Optical coherent tomography and fluorescent microscopy for the study of meningeal lymphatic systems

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ABSTRACT

The development of novel technologies for the imaging of meningeal lymphatic vessels is one of the amazing trends of biophotonics thanks to discovery of brain lymphatics over several years ago. However, there is the limited technologies exist for the study of lymphatics *in vivo* because lymphatic vessels are transparent with a low speed flow of lymph. Here we demonstrate the successful application of fluorescent microscopy for the imaging of lymphatic system in the mouse brain *in vivo*.

Keywords: cerebral lymphatic vessels, glymphatic system, optical imaging, optical coherent tomography, fluorescent microscopy

1. INTRODUCTION

For years, there was concept of the absence of lymphatic vasculature in the brain. The discovery of the cerebral lymphatic vessels was reported in two studies published in 2015 by Louveau et al. and Aspelund et al.[1,2] Authors clearly showed the predominant location of lymphatic vessels in the meninges nearby the dural sinuses. The understanding of physiology of cerebral lymphatic system sheds new light on the therapy of many neoroinflammatory and neurodegenerative diseases. However, there are challenges to study the cerebral lymphatic system in vivo due to optical transparency of lymphatic vessels (low scattering and absorption) and low speed and nonstationary flow of lymph. There are some technical solutions including digital microscopy,[3–6] speckle and Doppler imaging,[3–6] photoacoustics,[4,6–8] optical coherent tomography,[9,10] molecular fluorescent,[3,4] and NIRS imaging,[11] allowing one to analyze lymphatic vasculature of the peripheral circulation. To present, there are no available technologies that can be used for imaging of cerebral lymphatic vasculature in vivo, therefore the development of new approaches to study physiology of lymphatic system in the brain is an urgent problem.

Even fluorescent imaging already demonstrated its efficiency for the imaging of peripheral lymphatics, the promotion and dissemination of this method to in vivo studies of mengeal lymphatics is a challenging task. Here we present new approach based fluorescent microscopy for imaging of meningeal lymphatic system in the mouse brain.

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2. MATERIAL AND METHODS

2.1 Subjects

Male mice (20–25 g) were used in all experiments. The animals were housed under standard laboratory conditions, with access to food and water, *ad libitum*. All procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals". The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Saratov State University (Protocol H-147, 17.04.2001).

2.2 *Cisterna magna* Evans Blue clearance

The mice are anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg), administered intraperitoneally. The skin of the neck is shaved, and the mouse is then placed prone on the stereotaxic instrument (the head forms a nearly 120° angle with the body). The *dura matter* of the *cisterna magna* appears as a reversible triangle. Hamilton a 34-G inserted into the *cisterna magna* through the *dura matter*. Evans Blue (7 μl) was injected into the subarachnoid space at a 0.1 μl/min rate over 5 min with a syringe pump (Harvard Apparatus).

2.3 Fluorescent microscopy

Fluorescent microscope was used to image distribution of Evans Blue dye injected into lymphatics.[12] The homemade setup consists of microscope objective lens with magnification of 5.5 and numerical aperture of 0.12, and CMOS camera (DCC1545M, Thorlabs Inc, USA) attached to extension tube with switchable long pass filter. Continuous wave diode laser module (50000463, Laserlands.net, China) with 160 mW output power at 635 nm was used to excite the dye florescence. Laser beam was expanded with a cylindrical lens (f=50 mm) and then directed towards an object at 45° with respect to the microscope optical axis. The switchable long pass filter with 650 nm cut-off wavelength (FELH0650, Thorlabs Inc, USA) was used to block excitation radiation in order to observe Evans Blue fluorescence. With the switched off filter the setup allows for imaging of blood vessels using ring illuminator consisting of 8 green light-emitting diodes (DFP-5013PGC-20, China) with the wavelength range 500-560 nm. Anesthetized animal was positioned at the microscope stage using 3D printed homemade system.

2.4 Statistical analysis

The results were reported as mean $\pm$ standard error of the mean (SEM). Differences from the initial level in the same group were evaluated by the Wilcoxon test. Intergroup differences were evaluated using the Mann-Whitney test and ANOVA-2 (post hoc analysis with the Duncan's rank test). Significance levels were set at $p<0.05$ for all analyses.

