PROCEEDINGS OF SPIE

SPIEDigitalLibrary.org/conference-proceedings-of-spie

Fluorescent angiography of chicken embryo and photobleaching velocimetry

Anton A. Namykin, Elena S. Stiukhina, Ivan V. Fedosov, Dmitry E. Postnov, Valery V. Tuchin

SPIE.

Fluorescent angiography of chicken embryo and photobleaching velocimetry

Anton A. Namykin[a] , Elena S. Stiukhina[a], Ivan V. Fedosov[a], Dmitry E. Postnov[a], Valery V. Tuchin[a,b,c]

[a]Research-Educational Institute of Optics and Biophotonics, Saratov National Research State University named after N.G. Chernyshevsky, Saratov, 410012 Russian Federation
[b]Institute of Precision Mechanics and Control RAS, Saratov, 410028 Russian Federation
[c]Interdisciplinary Laboratory of Biophotonics, National Research Tomsk State University, Tomsk, 634050 Russian Federation

ABSTRACT

Fluorescent angiography approach in application to a living chicken embryo is discussed. It provides precise vessel wall detection and demonstrates usefulness for real time monitoring of vasoconstriction and vasodilatation related to self-regulation of vascular network as well as to response to external factors. On the other hand, high stability of fluorescence and long period of dye elimination makes variations of fluorescent intensity practically independent from fast variations of blood flow rate. Therefore, we proposed the improvement of fluorescent angiography technique by introduction of photobleaching fluorescent velocimetry approach. We have developed the imaging system for intravital microscopic photobleaching velocimetry and tested it by using a glass capillary tube as a model of blood vessel. We demonstrated high potential of the technique for instant flow velocity distribution profile measurement with high spatial and temporal resolution up to 2 μm and 60 ms, respectively.

Keywords: Photobleaching, fluorescein, chicken chorioallantoic membrane, fluorescent angiography, flow velocity.

1. INTRODUCTION

Fluorescent angiography (FA) is based on injection of fluorescent dye in blood stream with consequent monitoring of its propagation with optical imaging techniques. FA is a well established technique in ophthalmology for blood flow disorders diagnostics in ocular fundus.[1,2] It is well developed for imaging of both capillary network and large vessels like arterioles and venules in human eye as well as in animal models like rat, chicken and zebra fish embryos.[3,4] The similar technique based on injection of fluorescent dyes in blood flow is used to enhance contrast of blood vessels in confocal microscopy.[5] While fluorescent angiography is efficient for imaging of blood vessels on the background of surrounding tissues, it provides also data on functional state of blood vessels by means of observing dye propagation along intact vascular network. However it is not yet suitable for monitoring of fast changes of blood flow velocity, e.g. related to heart activity. This is because injected fluorescent dye remains in blood flow for relatively long time so no fluorescence intensity variations related to cardiac activity can be detected.

To detect fast variations of blood flow velocity another method referred to photobleaching velocimetry can be used.[6,7] It is based on the phenomenon of bleaching of fluorescent dyes caused by absorption of intensive laser radiation. It can be arranged either by means of a long term irradiation of dye by a low intensity light radiation or by a short pulse intensive light. Typical application of photobleaching velocimetry is aimed for velocity measurements in microscopic flows. In the current study, we proposed to apply photobleaching method for blood flow velocity monitoring in blood vessels of choroioallantoic membrane of chicken embryo. The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane providing gaseous and heat exchange, delivery of nutrients into the embryo. These functions are supported by dense vascular network. Because of its comprehensive capillary network and easy accessibility, the CAM has been broadly used to study morphology properties of angiogenesis *in vivo*. The CAM has long been a favored system for the study of cancer angiogenesis.[8]

In order to study blood flow dynamics in the CAM we had developed microscopic imaging setup for fluorescent angiography and used it for microangiography of the CAM blood vessels. We had demonstrated long lifetime of injected dye into circulating blood that prevents detection of instant blood flow velocity changes. In the second part of the work done, we present photobleaching velocimetry arrangement and software we developed for *in vivo* real-time monitoring of

