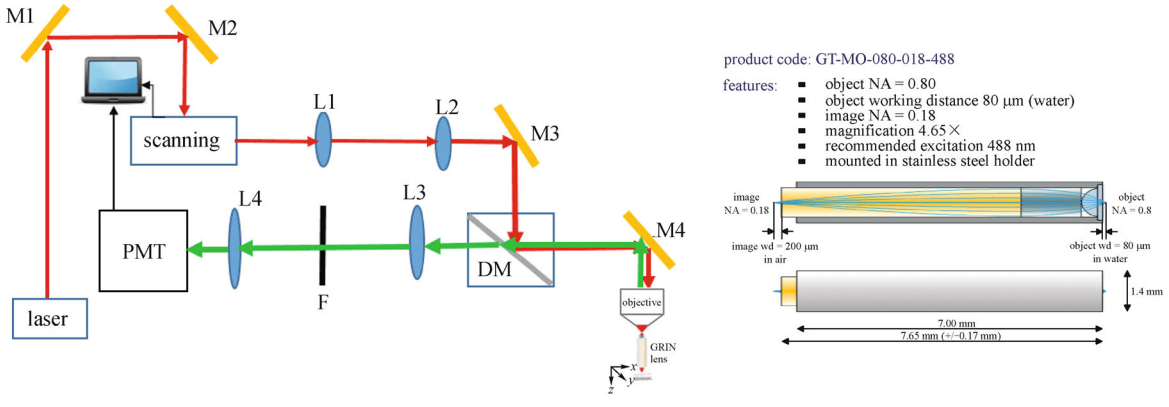

Fig. 1 Experimental setup of the fluorescence microendoscopy imaging system using GRIN lenses. The right panel is the GRIN lens and its parameters. Laser: Spectra-Physics, Mai Tai DeepSee, or a semiconductor laser; DM: dichroic mirror; F: blocking filter; PMT: photomultiplier tube, Hamamatsu H7422-40; Objective: Olympus 10 × /NA 0.3 dry objective

(H7422-40, Hamamatsu) was used for collecting fluorescence signal for constructing dynamic images. The control software was written in LabVIEW (National Instrument).

3 One-photon excited fluorescence microendoscopy imaging

To demonstrate the imaging capability of the above-mentioned fluorescence microendoscopy system, we first used slide samples to perform one-photon excited fluorescence imaging. Figure 2(a) is the fluorescence intensity image of a Convallaria rhizome slide. Figure 2(b) shows the inside of an Aloe stem by inserting the GRIN lens into the internal of plant Aloe stem sample, but its internal structure cannot be observed clearly. Figures 2(c) and 2(d) are fluorescence intensity images of breast cells slides, Fig. 2(c) depicts the normal breast cells, and Fig. 2 (d) shows the breast cancer cells. From Figs. 2(c) and 2(d), we can easily distinguish the normal breast cells from breast cancer cells, in the former, the cells distribution are uniform, and the boundary of nucleus is clear, while the boundary of nucleus is blurry in the latter.

The quality of the images obtained with the fluorescence microendoscopy imaging system using one-photon excitation is poor, especially for the *in vivo* sample, which agrees with past reports [4,5]. The main reason is that the focusing capability of one-photon excitation is weaker and light scattering is stronger in the internal sample, that is to say, the signal to noise ratio (SNR) is lower. Therefore, it is not possible to get high quality images using one-photon excitation.

4 Two-photon excited fluorescence microendoscopy imaging

Figure 3(a) shows a two-photon excited fluorescence image of cells on the same Convallaria rhizome slide. Compared with Fig. 2(a), we can clearly see that the image has better contrast and higher SNR. Figure 3(b) is the images of the internal structures of an Aloe stem. Compared with Fig. 2(b), Fig. 3(b) provides much sharper image of the internal structure of the Aloe stem. The reason is that laser beam in two-photon excitation mode is tightly focused, resulting in a higher degree of rejection of light from out of focus. In addition, longer wavelength excitation light is scattered less than that of shorter wavelength, providing higher contrast and sharper images.

