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Vyacheslav Kalchenko
Yuri Kuznetsov
Igor Meglinski
Alon Harmelin

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Vyacheslav Kalchenko,[a] Yuri Kuznetsov,[a] Igor Meglinski,[b] and Alon Harmelin[a]

[a]Weizmann Institute of Science, Department of Veterinary Resources, Rehovot, 76100, Israel

[b]University of Otago, Department of Physics, PO Box 56, Dunedin 9054, New Zealand

Abstract. The peripheral lymphatic vascular system is a part of the immune body system comprising a complex network of lymph vessels and nodes that are flowing lymph toward the heart. Traditionally the imaging of lymphatic vessels is based on the conventional imaging modalities utilizing contrast fluorescence materials. Given the important role of the lymphatic system there is a critical need for the development of noninvasive imaging technologies for functional quantitative diagnosis of the lymph vessels and lymph flow without using foreign chemicals. We report a label free methodology for noninvasive *in vivo* imaging of blood and lymph vessels, using long-exposure laser speckle imaging approach. This approach entails great promise in the noninvasive studies of tissues blood and lymph vessels distribution *in vivo*. © *2012 Society of Photo-Optical Instrumentation Engineers (SPIE)*. [DOI: 10.1117/1.JBO.17.5.050502]

The lymphatic vascular system plays a key role in the human body and works in close cooperation with the blood vascular system by returning the excess of interstitial fluid to the circulatory system.[1] The lymphatic system is essential for the tissue fluid homeostasis, fat absorption, and immune responses and also in pathological conditions, such as tumor metastasis, lymphoedema, and inflammation. Lymph itself is a fluid, which is composed of macromolecules, leukocytes, and activated antigen-presenting cells transported from the blind-ended lymphatic capillaries toward the collecting lymphatic vessels and returned to the blood circulation through lymph-venous junctions.[1] Despite its importance, the ultimate understanding of the lymphatic system and lymph flows is incomplete due to a lack of diagnostic modalities, suitable for *in vivo* noninvasive imaging, of lymphatic flow and lymph vascular microstructures.[1]

A number of diagnostic modalities for imaging of vascular network are available in clinical and preclinical practice, including x-ray and computer tomography (CT),[1] magnetic resonance imaging (MRI),[1] Doppler ultrasound,[2] laser Doppler blood flowmetry (LDF),[3] laser-scanning confocal imaging,[4] single photon emission computed tomography (SPECT),[5] capillaroscopy,[6]

optical/fluorescent imaging,[7,8] photo-acoustic imaging,[9] optical coherence tomography (OCT)[10,11] and others.[12] However, a noninvasive diagnostic technique for high-resolution *in vivo* imaging of vascular network, blood flow, and lymph microcirculation is not available for day-to-day medical practice, mainly due to the limitations of the techniques mentioned above. Doppler ultrasound allows tracking flow velocities at different locations in a tissue, however, long acoustic wavelength required for deep-tissue penetration limits spatial resolution to 200 μm. Application of the capillaroscopy technique requires the tissues to be thin enough (less than 400 μm) to be trans-illuminated. Images obtained using laser-scanning confocal microscopy can only be collected at a fraction of the normal video rate. Conventional and magneto resonance angiography provides information mainly on large blood vessels, such as the coronary artery. The disadvantages of OCT and photo-acoustic imaging are their high sensitivity to the unintended movements of the subject of investigation. Inability to monitor flow value in the lymph and blood vessels of 50-μm diameter or smaller with the flow rate less 100 μm/s is another drawback. LDF provides only an average characteristic of the skin blood flow, so-called perfusion, as the strong optical scattering in biological tissue significantly restricts visualization of spatially resolved blood vessels and blood flows.

