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Multimodal imaging of vascular network and blood microcirculation by optical diagnostic techniques

Y.L. Kuznetsov, V.V. Kalchenko, I.V. Meglinski

Abstract. **We present a multimodal optical diagnostic approach for simultaneous non-invasive *in vivo* imaging of blood and lymphatic microvessels, utilising a combined use of fluorescence intravital microscopy and a method of dynamic light scattering. This approach makes it possible to renounce the use of fluorescent markers for visualisation of blood vessels and, therefore, significantly (tenfold) reduce the toxicity of the technique and minimise side effects caused by the use of contrast fluorescent markers. We demonstrate that along with the ability to obtain images of lymph and blood microvessels with a high spatial resolution, current multimodal approach allows one to observe in real time permeability of blood vessels. This technique appears to be promising in physiology studies of blood vessels, and especially in the study of peripheral cardiovascular system *in vivo*.**

Keywords: *fluorescence intravital microscopy, dynamic light scattering, lymphatic and blood microvessels, microcirculation, vascular bed, multimodal approach.*

1. Introduction

Last years, due to the intense development of optical technologies, there is a steady trend towards an intensive use of laser and optoelectronic devices in biomedical diagnostics to visualise the internal structure of biological objects [1 – 3]. The examples of successfully solved diagnostic tasks are image reconstruction of the internal structure of the objects (optical tomography), non-invasive determination of various physiological parameters, including changes in the concentration of oxygen, glucose, various chromophores, etc. [3]. Special place in optical diagnostics takes the development of non-invasive imaging modalities and the methods of quantitative evaluation of microcirculation blood flow and/or lymph flow *in vivo*. Microcirculation is a general concept that combines a number of phenomena occurring in small peripheral vessels of blood and lymphatic vascular systems, as well as in the intercellular clefts within the tissues [4 – 11]. The group of blood vessels securing microcirculation is called the microcirculatory vascular bed, and includes capillaries, venules, arterioles, arteriolo-venulyarnye anastomoses and lymphatic capillaries [4 – 11]. The main function of microcirculation is to transport blood cells, nutrients and waste products of cells to tissues and from tissues. Besides, the microcirculation is involved in thermoregulation, the formation of colour and density of tissues.

Abnormalities in blood microcirculation and in lymph flow are often caused due to pathological processes associated with various diseases, including diabetes [12], atherosclerosis [13], varicose veins [14], anemia [15], coronary heart disease [16], etc.

Nowadays there are a number of optical and nonoptical imaging modalities that are used to visualise anatomy of cardiovascular system and to monitor functionality of blood and lymphatic vessels, e.g. magnetic resonance imaging [16], positron emission tomography [17], ultrasound [18], computer capillaroscopy [19], confocal microscopy [20], optical coherence tomography [21], optical Doppler tomography [22], laser Doppler spectroscopy [23], laser speckle contrast imaging [24], orthogonal polarisation spectral imaging [25], dynamic light scattering [26], diffusion-wave spectroscopy [27], and diffusing laser Doppler flowmetry [28]. It should be pointed out that the major drawback of the techniques mentioned above is the incapability of simultaneous monitoring of blood and lymphatic microvessels *in vivo*.

In this paper we present an effort of combined use of fluorescence intravital microscopy (FIM) and dynamic light scattering (DLS) for simultaneous visualisation of blood and lymphatic vessels, blood and lymph microcirculation, as well as to reduce the overall toxicity of the FIM approach.

2. Fluorescence intravital microscopy

The first application of intravital microscopy dedicated to microcirculation study goes back to 1846, when Waller studied the passage of red blood cells through the frog tongue microvessels [29]. Further developments of the technique revealed various improvements, such as the use of implanted transparent chambers that allow long-term monitoring of microcirculation during wound healing [30], epithelial tumour implantation [31], tumour progressive growth [32, 33], etc. Besides, various fluorescence agents (markers) injected into the body (biological tissue)

Y.L. Kuznetsov, V.V. Kalchenko Department of Veterinary Resources, Weizmann Institute of Science, Rehovot, 76100, Israel;
e-mail: a.kalchenko@weizmann.ac.il;
I.V. Meglinski. Department of Physics, University of Otago, Dunedin, 9054, New Zealand; e-mail: igor@physics.otago.ac.nz