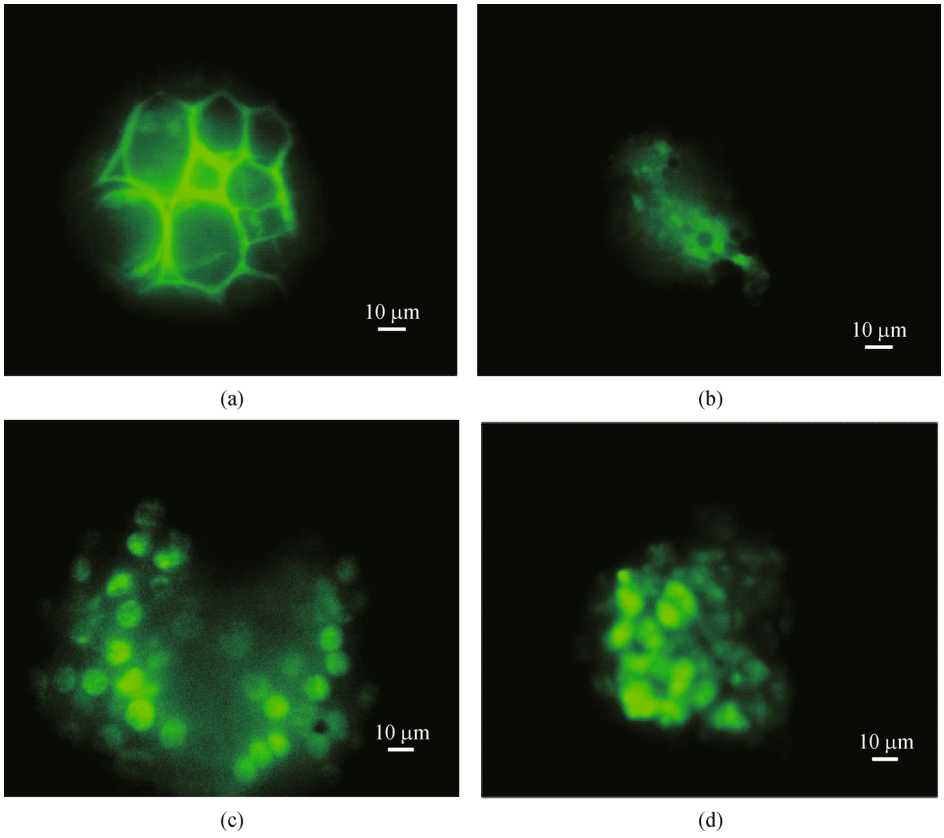

Fig. 2 One-photon excited fluorescence microendoscopic images of cells in (a) a Convallaria rhizome slide; (b) inside of an Aloe stem; (c) normal breast cells and (d) breast cancer cells in slides

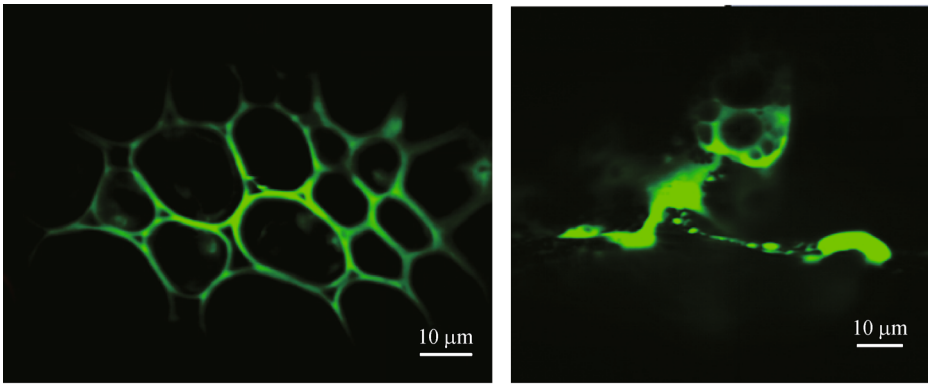

Fig. 3 Two-photon excited fluorescence images of cells in (a) a Convallaria rhizome slide; (b) inside of an Aloe stem

5 Dynamic fluorescence microendoscopy imaging

Furthermore, we performed dynamic fluorescence microendoscopy imaging with the two-photon excitation mode to monitor blood flow beneath the skin in anesthetized mice *in vivo*. Figure 4 shows the typical results of dynamic fluorescence imaging of blood flow beneath the skin of an anesthetized mouse. In order to image the movement of the blood flow, fluorescein