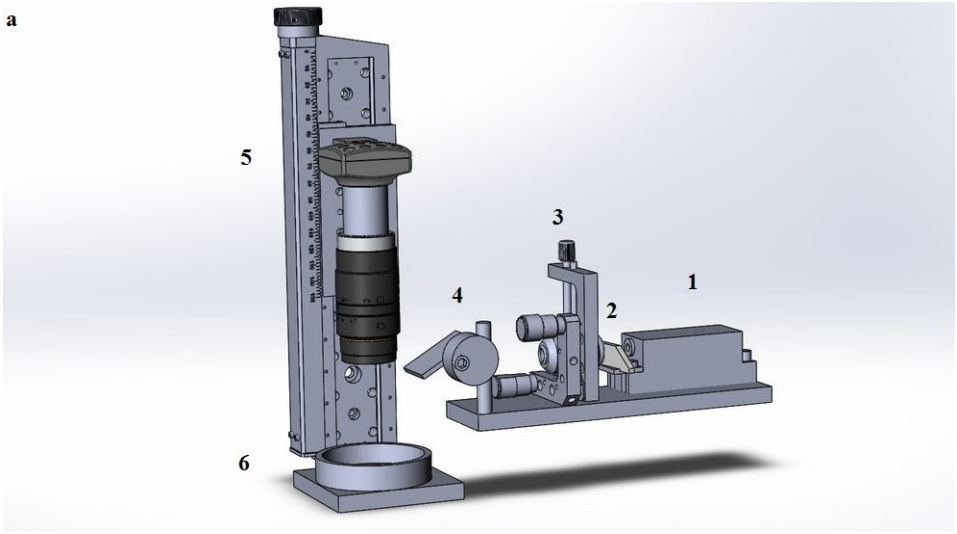
Saratov Fall Meeting 2016: Optical Technologies in Biophysics and Medicine XVIII, edited by Elina A. Genina, Valery V. Tuchin, Proc. of SPIE Vol. 10336, 103360V · © 2017 SPIE
CCC code: 1605-7422/17/$18 · doi: 10.1117/12.2267948

blood flow dynamics with high spatial and temporal resolution. We had tested the technique performance using a model of blood vessel.

2. MATERIALS AND METHODS

2.1. Fluorescent angiography of chicken embryo

Fluorescent angiography of CAM includes preparation of chicken embryos, injection of dyes and imaging of blood vessels. We used 5 – 8 days old embryos most suitable for our study because at that stage of development no sufficient movements of an embryo can complicate optical imaging of blood vessels. Cultivation process divide in two methods. The first, is *in ovo* (in the egg) method. To prepare the model, we incubated chick eggs from local breed "Rygayanesushka" at temperature of 38°C and humidity of 60%. An egg was turned on 180 degrees along its long axis twice a day. At day 5, and immediately before experiment the ovoscopy was performed. During the experiments, the egg temperature was maintained at 37±1°C. The second, is *ex ovo* method. To prepare the model, we incubated hen's eggs breed "Cross" Super Nick" at 38°C and 60% humidity. Eggs in the incubator were automatically rolled over along their long axis every two hours. On the third day, we poured of contents of the egg into a pre-washed, disinfected and dried glass Petri dish. This resulted in embryo appearance with the developed blood network on the surface of the yolk. After that Petri dish is closed by a glass lid to be further kept in an incubator under the same conditions. Dye injection into cultivated embryo blood stream was performed with 300 μm internal diameter needle (Ni-Pro Dental needle 30G) connected with an adapter to insulin syringe. We used 0.05% solution of fluorescent dye in water (soluble fluorescein, Russia), dye solution of 0.1mL was injected into vascular chicken embryo. If injection was made in the arterial downstream (2-3 cm), from the point of monitoring, fluorescence was observed in 2-5 s after injection. Injection was done without micromanipulation by using a hand-made injection. Optical imaging setup was built using monochrome CMOS camera (DCC1545M, Thorlabs, Germany) connected through 30 mm extension tube with 50 mm focal length C-mount objective lens (Tamron, Japan; 5, Fig. 1(a)). CW DPSS laser with 80 mW emission power at 473 nm (LS-1-N473/80, "LaS", Russia) controlled with laser driver module was used to excite fluorescence of the dye (see 1 in Fig. 1(a)). IR cutoff filter was used to suppress IR radiation of DPSS laser system. A microscope lens with (10x, NA 0.2) was used to expand laser beam deflected by a mirror toward object (2, Fig. 1(a)). A low pass light filter with 500 nm cutoff wavelength was placed before camera lens to reject excitation radiation.



