Light sheet microscopy of blood vessels in mouse brain in vivo

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ABSTRACT

A method for intravital light sheet microscopy of blood vessels of mouse brain cortex is proposed. The use of tilted microscope lens mount and long working distance of microscope lens ensure high contrast images in both scattered light and fluorescence modes without immersion. Fluctuations of laser speckles related with the blood flow has been observed in scattered mode. In fluorescence mode distribution of Evans Blue dye over blood vessel cross-section was visualized.

Keywords: light sheet microscopy, Evans blue dye, blood circulation, brain

1. INTRODUCTION

Fluorescent microngiography[1, 2] and two photon microscopy[3], are the suitable techniques for the blood brain barrier studies. But the practical use of the techniques for *in vivo* studies is limited with principal issues. In particular the former technique is very simple and robust it provides no reliable data related to localization of blood vessels and dye leakage in depth. Limited depth of focus of optical microscope allows for decision is a part of object in focus or not when the object has clearly detectable edges and it is located in transparent medium. But the blood vessel located in scattered medium such as the brain tissue could not be localized because of its diffuse appearance due to light scattering. Moreover the regions of the dye leakage through blood brain barrier (BBB) is diffuse by nature. Thus the conventional microscopy and microangiography fail to distinguish dye stain located of the surface and deep leakage of dye related with BBB opening. The capability of out of plane selection of multi photon, laser scanning and spinning disc microscopy potentially overcome the issue discussed above but at the cost of high numerical aperture; short working distance and long acquisition time because of the scanning principle of image formation.

Light sheet microscopy originating from one century old ultramicroscopy approach of dark field imaging of submicrometer particles has been developed during recent decade into the flexible and robust technique for biological imaging[4] Currently it is implemented for studies of biological structures in the spatial scales ranging from single molecular and single cell level accessible with super resolution methods to whole organs and organisms structural imaging by means of sophisticated optical clearing protocols. In the intermediate length scale in vivo light sheet microscopy application are established for transparent multicellular organisms like embryos or genetically stained adult zebrafish.[5] The adult mouse as an object for light sheet microscopy is typically considered as a donor of organs for ex vivo examination by means of optical clearing.[6] But the light sheet microscopy also provides an access to the in vivo brain activity studies at single neuron level[7] and it demonstrates the advantages over confocal techniques.

The goal of our current study is to develop the technique for the in vivo imaging of brain blood vessels stained with administration of Evans Blue Dye into systemic circulation. Our interest is to detect the leakage of the dye related with BBB opening. Thus there is no need for high resolution imaging but large field of view and capability for quick looking over the trephined skull area and referencing of images with anatomical features are critical. Additional requirement is to avoid any mechanical contact with mouse head because requirement to leave a skull wound intact when over viewing a large part of the brain. All these considerations result in the development of the instrument with single side access to

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trephined skull of a mouse. In the paper we describe the setup and report the first time in vivo light sheet fluorescence microscopy of mouse brain blood vessels. We demonstrate a set of brain cros-sections asquired with the instrument and we propose the technique for blood vessel contrasting based on the movement of red blood cells that can be detected with respect to the stationary background.

2. MATERIALS AND METHODS

2.1 Optical arrangement for in vivo light sheet microscopy

Optical setup for intravital light sheet microscopy of mouse brain is presented on the fig.1. The setup was built around upright microscope Axio Scope A1 (Zeiss, Germany). The microscope was mounted over optical vibroinsulated breadboard table (Standa, Lithuania). The microscope was equipped with EM CCD camera Ixon Ultra DU 897 (Andor, Great Britain) for high sensitivity in vivo fluorescent imaging of biological objects. To enhance spatial resolution of camera 3.5× homemade camera adapter was used. Microscope table was shifted down along its mount to provide enough space for fixation of an animal under investigation. Because on single side access to the brain of the animal both illumination and imaging were performed at 45° with respect to microscope optical axis. For this purpose custom built 45° microscope lens adapter was attached to the microscope nosepiece (fig.1) with M27 mounting thread. Its optical path includes half pentaprism and an RMS thread mount for imaging lens. In the current study 5× 0.12 microscope lens (PZO, Poland) was used to ensure large enough working distance and low NA for visualization of blood vessels of mouse brain.