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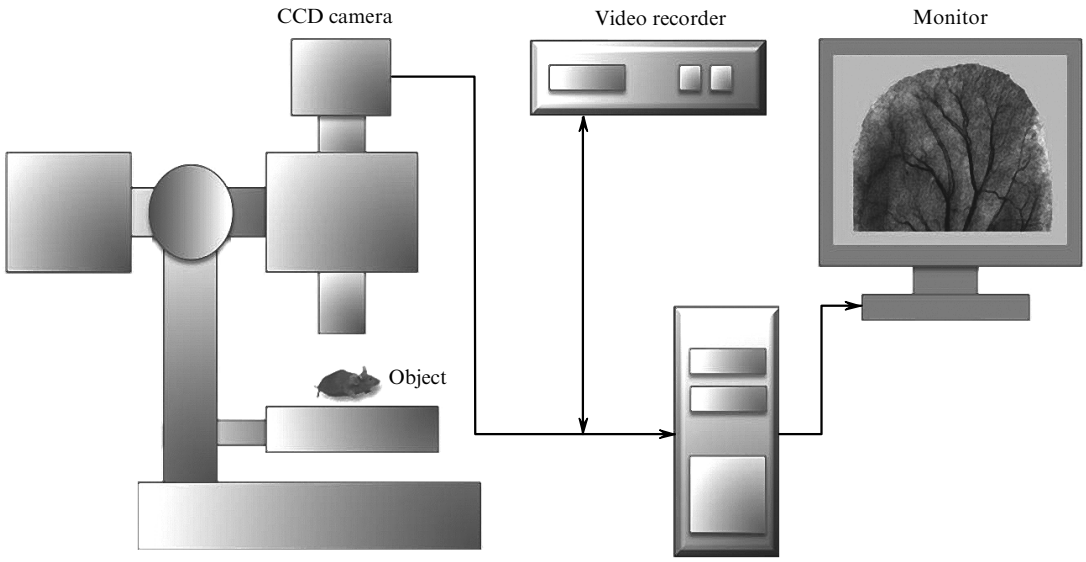

Figure 1. Experimental setup for FIM modality.

has been actively used to excite fluorescent of the marker in response to the external light exposure*. Since this time the method has been known as fluorescence intravital microscopy [34, 35]. Due to the ability of markers to bind specific molecules the visualisation of fluorescent light allows identifying the spatial localisation of fluorophores within the biological tissues *in vivo*. Thus, the technique provides a possibility to visualise separate microvessels with a spatial resolution up to 3–5 μm. Fluorescence intravital microscopy is widely used in the study of resorption and excretory functions of various organs, allowing to receive valuable information about the physical and chemical conditions and physiological processes occurring in living cells [36]. With the development of atraumatic microsurgical technologies, FIM is becoming widely used in biomedical research, including imaging of blood microcirculation in human skin, but also other living organs, including liver [37], kidney [38], pancreas [39], lung [40], heart [41], brain [42], lymph nodes [43] and others.

It should be pointed out that classic optical microscopy used in various studies in biology and medicine usually deals with an individual cell, cell culture or histological sections *in vitro*. Herewith, the used biological samples are subjected to special chemical or thermal treatment, including so-called histological staining, resulting in total termination of all the living processes. Upon that, even working with the living cell objects the technique, typically, applies to the individual cells, rather than to a multi-cellular organism.

In contrast, FIM and its various modifications are used for monitoring the physiological processes *in vivo*, i.e. in the living cell objects or, even, in the living multi-cellular organism with no termination of its life-sustaining activity, noninvasively. Therein, the examined biological object is not subject to preliminary histological and/or histochemical treatment.

Therefore, the significant advantage of FIM is the ability to obtain images of the internal structure of biological tissues in real time with high spatial and temporal resolution that are particularly required in the studies of anatomy and physiology of blood and lymph vessels, especially in context of their vascular systems relation.

Figure 1 shows the FIM experimental setup, where the fluorescence is used as an approach of marking (contrasting) tissues microstructure. The scheme includes a microscope, adapted for study of live objects; a device for image recording – a CCD camera, the signal from which goes to the video recorder (storage), and a personal computer used for data processing and an output of the images on the screen of the computer monitor. The arsenal of FIM's visualisation includes a variety of imaging techniques, ranging from standard fluorescent microscopy, when for the excitation of fluorescence an external light source (mercury, xenon, at least – a halogen lamp) is used, to the confocal or multi-photon microscopy that allows one to observe the tissue structure with high quality [44]. As was mentioned above, in practice the optical imaging of blood and lymphatic capillaries typically is carried out by injection of a high molecular weight fluorescent contrast agent with a basis on albumin and/or dextran. The circulation time of such markers in the vascular bed is quite long (up to several hours) that allows to identify the area of extravasation of dye and the area of obstruction with high accuracy [45].

3. Dynamic light scattering

The development of the DLS technique with respect to the diagnostics of optically inhomogeneous dynamic scattering media bagan at the end of the 1960s [46, 47]. The technique is based on the statistical analysis of the intensity of laser radiation scattered by moving particles [48]. Due to the relatively low velocity of scattering particles, the Doppler shift is sufficiently small in comparison with the eigenfrequency of incident light. The DLS method is also called the method of quasi-elastic light scattering [49], optical mixing spectroscopy and photon correlation [47], light beating spectroscopy [50], laser correlation spectroscopy [51], laser heterodyning [52]. All of the above names reflect some methodological and technical details, but in fact correspond

*This flouorescent agent or marker is a fluorophore, i.e., a fluorescent substance (dye) which can absorb radiation at a given wavelength and re-emit light at a longer wavelength.

to the same type of experiment, namely the DLS, when the fluctuations of intensity of optical radiation scattered with a medium are analysed.