3. RESULTS AND DISCUSSION

Here we analyzed the ability of using the fluorescent microscopy and OCT for the imaging of cerebral lymphatic vessels. With this aim, we made the injection of Evans Blue into the *cisterna magna*, which is subarachnoid space of the meningitis collecting cerebrospinal fluid produced by the forth ventricle. The mouse fixed in stereotaxic system so that the head forms a 120° angle with the body (Figure 1A). Then under the stereoscopic microscope, the Evans Blue inserted in the cisterna magna using special surgical navigation such as clear reverse triangle through which the medulla oblongata and a major blood vessel (arteria dorsalis spinalis) are visible (Figure 1B). The injection of tracers into the cisterna magna is well-recognized method for the imaging of cerebral lymphatic vessels.[1,2]

Figure 2A schematically illustrates the pathway of Evans Blue in meningeal lymphatic vessels and sinuses. Figure 2B demonstrates results received by fluorescent microscopy: First blood vessels were captured in green illumination without the long-pass filter (fig. 3A). Because of the hemoglobin absorption blood vessels appear here as dark regions. Then the fluorescence of lymphatic vessels was captured over the same field of view (fig. 3B). In the figure, lymphatic vessels appear as right structures. Superposition of these images false color (blood vessels – red color, lymphatic vessels – green color) is shown in fig. 2B. The mean diameter of lymphatic vessels was 40 μm.

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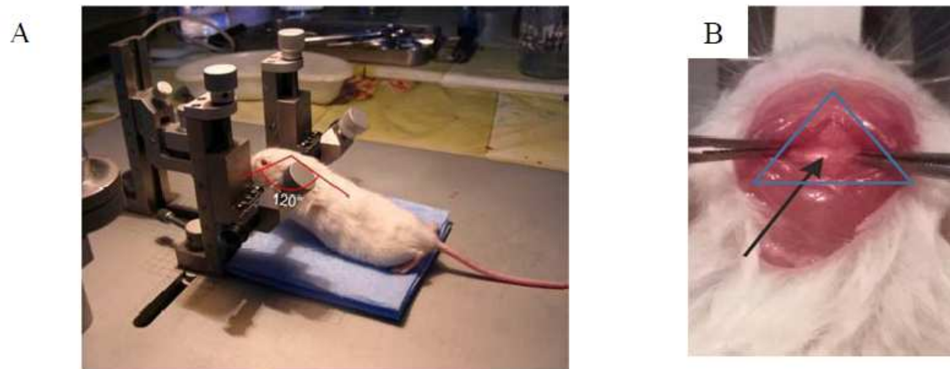
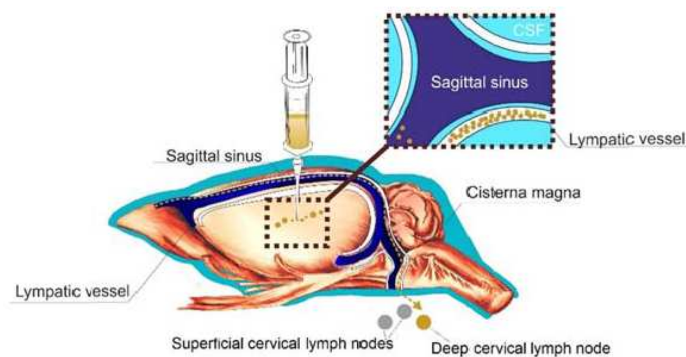


Figure 1. The injection of Evans Blue into the *cisterna magna*: A – the right position of mouse in stereotaxic system with fixation of head so that angle between head and body is about 120°; B – surgical navigation for the injection of Evan Blue into the *cisterna magna*.