A short pass filter with 650 nm cut on wavelength was introduces into the microscope optical path to detect Evans Blue dye fluorescence. EMCCD camera resolution of 512×512 corresponded to 370×370 μm field of view at the object plane. High dynamic range and 16 bit grayscale camera output ensure in depth imaging trough scattering media of mouse brain through trephined skull. Image acquisition and processing was performed with custom software developed in LabVIEW (National Instruments, USA).

Optical system for light sheet illumination was built of semiconductor laser diode module with 160 mW output at 638 nm (Wuhan Laserlands Laser Equipment Co., ltd, China). Laser beam was attenuated with neutral density filter to ensure minimal possible laser irradiation of the object, that typically do not exceed 1W/cm^2 at the waist of light sheet beam in object plane. Laser was synchronized by means of TTL control port with "Fire" output of the EM CCD camera. It was turned on only for image accumulation period to avoid frame transfer related fringes at high speed operation mode of CCD. Light sheet was produced with a cylinder lens with 50 mm back focal distance. Mounting of the laser and light sheet optics was designed to ensure quick and intuitive adjustment of light sheet position during the experiment.

2.2. Animals

Male mice (20 to 25 g, n=6) were used in all of the experiments. The animals were housed under standard laboratory conditions with access to food and water ad libitum. All of the procedures were performed in accordance with the “Guide for the Care and Use of Laboratory Animals.” The mice were anaesthetized byinhalation anesthesia with 2% isoflurane at 1 L⁄ min N2O⁄O2 – 70:30 and were fixed in a stereotactic frame. A midline skin incision was made to reveal the skull bone, which was thinned with a dental drill to 2 mm lateral and 2.5 mm caudal to the bregma. The optical window (ϕ 2 mm) was prepared incoordinates of 1 to 4 mm caudal and 1 to 4 mm lateral. During the imaging, the mice were kept under inhalation anesthesia. The body temperature was maintained at 37.5°C by a homoeothermic blanket system with a rectal probe (Harvard Apparatus, Holliston, Massachusetts). The brain temperature was kept at 37°C using an objective heating system with a temperature probe (Bioptechs Inc., Butler, Pennsylvania). A polyethylene catheter (PE-10 tip, Scientific Commodities Inc., Lake Havasu City, Arizona) was inserted into the right femoral vein for EBd (Sigma