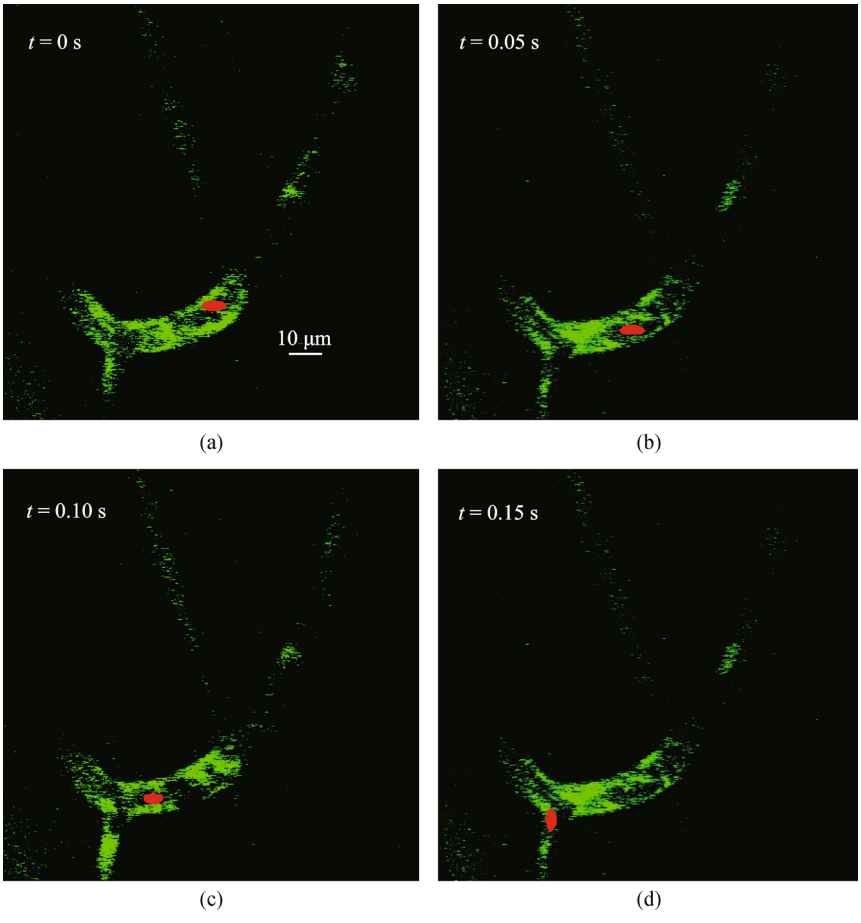

Fig. 4 Dynamic fluorescence images (imaging speed 20 frames/s, 512 × 512 pixels per frame) of blood flow beneath the skin of an anesthetized mouse. The blood flow was enabled to be visible by staining with fluorescein isothiocyanate-dextran (average mol wt 2,000,000). The images were captured at $t = 0$ s (a), 0.05 s (b), 0.10 s (c) and 0.15 s (d), respectively. The marked red spot is an aggregate of the dye whose movement was clearly observed during the dynamic imaging process

isothiocyanate-dextran (average mol wt 2,000,000) was injected into mice by tail-vein about 20 min before imaging. From the captured dynamic images, the circulation of fluorescein in the blood flow of the vascular can be clearly observed. Especially as a lot of fluorescein was aggregated together, they were shown as a marked red spot (Fig. 4). The movement of the blood flow can be monitored from these dynamic images and its speed can be calculated according to the movement of the red spot. These results suggested that this two-photon excited fluorescence microendoscopy can be a very useful tool in the blood flow imaging.

6 Conclusions

In this paper, we presented a home-built fluorescence microendoscopy imaging system using GRIN lenses with either one-photon or two-photon excitation modes. First, we demonstrated that the system can work with one-photon excitation mode, by which fluorescence microendoscopy imaging of slide samples and *in vivo* sample (inside of an Aloe stem) was performed. Then, we performed two-photon fluorescence microendoscopy imaging of samples using the same system with updated laser system and dichroic mirror. Comparing images obtained with one-photon and two-photon excitation modes, we can find that two-photon system can provide higher quality images, with higher contrast and SNR. Furthermore, we used the two-photon system to dynamically monitor the blood flow beneath the skin of an anesthetized mouse, and could also calculate the speed of blood flow from the dynamic frames. The fluorescence microendoscopy imaging system based on GRIN lenses with both one-photon and two-photon excitation modes we described here may provide a useful tool for biologic investigations of small animals or plants *in vivo*.

