Depth-resolved NIR-II fluorescence mesoscope

JIULING LIAO,1,2 YIXUAN YIN,1,2 JIA YU,1,2 RONGLI ZHANG,1,3 TING WU,1,2 HUI LI,1,2 QINCHAO SUN,1,2 LABAO ZHANG,4,5,6 and WEI ZHENG1,2,5,7

1Research Laboratory for Biomedical Optics and Molecular Imaging, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China
2CAS Key Laboratory of Health Informatics, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, China
3Guangdong Province’s People Hospital, School of Medicine, South China University of Technology, Guangzhou 510055, China
4Research Institute of Superconductor Electronics of Nanjing University, Nanjing, 210023, China
5Corresponding author
6lzhang@nju.edu.cn
7zhengwe@siat.ac.cn

Abstract: NIR-II fluorescence imaging is a promising method for visualizing biological structures in deep tissue, owing to the advantages of significantly suppressed optical scattering and diminished autofluorescence in biological tissues. However, few NIR-II fluorescence imaging approaches can simultaneously achieve a large field of view, high resolution and superior penetration depth, while exhibiting optical sectioning capability. In this paper, we present a novel NIR-II fluorescence mesoscopy system based on the f-θ scanning scheme and confocal detection to overcome these limitations. When used with NIR-II fluorescent dyes, our setup performs NIR-II fluorescence imaging on samples as large as 7.5×7.5 mm² with a lateral resolution of 6.3 µm. In addition, our system provides a depth-resolved imaging ability and zooming function. We successfully demonstrate in vivo cerebrovascular imaging of a mouse with local ischemia. Thus, our system provides new opportunities to explore the mechanism of cerebrovascular disease.

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1. Introduction

Exploring and understanding the biological events occurring in its native tissue environment remains the most coveted and challenging goal in biological studies. The depth-resolved fluorescence imaging provides a compelling means for interrogating events in biological tissues [1]. However, before selecting or developing an appropriate fluorescence imaging approach, several important factors should be considered including spatial resolution, which determines the smallest feature resolved in the image, the field of view (FOV), which determines the largest imaging area, and penetration depth, which defines the deepest imaging depth [2]. Thus, systems or methods that provide fluorescence imaging with features of both deep imaging penetration and large field of view with the relatively high spatial resolution are thus of great practical interest in biological studies [3].

The fluorescence imaging in the second near-infrared region (NIR-II, 1000-1700 nm) has shown great promise in improving the image penetration depth [4–9]. Compared to the visible wavelength window (400-750 nm) and the first near-infrared wavelength window (NIR-I, 750-900 nm) [5,9], the NIR-II fluorescence imaging acquires deeper imaging depth owing to significantly suppressed optical scattering and diminished autofluorescence in biological tissues [6]. Currently, most of the NIR-II fluorescence imaging approaches are based on the combination of a CCD camera with a camera lens or a photomultiplier tube with an objective lens [4,5,7]. Given the inherent tradeoff in spatial resolution and imaging area, these imaging approaches are difficult to achieve large field of view (FOV) with relatively high spatial resolution. In addition, the
epi-illumination configuration used in these imaging systems will accumulate the out-of-focal plane fluorescence signals, resulting in the difficulty to extract the focal plane image [10]. The NIR-II light sheet microscopy can provide rapid three-dimensional imaging capability with high spatial resolution [11]. However, the configuration of two perpendicular objectives in light sheet microscopy not only increases the complexity of the system but also limits the imaging area.

Recently, the NIR-II confocal microscopy has been reported to overcome some of the limitations of previous approaches [9,12,13]. In the NIR-II fluorescence confocal microscope, a pinhole at the conjugate position of the focal point rejects the out-of-focus light, thus accumulating only in-focus signals leading to improved spatial resolution and optical-sectioning capability. However, confocal microscopy suffers from limited FOV (typically less than 1 mm [13]). The maximum depth of penetration in this system is also limited and is reported to be only 1.3 mm [12]. These limitations restrict the application of NIR-II confocal microscopy in many preclinical and clinical applications [14], e.g., the study of the evolution of stroke [15]. There is a need for developing depth-resolved NIR-II fluorescence imaging tools to achieve large FOV, high resolution and deep penetration depth.

In this paper, we develop a novel NIR-II mesoscopy system, which achieves the desired goals. The FOV of the developed system is $7.5 \times 7.5$ mm$^2$, and the lateral resolution is 6.3 µm. The imaging depth could reach up to 2.5 mm below the mouse skull surface. Due to the confocal configuration, our system provides depth-resolved imaging capacity and FOV-scalable capability. Using this system, intravitral 3D cerebrovascular imaging of a mouse was conducted and dynamic cerebrovascular imaging of local ischemia was demonstrated.