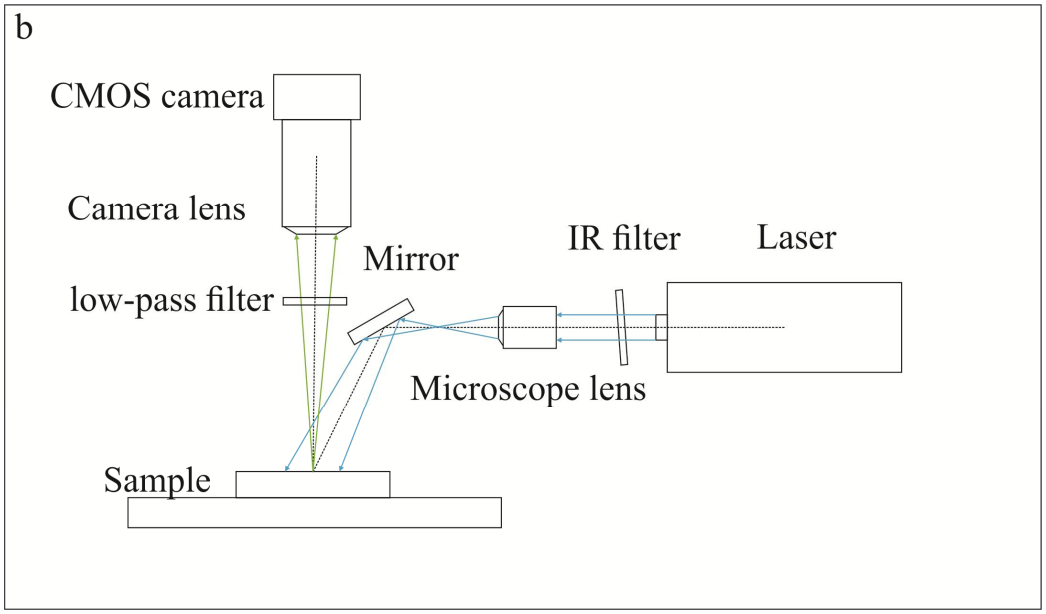


Figure 1. 3D model (a) and optical scheme (b) of imaging system for fluorescent angiography of chicken embryo (mounts, modules, and breadboard produced by thorlabs.com and standa.lt were used for the artwork).

2.2. Photobleaching micro velocimetry

Photobleaching velocimetry method was used for measurements of the instant flow velocity distribution profile in blood vessel models. A glass capillary circular tube with 100 μm internal and 1 mm external diameter was used as a model of chorioallantoic membrane blood vessel. In order to eliminate refraction at external surface of the tube and to reproduce optical geometry of CAM imaging, tube was covered by a 0.17 mm thick microscope coverslip and placed into immersion oil with refraction index close to that of borosilicate glass. The sodium fluorescein solution in water was pumped through the tube with two dishes connected with a tube with PVC hoses and placed one above the other. Flow velocity was controlled by variation of vertical positions of dishes. The custom-built microscopy setup was used for photobleaching and imaging of the model flow (Fig. 2).

A 405 nm cw diode laser with 150 mW output power was used as a bleaching light source. Laser radiation focused with cylinder lens (f = 70 mm) was introduced into optical path of the microscope with dichroic mirror and then focused into a line in the object plane with 3.7x (NA= 0.12) microscope lens. The same lens was used for imaging of the object with CMOS camera (DCC1545M, Thorlabs, Germany) through low-pass dielectric filter with cutoff wavelength of 500 nm used to reject excitation radiation. Fluorescence of dye was excited with ten 405 nm LED arranged into 3D printed illuminator ring (Fig. 2). Image processing was performed with the developed software using NI LabVIEW Development system (National Instruments, USA).