Optical imaging approach, known as a fluorescent lymphangiography, has become popular since the last decade and now is often used in image-guided surgery.[7] The fluorescent lymphangiography requires intradermal injection of fluorescent marker, which significantly limits the observation time due to its biochemical properties. Therefore, development of a noninvasive and label free imaging modality could have many benefits including toxicity reduction.[13,14]

In our current study, particular attention is given to the development of noninvasive label free optical micro-angiography modalities for quantitative evaluation of blood and lymph microcirculation *in vivo*. In this paper, we introduce a long-exposure time laser speckle imaging (LSI) based approach for noninvasive label free imaging of blood and lymphatic vessels *in vivo*.

LSI is based on the analysis of the intensity fluctuations of laser light scattered within the medium containing the moving scattering particles, provided sufficiently small Doppler shift in comparison with the frequency of incident light.[3]

Applying to biomedical diagnostic applications, LSI is widely used to monitor capillary blood flow,[15] analysis of blood samples *in vitro*,[16] imaging of blood coagulation,[17] and blood perfusion mapping.[18] Due to motion of tissues scattering particles, such as blood cells, the intensity of scattered laser radiation is fluctuated and observed as a time-varying speckle pattern called speckles. The motions of scattering particles lead to blurring the speckles, which is analyzed quantitatively by speckle contrast:[19]

$$K(T) = \sigma(T)/\langle I \rangle. \tag{1}$$

Here, K is speckle contrast, σ is the temporal standard deviation of the speckle intensity, T is the exposure time, and $\langle I \rangle$ is the average intensity. This single-exposure LSI technique has been widely used to measure blood flow changes in biological tissues. Recently, by taking advantage of the dependence of the speckle contrast on the camera exposure, the technique has been significantly improved and can be operated with multi-exposure time[20] or dynamic LSI.

Address all correspondence to: Alon Harmelin and/or Vyacheslav Kalchenko, Weizmann Institute of Science, Department of Veterinary Resources, Rehovot, 76100, Israel. Tel: +972 89344520; Fax: +972 89344133; E-mail: Alon.Harmelin@weizmann.ac.il; a.kalchenko@weizmann.ac.il
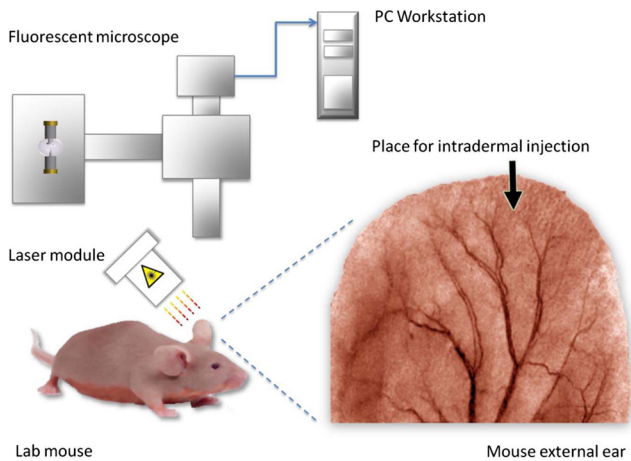
Fig. 1 Schematic presentation of hybrid LSI/FIM experimental imaging system, used in the current study.

In the current study, we apply a hybrid dual-mode experimental system, presented in Fig. 1. The system utilizes multiexposure time LSI and fluorescent intravital microscopy (FIM) modes for simultaneous imaging of the same area of biological tissues.[8,13,14]

In the LSI mode a diode laser module (LDM808/3LJ, 808 nm, 3 mW, Roithner Lasertechnik, Austria) is used. A coherent laser beam passes through ground grass diffuser (Thorlabs, Newton, New Jersey, USA) and illuminates the mouse ear. The laser speckles, produced by diffusively reflected coherent laser light, are registered by charge coupled device (CCD) camera (Pixelfly QE, PCO, Germany) setting with the short (~33 ms) and long (~650 ms) exposure times. Camera control and image acquisition are performed through CamWare (PCO, Germany).