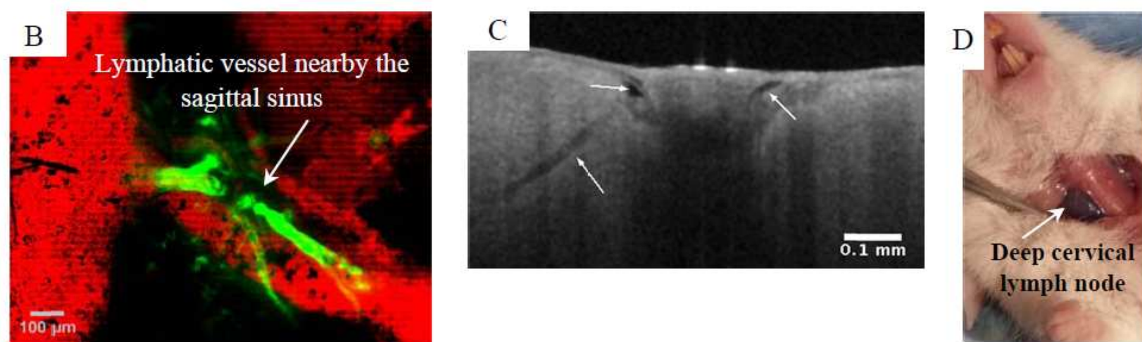


Figure 2. The vizualization of meningial lymphatic vessels: A – schematic illustration of Evans Blue pathway in the meningeal lymphatic vessels and sinuses; B – the results of fluorescent microscopy: green – lymphatic vessels, red – cerebral vessels; C – the OCT imaging of the meningeal lymphatic vessels (white arrows); D – Evans Blue detected in the *deep cervical lymph node* in 30 min after injection into the *cisterna magna*.

Figure 2C shows the OCT image of meningeal lymphatic vessels indicated by white arrows. Commercial OCT system (Thorlabs Ganymede II) was used as an imaging tool. Lateral and transverse resolution of this system are 6.2 μm (on air) and 8 μm, respectively. Central wavelength of illumination source is 930 nm. Each B-scan consists of 1024 A-lines. Time between adjacent B-scans is around 40 ms (30 kHz A-line rate). Each B-scan for the imaged sample was stabilized using intensity based correlation algorithm to reduce motion artifacts. Four consequence B-scans were averaged to reduce speckles and random noise. Lymphatic vessels are clearly seen on both side of sagittal sinus as "empty" structures with low SNR ratio. Another lymphatic vessel is located on the

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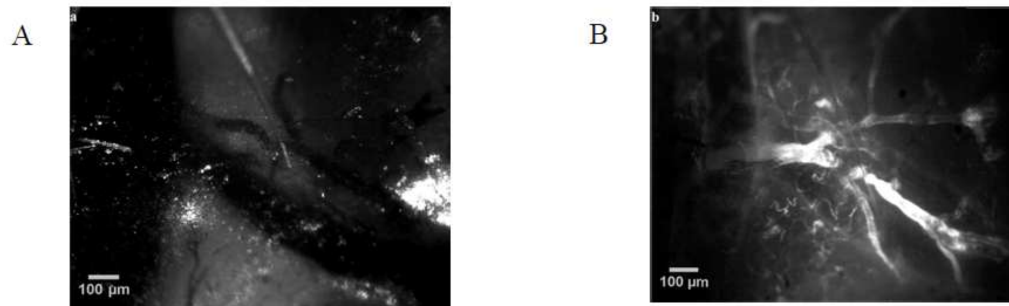

Figure 3. Florescence microscopy: A – image of blood vessels in green light illumination, B – fluorescent image of lymphatic vessels.

left side of the image and goes deep into the brain tissue.

Here we paid attention for three paired cervical lymph nodes, which accumulate the lymph from the brain the deep cervical lymph node and from the peripheral lymphatic system – two superficial lymph nodes.[13–15] We studied the accumulation of Evans Blue in these nodes using stereoscopic microscope. Our observation demonstrates that the Evans Blue injected into the cisterna magna detected in the deep cervical lymph node in 30 min after injection (Figure 2D), while in the superficial lymph nodes – in 1.5 h after injection.

4. CONCLUSION

These findings for the imaging of meningeal lymphatic system using fluorescent microscopy open new strategies for further in vivo studies of role of lymphatic systems in the etiology and treatment of many neuroinflammatory and neurodegenerative diseases.

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