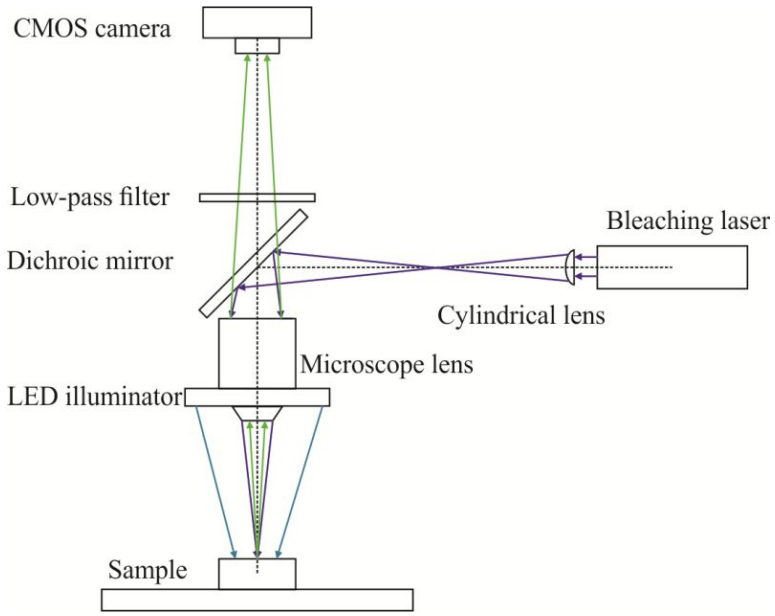


Figure 2. Optical arrangement for photobleaching velocimetry

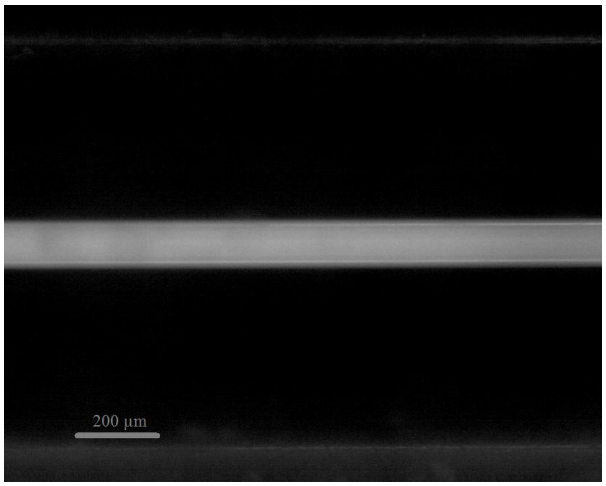


Figure 3. Unbleached fluorescent image of glass capillary tube placed into immersion oil and filled with sodium fluorescein water solution

To enhance photobleached dye detection and to exclude effect of non-uniform image brightness, the procedure of an averaged image subtraction was performed. Each image of tube with bleached dye was subtracted from an average of 10 background images captured without photobleaching (Fig. 3). As a result, we obtained a negative image of bleached region on a dark background (Compare Figs. 4 (a) and (b) and (c) and (d)). This procedure has optimized signal to noise ratio of the image and made it possible to measure flow velocity over 5 – 6 frames (360 ms) after the bleaching laser fire.