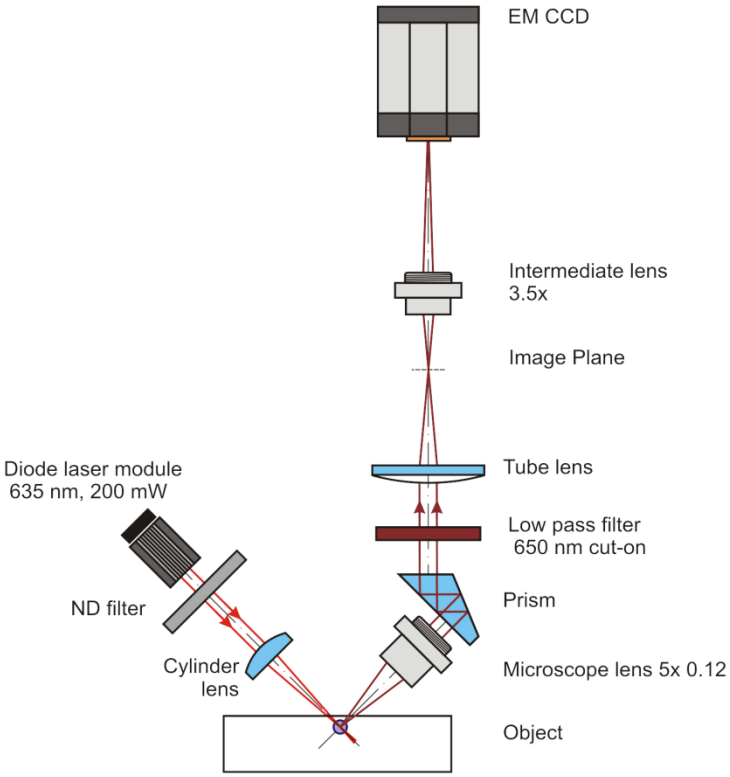


Figure 1. The setup for the intravital light sheet microscopy of mouse brain blood vessels

3. RESULTS AND DISCUSSION

3.1 Imaging of mouse brain blood vessels

Figure 2 shows results of in vivo mouse brain imaging of blood vessels of the brain. Because there are no immersion between microscope lens and the brain, actual position of the cross-section is shifted with respect to that in air because of refraction in brain tissues. Therefore the actual 3D structure of the object can be reconstructed only approximately. But the actual position of the light sheet can be referred to the anatomical features of the brain surface by means of the panoramic imaging of the light sheet position with additional camera. As it can be seen from fig.2 the imaging depth do not exceeds 200 μm. We performed ray optics based analysis of the effect of light refraction at the surface of the brain for the microscope lens with numerical aperture of 0.12 we used in the experiments. When optical axis of the lens is placed at the angle of 45° with respect to brain surface normal the object plane (the plane optically conjugated with the camera image sensor) inside the medium forms the angle about 30° with the normal to the surface. Because of the refraction object plane tends to turn toward the perpendicular to the brain surface. The 15 degree tilt of object plane can be taken into consideration and, thus it is not critical for 3D reconstruction of the object. The refraction on the tilted surface of the brain also causes the aberrations that potentially reduce the image quality. But in practice for low NA lens such as that used in our instrument the aberrations are negligible in comparison to the effect of light scattering in the brain tissues.

Because of the refraction on the brain surface actual position of the light sheet also changes so the additional system alignment is required to ensure the coincidence of the light sheet and tilted object plane of the microscope. The coarse alignment was performed using a small dish covered with a cover slide and filled with 40 nm colloidal gold solution

Sigma, Germany). The light sheet was positioned to ensure bright and sharp images of gold particles over the microscope field of view. Fine alignment of the light sheet was performed manually during mouse brain imaging.