2. Methods

2.1. Mesoscopy system setup

The schematic diagram of our NIR-II fluorescence mesoscopy system is shown in Fig. 1(a). The excitation light from a femtosecond laser (Chameleon Ultra, Coherent) at 808 nm was expanded to 4 mm diameter using expander lenses. The reflected laser beam by a dichroic mirror (DM) (DMLP1000, Thorlabs) was steered by a two-dimensional galvanometer scanner (GM) (GVSM002/M, Thorlabs) for raster scanning. The laser beam was then delivered to the back aperture of a scan lens (LSM02-BB, $f=18$ mm, Thorlabs). By changing the angle of incidence, the focal spot of the scan lens over the sample can be varied. Compared to the common microscope objective with short focal length, the scan lens created a larger planar imaging field where the spot size (resolution and intensity) was nearly constant across the entire imaging plane due to its low f-theta distortion [16]. The excited epi-fluorescence light from the sample was collected by the same scan lens, de-scanned by the galvanometer scanner, and was passed through the dichroic mirror. The fluorescence signal was eventually focused on a multimode fiber and detected by a home-built superconducting nanowire single-photon detector (SNSPD) with a system detection efficiency of $>50\%$ at 1064 nm (unpolarized). The SNSPD works under 2.5 K temperature and can keep 100 count per second dark count rate [17]. To remove the residual excitation light, a 1000 nm longpass filter (FEL1000, Thorlabs) was placed in front of the fiber. The core of the multimode fiber (diameter of 62.5 µm) served as the confocal pinhole to reject the out-of-focus signal, thereby achieving depth-resolved imaging within a thick specimen. To acquire the specimen information at different depths, we mounted the specimen on a motorized z scanner (MTS25, Thorlabs) for depth scanning.

A small-molecule organic dye IR-820 derivative was used in this study. The IR-820 has already been used successfully for in vivo NIR-II fluorescence functional cerebrovascular imaging of mice in the previous study [18]. Fluorescent beads with a diameter of 1 µm and labeled by dye IR-820 were imaged to evaluate the system resolutions. As shown in Fig. 1(b) and (c), the measured lateral and axial resolutions were 6.3 µm and 160 µm, respectively. The theoretical lateral resolution was calculated as 3.7 µm (confocal resolution $0.51.l/NA \approx 0.51.l*2f/D$). The
measured lateral resolution is some larger than the theoretical value, probably attributing to the optical aberration of the system. From the image of a steel ruler, the FOV of our system was determined to be 7.5 mm as shown in Fig. 1(d).

2.2. Fluorescent bead preparation

A 100 µL aqueous suspension of polystyrene (PS) beads (1.0 µm, 2.5%) was diluted by water to 1 mL. Then 1 mg IR820 in 0.5 mL dichloromethane (DCM) and 5 mg sodium dodecyl sulfate (SDS) in 0.5 mL water were added. The mixture was stirred at 40 °C for 3 hours. The dyed PS beads were collected by centrifugation and resuspended by 1 mL water. The SDS could prevent particle aggregation [19], so the final mixture was stable aqueous suspension of fluorescent beads with diameter of 1 µm.

A 10 µL aqueous suspension of fluorescent beads was added into 1 mL of 1% agarose solution. The mixture was vortexed vigorously and then deposited on a petri dish. When the mixture in the dish cooled and solidified, it was transferred to mesoscope stage to conduct resolution measurement. The irradiation laser power into the fluorescent beads was 1.8 mW.

2.3. Cell preparation

In our study, we used the C6 malignant glioma cell line. The C6 cells were labeled and fixed as per the following methodology. First, the cells were seeded on a culture dish and left overnight for them to adhere to the bottom of the dish. Second, the culture medium in the dish was removed and filled with Dulbecco’s modified Eagle medium (DMEM) containing 10% IR-820 (0.5 mg/mL). Third, the cells were incubated for 6 h at 37°C in the presence of 5% CO2. Finally, the cells were fixed in 4% paraformaldehyde for 10 min, at 4°C, followed by washing 3 times, each for 10 min,
with a stock solution composed of phosphate-buffered saline (PBS). The excitation laser power of 2.9 mW was used for cell imaging.

2.4. Animal preparation

The BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Experiments on mice were conducted in accordance with National Institutes of Health guidelines, and procedures were approved by the Shenzhen Institutes of Advanced Technology Chinese Academy of Sciences Animal Committee.

The local ischemia model was induced by Rose Bengal (Sigma-Aldrich) photothrombosis [20]. Mice were anesthetized by chloral hydrate (400 mg/kg, i.p.). The skull was exposed and fixed on stereotaxic apparatus for study. Rose Bengal was intravenously administered at 150 mg/kg in saline (i.v.). After 5 minutes of administering Rose Bengal, the exposed skull was placed in the cold light illuminator (150 W intensity) for 25 min. The illuminated square area was 1x1 mm². After 25 min, the light source was removed and the mouse was transferred to the mesoscope platform. Before imaging, the anesthetized mouse was intravenously injected with 200 µL PBS dispersion of IR820-BSA (0.5 mg/mL). The skull, with scalp removed, remained intact throughout the experiment. The laser power was ~6 mW for all in vivo imaging and no obviously adverse effects were observed in the mice.