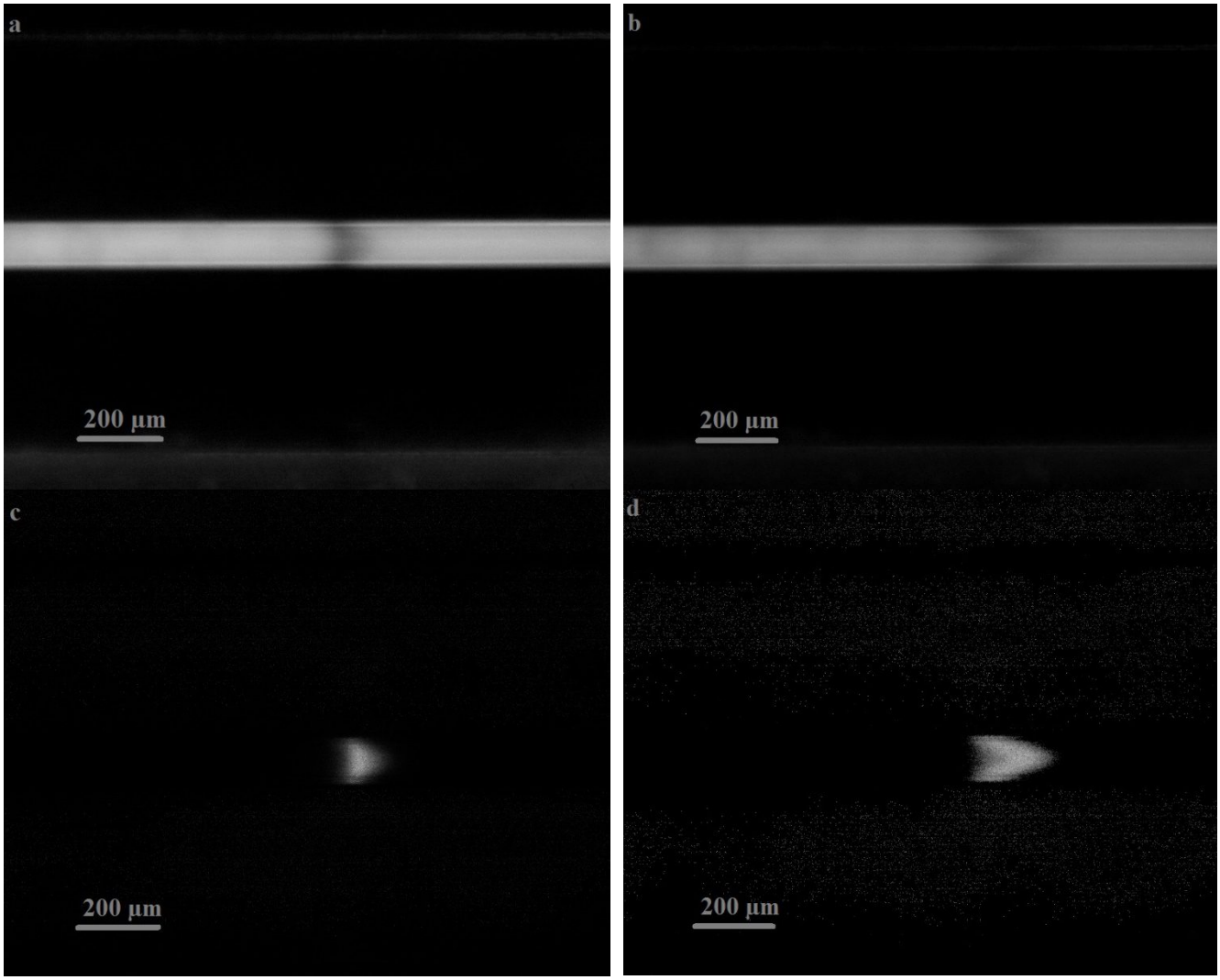


Figure 4. Images of photobleached dye flowing through capillary tube at 60 ms (a) and 360 ms (b) after bleaching by a laser fire; and the same images subtracted from averaged background image (c) and (d), respectively.

3. RESULTS AND DISCUSSION

Fluorescent angiography approach was used to visualize blood vessels of chicken embryo CAM. The injected dye was clearly detected inside both large and small vessels of embryo (Fig. 5). Immediately after injection, an increase of fluorescence intensity corresponding to propagation of dye along blood stream can be observed.

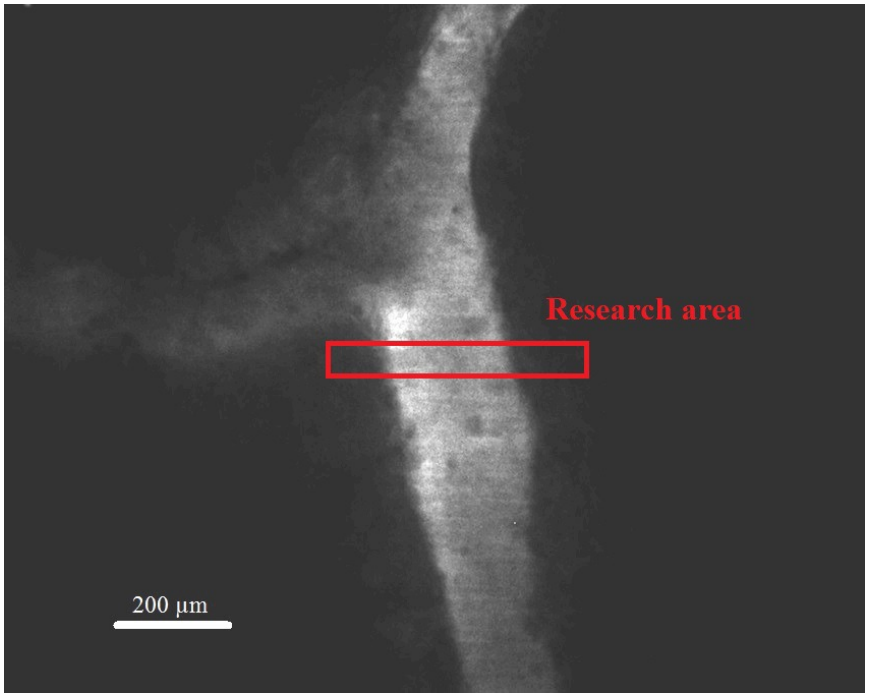


Figure 5. Image captured in 5 s after the injection of dye into blood stream of chicken embryo

Such intensity changes can be used for mapping of blood vessel network and functional imaging of the blood stream. After some time interval, the injected dye mixes with blood and produces stable fluorescence over whole network of blood vessels. This stage of blood fluorescence labeling is typically used for blood pH measurements.[9] Peak intensity was achieved in 5 s after the injection. The intensity profile corresponding to blood vessel shown in Fig. 5 is presented in Fig. 6(a). Then the intensity of fluorescence decreases slowly because of both metabolic elimination of dye The same intensity profile captured in 3 min after the injection shows only 10% decrease of fluorescence intensity.