A specifically designed plugin for ImageJ (Rasband, W.S., U.S. National Institutes of Health, Bethesda, Maryland, USA) software is used for the image processing and analysis of acquired image sequences; typically, 300 frames.[21]

CD1 nude female mice aged six to eight weeks from Harlan Laboratories were used in the experiments. Each animal was intravenously anesthetized with the ketamine (Fort Dodge, Iowa) and xylazin (Kepro, Deventer, Holland) 10 mg/100 mg/kg and placed on a thermally controlled stage. The external ear of the mouse is gently attached (using double-sided glue tape) to the plastic platform for better imaging.

The images obtained by LSI mode are presented in Fig. 2 for the short and the long exposure times, respectively. As one can see, when the higher exposure time (650 ms) is applied, the "white vessels" appear [Fig. 2(b)].

To understand the formation of the "white" speckle patterns, we consider speckle contrast K as a function of the exposure time T of the camera and speckle correlation time τ_c. Previously it was reported that:

$$K = \left\{ \frac{\tau_c}{2T} \left[1 - \exp\left(\frac{-2T}{\tau_c} \right) \right] \right\}^{1/2}. \quad (2)$$

This relationship, originally suggested by Briers,[22] has been widely used in the literature for a number of years. Recently, a more accurate expression has been introduced:[20]

$$K = \left\{ \beta \frac{\exp(-2x) - 1 + 2x}{2x^2} \right\}^{1/2}, \quad (3)$$
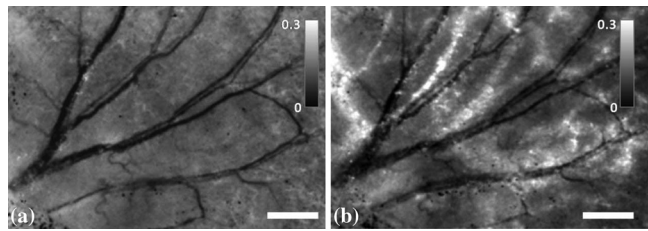
Fig. 2 Speckle contrast images of the external ear of nude mouse obtained *in vivo* with the exposure time 33 ms (a) and 650 ms (b); the white bar is 1 mm.

where $x = T/\tau_c$, β is the coefficient ($0 \leq \beta \leq 1$) determined by the ratio of speckle size and pixel size at the detector as well as by polarization and coherence properties of incident light. Furthermore, the contributions from statically scattered light and noise of sources have been added:[23]

$$K = \left\{ \beta\rho^2 \frac{\exp(-2x) - 1 + 2x}{2x^2} \right.$$
$$\left. + 4\beta\rho(1-\rho) \frac{\exp(-x) - 1 + x}{x^2} + \nu_{noise} \right\}^{1/2}. \quad (4)$$

Here, ρ is the fraction of light scattered dynamically on the particles flowing in the medium, ν_{noise} is the spatial variance defined by camera noise, short noise, etc.

The sensitivity of LSI depends on the exposure time and speckle correlation time and can be estimated as a ratio of the relative speckle contrast change to the relative flow velocity change:[24]

$$S = \tau_c \frac{x}{2K} \left[\frac{1}{2x^2} - \frac{(2x+1)}{2x^2} \exp(-2x) \right]. \quad (5)$$

Figure 3 presents a plot of LSI sensitivity Eq. (5) as a function of $1/\tau_c$ counted for a long detector exposure time used in the experiment. The results show that higher sensitivity is achieved at higher exposure time (over 200 ms) for longer τ_c, corresponding to the slow motion of scattering particles, i.e., presumably for blood flow in capillaries or lymph flow.

To confirm the functionality of the "white vessels," whether they are blood or lymph, we apply the FIM mode, which is a part of our dual-mode imaging system developed earlier.[8,13,14] In the FIM mode the mercury short arch lamp is used as a light source. The excitation light, adjusted by optical filter at 460 to 490 nm, is directed to the same area of the mouse ear via a diachronic mirror. The fluorescence light that passed through the emission bandpass filter at 510 to 550 nm is detected by the same CCD camera (see Fig. 1). The imaging of lymph vessels of the mouse ear in FIM mode is assisted by intra-dermal injection of 2 μl of Dextran-FITC (0.5 M 10 mg/ml).