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References

1. Lakowicz J R. Principles of Fluorescence Spectroscopy. 3rd ed. New York: Springer, 2006
2. Denk W, Strickler J H, Webb W W. Two-photon laser scanning fluorescence microscopy. Science, 1990, 248(4951): 73–76
3. Peter T C S. Two-photon fluorescence light microscopy. Encyclopedia of Life Science, 2002, Macmillan Publishers Ltd, Nature Publishing Group. www.els.net
4. Jung J C, Schnitzer M J. *In vivo* epi-fluorescence micor-endoscopy. Biophysical Journal, 2002, 82: 496a–497a
5. Levene M J, Dombeck D A, Kasischke K A, Molloy R P, Webb W W. *In vivo* multiphoton microscopy of deep brain tissue. Journal of Neurophysiology, 2002, 91: 1908–1912
6. Elangovan M, Wallrabe H, Chen Y, Day R N, Barroso M, Periasamy A. Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy. Methods (San Diego, Calif.), 2003, 29(1): 58–73
7. Jung J C, Mehta A D, Aksay E, Stepnoski R, Schnitzer M J. *In vivo* mammalian brain imaging using one- and two-photon fluorescence micorendoscopy. Journal of Neurophysiology, 2004, 92: 3121–3133
8. Campagnola P J, Loew L M. Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. Nature Biotechnology, 2003, 21(11): 1356–1360
9. Qu J, Liu L, Shao Y, Niu H, Gao B Z. Recent progress in multifocal multiphoton microscopy. Journal of Innovative Optical Health Science, 2012, 05(03): 1250018
10. Pillai R S, Lorenser D, Sampson D D. Deep-tissue access with confocal fluorescence microendoscopy through hypodermic needles. Optics Express, 2011, 19(8): 7213–7221
11. Li X, Yu W. Deep tissue microscopic imaging of the kidney with a gradient-index lens system. Optics Communications, 2008, 281(7): 1833–1840
12. Saunter C D, Semprini S, Buckley C, Mullins J, Girkin J M. Microendoscope for *in vivo* widefield high spatial resolution fluorescent imaging. Biomedical Optics Express, 2012, 3(6): 1274–1278
13. Kim P, Puoris'haag M, Côté D, Lin C P, Yun S H. *In vivo* confocal and multiphoton microendoscopy. Journal of Biomedical Optics, 2008, 13(1): 010501
14. Huland D M, Brown C M, Howard S S, Ouzounov D G, Pavlova I, Wang K, Rivera D R, Webb W W, Xu C. *In vivo* imaging of unstained tissues using long gradient index lens multiphoton endoscopic systems. Journal of biomedical optics, 2012, 3(5): 1077–1085
15. Yang C, Hou V, Nelson L Y, Seibel E J. Mitigating fluorescence spectral overlap in wide-field endoscopic imaging. Journal of Biomedical Optics, 2013, 18(8): 086012
16. Kyrish M, Utzinger U, Descour M R, Baggett B K, Tkaczyk T S. Ultra-slim plastic endomicroscope objective for non-linear microscopy. Optics Express, 2011, 19(8): 7603–7615
17. Wang C, Ji N. Characterization and improvement of three-dimensional imaging performance of GRIN-lens-based two-photon fluorescence endomicroscopes with adaptive optics. Optics Express, 2013, 21(22): 27142–27154
18. Qi J, Shao Y, Liu L, Wang K, Chen T, Qu J, Niu H. Fast flexible multiphoton fluorescence lifetime imaging using acousto-optic deflector. Optics Letters, 2013, 38(10): 1697–1699
19. Krasieva T B, Stringari C, Liu F, Sun C H, Kong Y, Balu M, Meyskens F L, Gratton E, Tromberg B J. Two-photon excited fluorescence lifetime imaging and spectroscopy of melanins *in vitro* and *in vivo*. Journal of Biomedical Optics, 2013, 18(3): 031107
20. Yan W, Peng X, Qi J, Gao J, Fan S, Wang Q, Qu J, Niu H. Dynamic fluorescence lifetime imaging based on acousto-optic deflectors. Journal of Biomedical Optics, 2014, 19(11): 116004

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