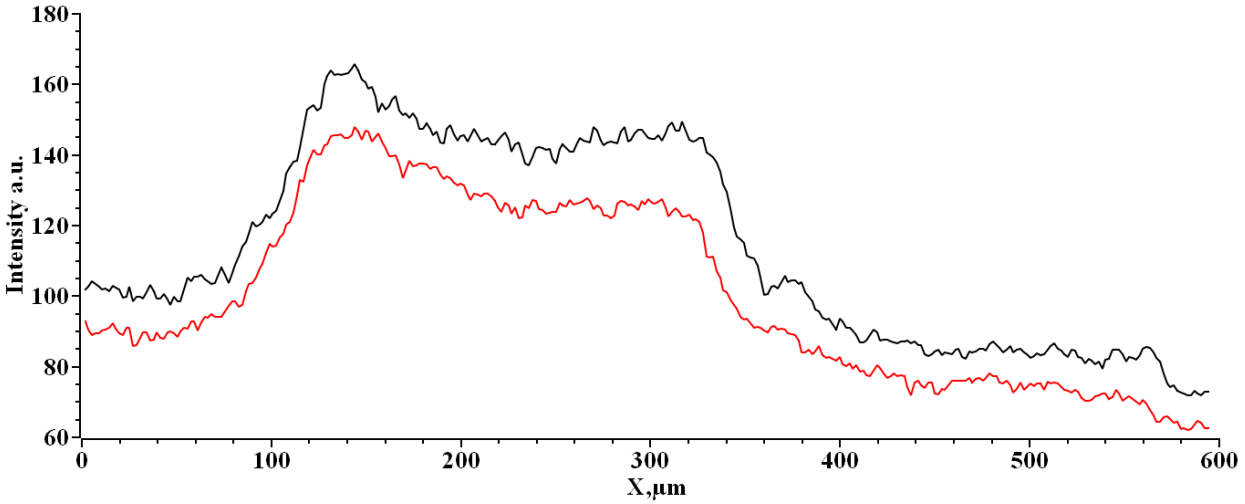


Figure 6. Intensity profile in direction perpendicular to chicken embryo blood vessel. 5 s (black) and 3 min (red) after dye injection.

High stability of fluorescence makes it possible for long term contrasting of blood vessels but it prevents monitoring of blood dynamics that should be performed repeatedly with high temporal resolution. On the other hand, such a high stability of fluorescence can be utilized for a photobleaching-based velocimetry (PBV).

To investigate the potential of technique for intravital real time monitoring of instant blood flow velocity we used a blood vessel model described in section 2.2. One way to measure flow velocity by means of PBV is to track the movement of a bleached portion of dye after a short pulse of bleaching laser focused into a narrow line perpendicular to the vessel direction. Figure 7 represents the intensity profile corresponding to a centerline of the flow shown in Fig. 4 (a, b). The intensity profile was taken after the subtraction of each frame from averaged background, so the higher values of intensity corresponds to the darker regions of bleached portion of dye (compare Figs. 4 a and c). As it can be seen, the intensity profile follows the flow and broadens because of dye diffusion and non-uniform velocity distribution in the flow. We should to note that the intensity profile corresponds to the longitudinal cross-section of the flow and it contains all velocity range from a high at the centerline of the cylindrical tube flow to a slow one in the near wall regions. These measurements were affected by the limited depth of focusing of the microscope and should be further investigated. However at the first approximation the movement of the bleached fluorescence intensity profile maximum can be used as a flow tracer for flow velocity assessment.