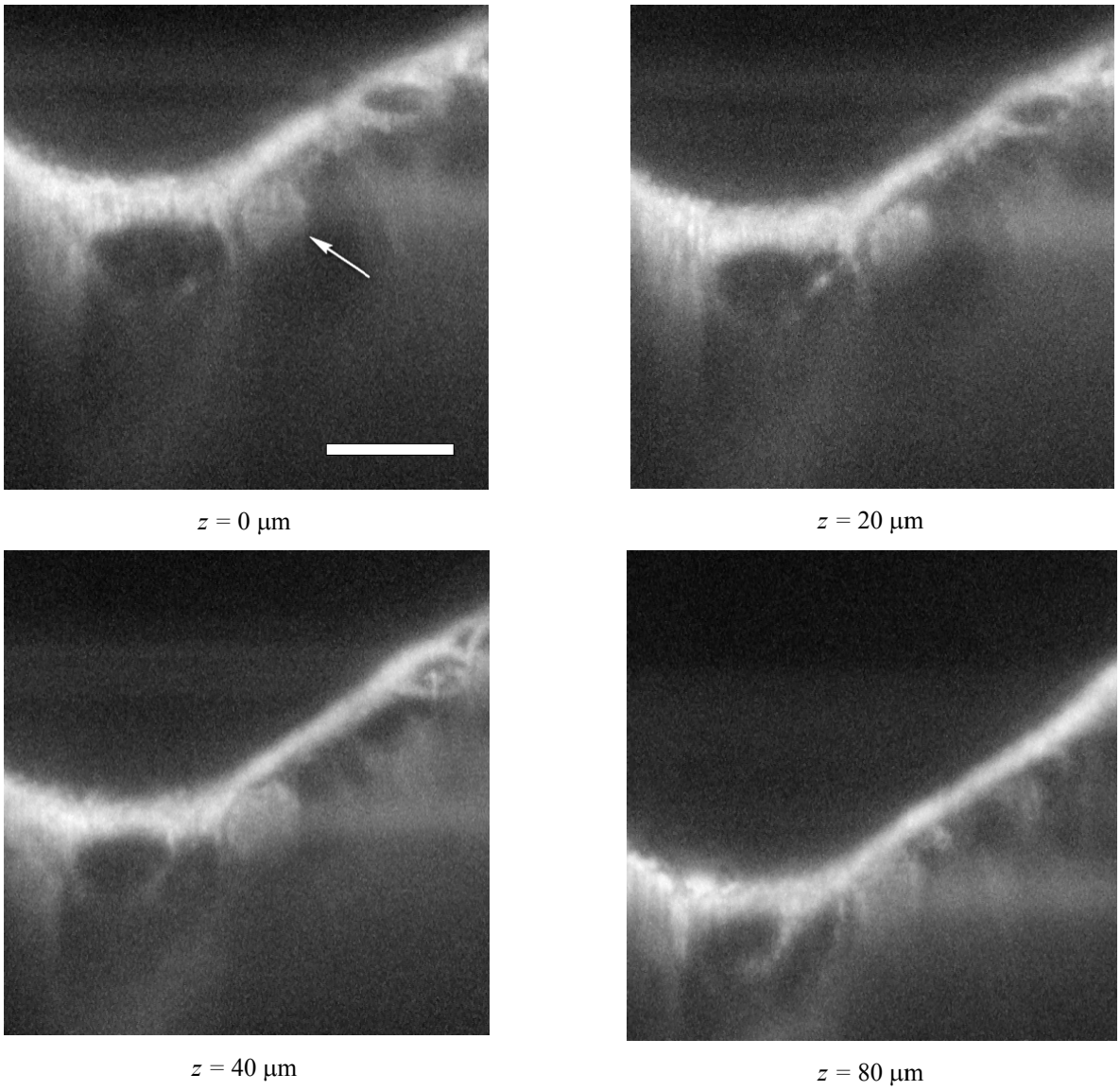
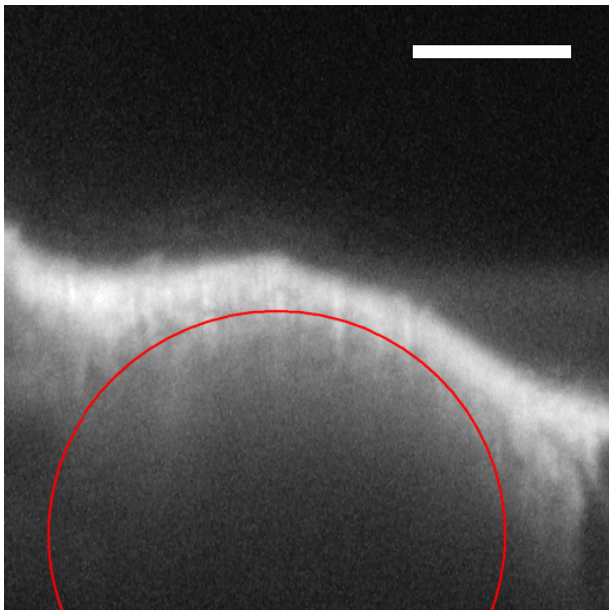


Figure 2. Images of the mouse brain acquired in vivo at different vertical positions of the microscope stage z. Grayscale corresponds to $\log_{10}$ of the fluorescence intensity detected with the camera. Scale bar corresponds to 100 μm. White arrow denotes brain blood vessel.