3. Results and discussions

3.1. In vivo cerebral vasculature imaging

To evaluate the performance of our mesoscope, we imaged the cerebral blood vessels of a BALB/c mouse in vivo and images are shown in Fig. 2. All images in Fig. 2(a) are of size 512x512 pixels and were acquired at 5.12 s/frame. Due to weak autofluorescence and low tissue scattering, the imaging depth could reach up to 2.5 mm below the skull surface. The microvasculature, including both major blood vessels and capillaries, can be clearly visualized in Fig. 2(b). The signal to background ratio (SBR) reached up to 14.3, as shown in Fig. 2(c). By stacking optical sectioning images in the brain together, a reconstructed 3D image of the vasculatures can be formed, as shown in Fig. 2(d). These high-quality depth-resolved imaging results with high SBR demonstrate that our system is suitable for imaging large areas of tissue and also to acquire images with high spatial resolution. Since the NIR-II fluorescence mesoscopy system is based on point-by-point excitation and single-point detection, the system can be readily combined with time-correlated single-photon counting technique to realize fluorescence lifetime imaging. In the near future, we plan to combine these two systems. Such a combined system will be not only able to provide morphology data, but also biochemical information of its environment as well [21].

3.2. Feasible zooming in and out capability

To verify the zoom in function of our system, we imaged the C6 cells cultured on a dish labeled with IR-820. The different FOVs of the cells fixed on the dish are shown in Fig. 3(a-c). In contrast to replacing an optical component, which is commonly used in wide-field NIR-II microscopy, the different FOV in our system can be achieved simply by clicking the mouse to change the scanning angle of the galvanometer. The scalable FOV feature of our system will provide information from a local to a global region of interest. For example, the FOV in Fig. 3(a) is appropriate for evaluating cell distribution on the dish bottom while the FOV in Fig. 3(c) is more appropriate to visualize the growth details of a single cell.

3.3. Dynamic imaging of cerebral blood kinetics

The ability of a system to monitoring biological events with time-lapse imaging has great practical importance. Thus, to demonstrate time-lapse imaging capability of our system, we adopted a
Fig. 2. *In vivo* through skull NIR-II fluorescence imaging of mouse cerebral vasculature. (a) The cerebral vasculature images of mouse at various depths. (b) Magnification graph of the solid rectangle in (a). (c) The cross-sectional intensity profiles along the capillary vessels indicated by the red line in (b). (d) 3D reconstruction of vasculatures in brain. (e) Magnification graph of the solid rectangle in (d).

Fig. 3. Images of IR-820 labeled C6 cells at different scanning range. (a) The largest scanning image of cells. (b) Zoomed image of the dashed rectangle area of (a). (c) Zoomed image of the solid rectangle area of (b). All images were acquired by only altering the scanning angles of the galvanometer, without changing any optical components.

local ischemia model induced by Rose Bengal photothrombosis in a mouse. Immediately after the vein injection of 200 µL IR-820 (0.5 mg/mL) into a left cerebral hemisphere ischemia mouse, we performed time-lapse imaging of cerebral blood at a time interval of 1.28 s. As can be seen in Fig. 4, after injection, the dyes first flowed into capillaries and then filled up the major blood vessels. It is important to note that several blood vessels (as indicated by the arrows in Fig. 4(a))
were invisible within the first 5.12s post-injection. This could be because these vessels were blocked at the beginning and the blood flow was restored 5.12s post-injection. The max intensity project image of the cerebral vasculature from 0 mm to 2.5 mm deep is shown in Fig. 4(b). The left hemisphere exhibited almost no blood flow, which is quite different compared to the right hemisphere (circled in Fig. 4(b)), indicating that the left cerebral hemisphere was ischemic. It should be noted that the imaging speed of our mesoscopy system is similar to those of confocal setup with scanning galvanometer [9], but is slower than the NIR-II imaging system using a CCD camera as the detector [7,18]. The imaging speed here is restricted by the galvanometer scanner used in our system. In the future, we will replace the galvanometer mirror with a resonant scanner to improve the imaging speed and to trace even faster biological events such as the blood flow kinetics.

![Fig. 4.](image)

**Conclusion**

In summary, we proposed a novel NIR-II fluorescence mesoscopy system with a large FOV and depth-resolved capability. The largest FOV our system could reach was 7.5×7.5 mm², and lateral and axial resolutions achieved were 6.3 µm and 160 µm, respectively. The imaging depth could reach up to 2.5 mm below the mouse skull surface. The depth-resolved imaging capability of our system in deep tissue was demonstrated by *in vivo* NIR-II fluorescence imaging of mouse cerebral vasculature. The zoom in and zoom out features were demonstrated by imaging IR-820 labeled cells. The system is also capable of performing time-lapse imaging. With all these features, our system provides new opportunities to explore the mechanism of cerebrovascular disease. Our future work will focus on developing fluorescence lifetime imaging function and improving the temporal resolution, all of which could lead to further advances in intravital optical imaging.

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Disclosures

The authors declare no conflicts of interest.

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