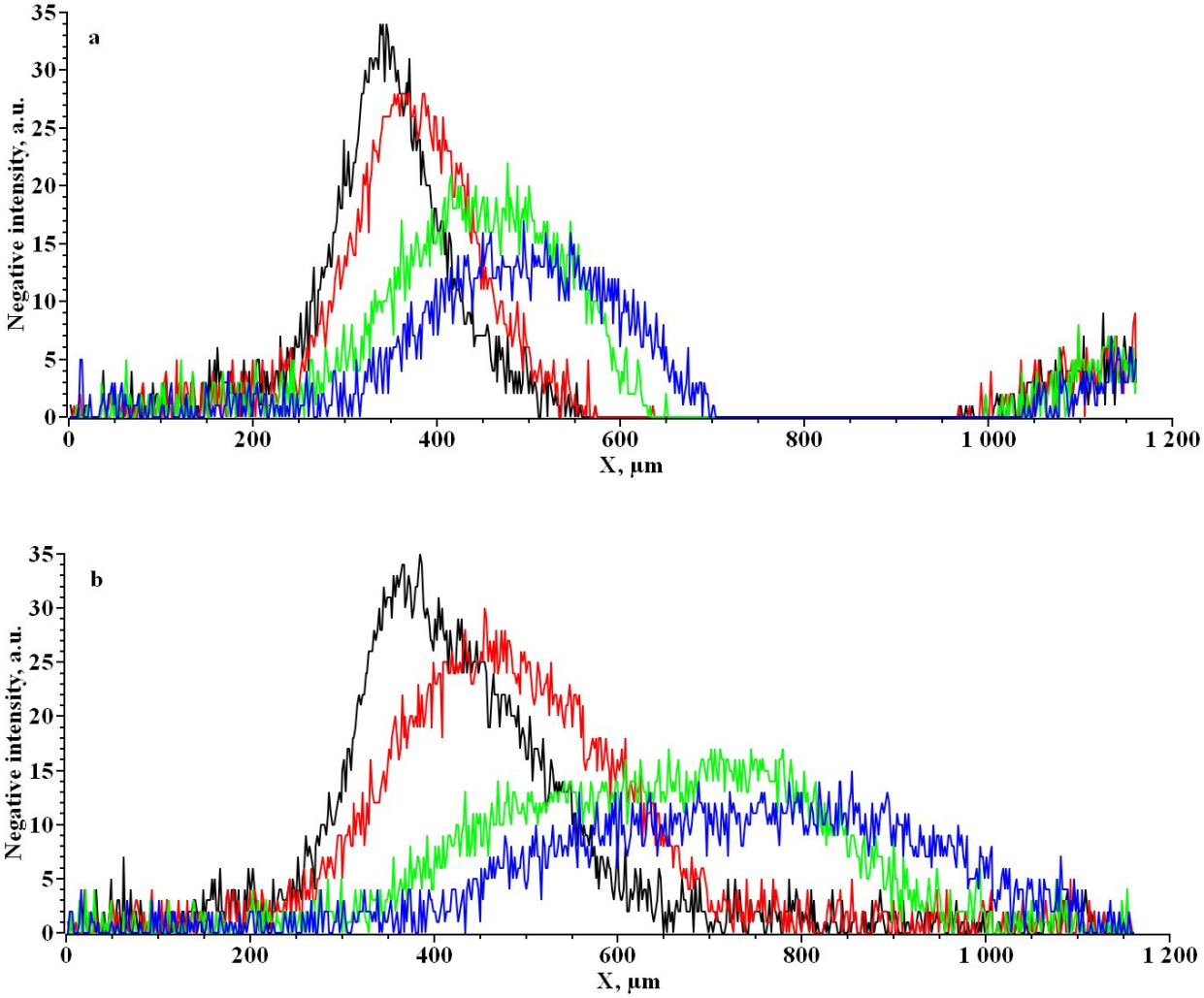


Figure 7. Centerline flow of the bleached fluorescence intensity profiles along vessel taken at four instants after bleaching laser pulse at flow velocity of 200 µm/s (a) and 550 µm/s (b). Time lapsed after bleaching: 60 ms (black), 180 ms (red), 540 ms (green), 840 ms (blue).

The intensity profile shown in Fig. 7 is obtained as a single row of pixel values along tube centerline. In the same manner each image row corresponding to tube area can be processed. After automated detection of intensity maximum it gives the current position of bleached region at each row at fixed instant of time. Repeating the procedure for the next frame and subtracting corresponding positions, we can calculate displacement of bleached region for time interval separating both frames. Calculating one point per row we obtained flow velocity profile with spatial resolution of 2 μm. Figure 8 represents the averaged velocity profiles corresponding to cases shown in Fig.7 a and b. Each point of both profiles was calculated as an average over six displacements calculated for six pairs of frames. Error bars show standard deviation estimated over the same data.

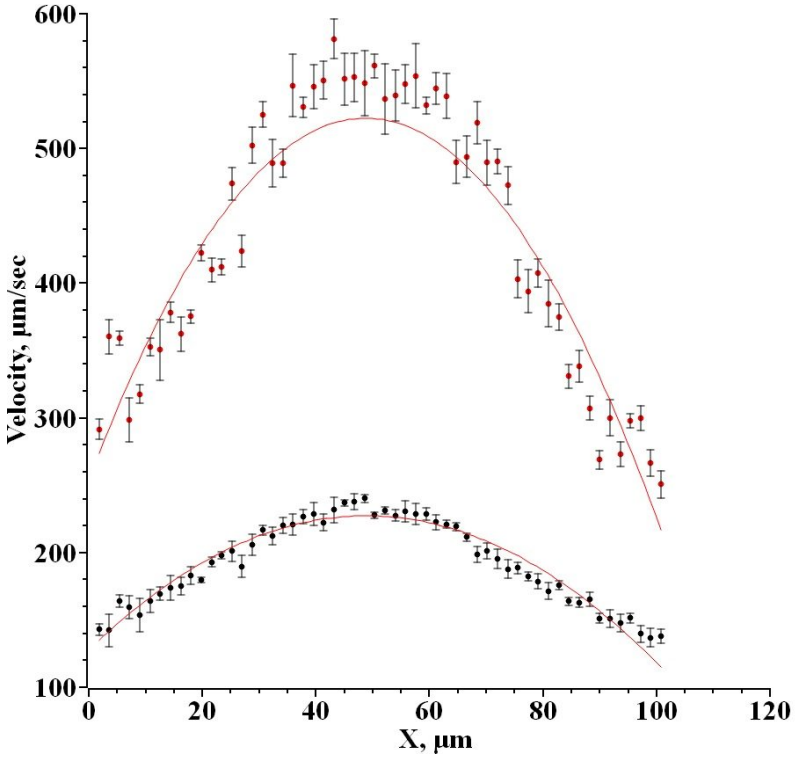


Figure 8. Averaged flow velocity profiles across the capillary tube. Black and red dots and lines correspond to the cases shown in Figs. 7 (a) and (b). Error bars denotes standard deviation calculated over 6 measurements at each point.