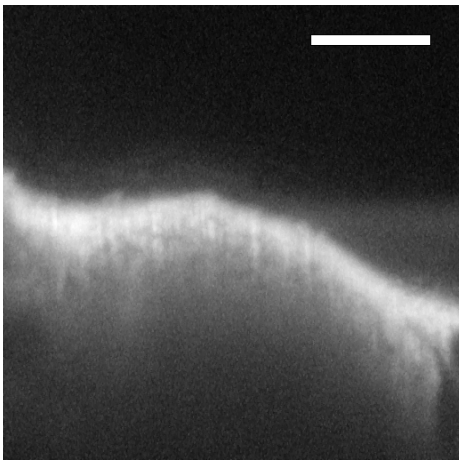
3.2 Detection of moving red blood cells

Figure 3 (a) shows the image of mouse brain saggital sinus. Actual position of sinus is shown with red circle. Because the sinus is about 400 μm in diameter, its whole cross section cannot be visualized with light sheet microscope. Only the superficial layers are available for the observation. When looking trough the video sequence of the sinus images moving red blood cells are clearly visible as dark spots over the light background corresponding to the blood plasma mixed with fluorescent Evans Blue dye. Movement of red blood cells (RBCs) enables to detect actual position of blood vessel. It is necessary when one have to discriminate between blood plasma mixed with dye and flowing through a vessel and a leakage of protein into the brain tissue caused by BBB opening, We proposed two stage procedure to detect moving RBCs into the blood vessel. First stage includes digital stabilization of a sequence of blood vessel images. The

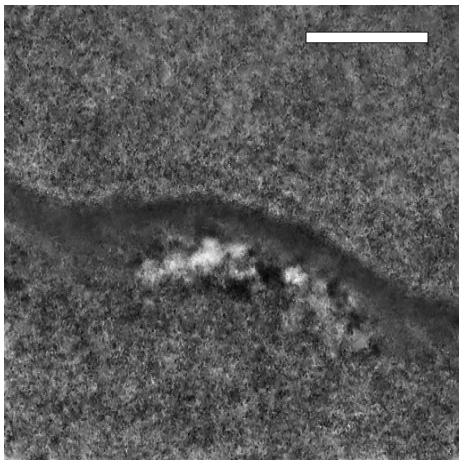
stabilization procedure is correlation based correction of spontaneous image displacements that compensates object movements related with cardiac activity etc. The second stage includes of averaging of stabilized images to determine stationary background. The averaged image is then subtracted from each image of series and moving red blood cells appear as contrast light and dark spots over the neutral background (fig.3(c)) thus revealing the actual blood vessel location. On the figure 3 the difference image is shown as negative and equalized to enhance the visibility.



a)



b)



c)

Figure 3. Image of the saggital sinus of mouse brain (a). Red circle corresponds to the actual sinus position. Comparison between single fluorescence image of saggital sinus (b) and the same image after subtraction of the averaged one. White spots corresponds to moving RBC detected over stationary background.

4. CONCLUSION

In the paper we report for the instrument for non contact in vivo fluorescent light sheet microscopy of mouse brain blood vessels. We developed the setup that does not require immersion and contact with trephined skull and ensures 20 mm working distance of the optical setup. The refraction of light on the interface between air and the brain tissue was compensated by appropriate positioning of the light sheet. Because of the use of low NA imaging lens the effect of aberrations induced with oblique incidence of light in the brain tissues was negligible in comparison with image degradation related with the scattering of light. We demonstrated for the first time the in vivo imaging of mouse brain blood vessels and superficial brain structures. We also proposed a method for contrasting of blood vessels based on the digital detection of moving red blood cells over a stationary background. The proposed instrument and image processing was successfully tested using biological object and can be applied for blood circulation and BBB function studies.

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