The image of lymphatic vessels of the mouse external ear, obtained *in vivo* using FIM mode is presented in Fig. 4(a). In Fig. 4(b) the LSI image superimposed with a mask of lymph vessels derived from the FIM image.

As one can see, the white vessels, observed by LSI [see Fig. 2(b)], and the pattern of lymphatic vessels, derived from FIM mode, are matching each other [see Fig. 4(b)], but are not identical. This can be explained by the fact that FIM imaging of lymphatic vessels [see Fig. 4(a)] corresponds to the contrast
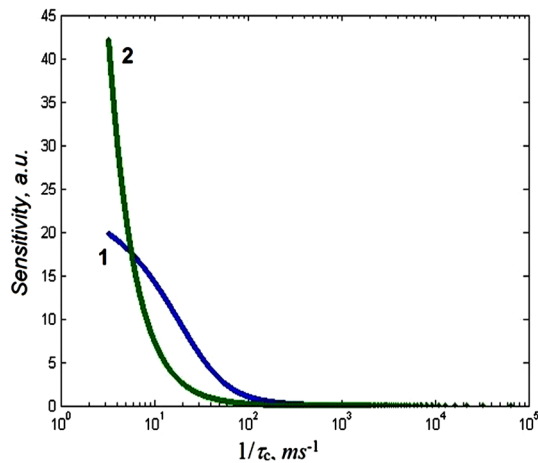
Fig. 3 Sensitivity of LSI as a function of inverse correlation time τ_c counted by Eq. (5) for the speckle contrast Eq. (4) at the fixed exposure time T: 33 ms (1) and 650 ms (2). Fraction of dynamically scattered light is taken 50%.
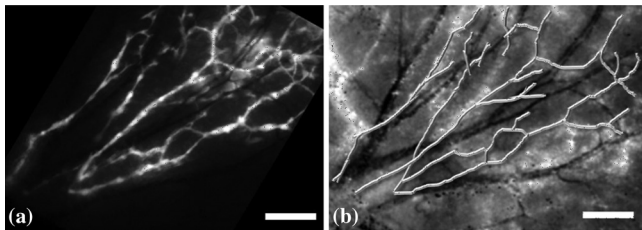
Fig. 4 Image of lymphatic vessels performed by FIM mode (a), and long-exposure LSI image [see Fig. 2(b)] superimposed with the FIM pattern of lymphatic vessels (b). White bar is 1 mm.

agent distribution within the vessels, which is limited due to complex inhomogeneous pulsatile character of lymph flow, capillaries distribution, and flow direction. Therefore, the pattern of lymphatic vessels images, obtained by FIM, is seeing irregularly with the presence and absence of a contrast agent.

To sum up, we demonstrated that a long-exposure time LSI imaging approach allows visualizing not only the blood vessels but also lymphatic vessels of the mouse ear *in vivo*. We conclude that LSI method allows rendering lymphatic vessels (about 50 to 150 μm in diameter) with the pulsatile flow inside the corresponding values of correlation times from 200 ms. Thus the long-exposure LSI imaging approach can be used to define and demarcate lymphatic and blood vessels. Along with the ability to obtain free-label images of vascular bed, the current technique could be a promising method for the noninvasive physiological studies of blood and lymph vessels *in vivo*. The current approach will be extremely useful in a number of biological studies where the mouse ear model is typically employed, including lymphangiogenesis during wound healing, quantitative lymphatic vessels, and tumor progression, evaluation of the efficacy of molecular treatment strategies for lymphatic vascular dysfunctions and many others applications. The long-exposure time LSI can be also a step forward in studying the lymphatic system on longitudinal study when the use of contrast material is highly restricted or limited.

Acknowledgments

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