Although we can expect parabolic velocity distribution profile for laminar flow of water through cylindrical tube, the resulting one appears "flattened". The possible reason of the profile deformation may be related to light refraction at glass tube wall, finite numerical aperture of microscope lens and dye diffusion. On the other hand, the obtained data can be used for volumetric flow rate estimation in real time using only one pair of frames.

4. CONCLUSION

The goal of our study was to assess the potential of fluorescent imaging techniques for the purposes of blood flow dynamics analysis in chorioallantoic membrane of chicken embryo which we used as a biological model for angiogenesis and vascular regulation. We had applied fluorescent angiography approach to a living embryo that allowed us a high contrast imaging of blood vessels. We had shown that it is suitable for vessel wall detection and vessel diameter assessment. The fluorescent dye introduced into blood stream can be used for automated mapping of blood circulatory network. It provides precise vessel wall detection and thus it is useful for real time monitoring of vasoconstriction and

vasodilatation related to self-regulation of vascular network as well as to its response to the external factors. Blood plasma contrasting with green fluorescent dye also enhances contrast of red blood cell that appear as dark spots over fluorescent vessel background. On the other hand, high stability of fluorescence and long period of dye elimination makes variations of fluorescent intensity practically independent from fast variations of blood flow rate. Therefore we proposed the improvement of a well-established fluorescent angiography technique by using of photobleaching fluorescent velocimetry. We have developed the imaging system for intravital microscopic photobleaching velocimetry and tested it by using a glass capillary tube as a model of blood vessel. We demonstrated high potential of the technique for instant flow velocity distribution profile measurement with high spatial and temporal resolution up to 2 μm and 60 ms, respectively.

ACKNOWLEDGEMENTS

The work was supported by Russian Science Foundation, project 16-15-10252. The authors are grateful to Oleg V. Grishin, Arkady S. Abdurashitov, and Natalya Tkachenko for their assistance. VVT was supported by Russian Presidential grant NSh-7898.2016.2 and the Russian Governmental grant 14.Z50.31.0004.

REFERENCES

[1] Kwon, Y. K., Kang, D. J., Lim, J. C., Kim, J. H., Park, D. H., and Shin, J. P., "Fundus Autofluorescence, Fluorescein Angiography and Spectral Domain Optical Coherence Tomography Findings of Retinal Astrocytic Hamartomas in Tuberous Sclerosis," J. Korean Ophthalmol. Soc., **57**(1), 134-140 (2016).

[2] Gass, J. D. M., "Stereoscopic atlas of macular diseases: diagnosis and treatment." Mosby Inc. (1997).

[3] Nowak-Sliwinska, P., Segura, T., and Iruela-Arispe M.L., "The chicken chorioallantoic membrane model in biology, medicine and bioengineering," Angiogenesis **17**(4) 779-804 (2014).

[4] Nowak-Sliwinska, P., Ballini, J. P., Wagnières, G., and Van Den Bergh, H., "Processing of fluorescence angiograms for the quantification of vascular effects induced by anti-angiogenic agents in the CAM model," Microvasc. Res. **79**(1), 21-28 (2010).

[5] Kim, T. N., Goodwill, P. W., Chen, Y., Conolly, S. M., Schaffer, C. B., Liepmann, D., and Wang, R. A. "Line-scanning particle image velocimetry: an optical approach for quantifying a wide range of blood flow speeds in live animals." PloS One **7**(6), e38590-1-13 (2012).

[6] Mosier, B., Molho, J., and Santiago, J., "Photobleached-fluorescence imaging of microflows." Exp. Fluids **33**(4), 545-554 (2002).

[7] Molho, J. I., Herr, A. E., Mosier, B. P., Santiago, J. G., Kenny, T. W., Brennen, R. A., and Mohammadi, B., "Optimization of turn geometries for microchip electrophoresis," Analyt. Chem. **73**(6), 1350-1360 (2001).

[8] Mason, W. T. (Ed.), *Fluorescent and luminescent probes for biological activity: a practical guide to technology for quantitative real-time analysis*, Academic Press, London (1999).

[9] Lokman, N. A., et al., "Chick chorioallantoic membrane (CAM) assay as an *in vivo* model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis," Int. J. Mol. Sci. **13**(8) 9959-9970 (2012).