The basis of the DLS technique is that the incident laser light (the optical frequency $\sim 5 \times 10^{14}$ Hz) is scattered in the medium. As a result, due to the scattering of light by various macro- and micro-inhomogeneities localised within the medium on the surface of photodetector a complex interference of waves arises. Therefore, a stationary interference picture (so-called speckle pattern) is observed [53]. When the scattering particles and/or inhomogeneities are moved, the interference pattern changes – the intensity of radiation at each point of photodetector fluctuates [51]. In other words, during the process of scattering a new wave appears. The central frequency of this wave is the same as that of the incident wave, but the amplitude and phase modulated synchronously with the fluctuations of the dielectric constant of the medium. To extract the information contained in the modulation, it is necessary to demodulate the scattered light and obtain the power spectral density of intensity modulation.

To analyse the time dependence of the intensity fluctuations of scattered radiation caused by the motion of scattering particles it is necessary to analyse the spectrum of scattered light. Thus, in the DLS methods the measured quantity is the power spectrum of photocurrent fluctuations $S(\omega)$, or temporal intensity autocorrelation function of scattered radiation $G_2(\tau)$. According to the Wiener–Khintchine theorem these two quantities are related by the Fourier transform [47, 50] and provide absolutely same information about the medium, but for their measurements different experimental approaches are used [1, 51–56].

Presently, there are a number of publications describing various aspects of the DLS approach and its application for noninvasive measurements of blood flow and blood microcirculation *in vivo* [1, 27, 57–60]. Another example of using the intensity fluctuations of radiation scattered by the environment for visualisation of blood flow in tissues is laser speckle contrast imaging [26]. The basis of this technique is consistent registration with the camcorder the dynamics of speckles that is directly related to the bloodstream. The measured quantity is the contrast of speckles, which characterises the depth of spatial modulation of the scattered speckle fields [26] and defined as the ratio of the standard deviation of the intensity fluctuations δ, measured by changing realisations of the scattering object, and the averaged intensity $\langle I \rangle$. Using a special developed computer Camware program (Germany) the images obtained by this technique are converted to the speckles distribution averaged over time (usually in the range 5–50 ms).

4. Materials and methods

The design of experimental multimodal imaging system combining the simultaneous use of FIM and DLS techniques, and applied for visualisation of blood microvascular bed and lymphatic vascular system is schematically presented in Fig. 2.

The setup is based on the fluorescent SZX 12 RFL2 stereomicroscope (Olympus, Japan), equipped with a high-sensitivity monochrome CCD camera (PIXELFLY QE, PCO, Germany) with a 12-bit dynamic range. The system includes two light sources applied for object illumination: a

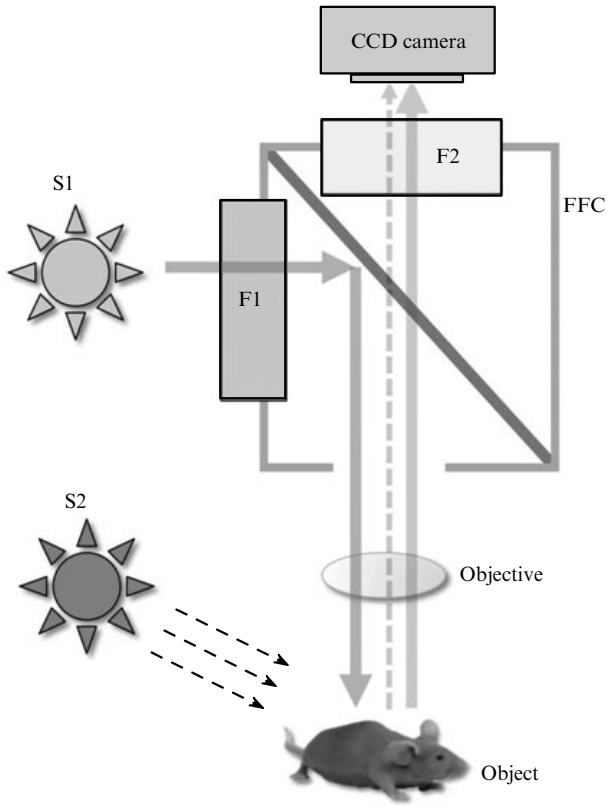

Figure 2. Setup of combined use of FIM and DLS techniques: (S1) mercury gas-discharge lamp, and (FFC) fluorescence filter cube (is a part of standard fluorescent lighter of stereomicroscope) containing filters F1 and F2; (S2) laser source.

50-W mercury discharge lamp (part of a standard fluorescent microscope illuminator) and a 670-nm, 10-mW laser (ELFI-C, Israel).

The external ear of a 'naked' mouse has been used as an object of investigation of lymphatic and blood microvascular circulatory systems [26]. The surface of the external mouse ear has been illuminated in two successive regimes, i.e. with FIM using the fluorescence filter cube (fluorescence is excited by a light source in the range of wavelengths 460–490 nm, the registration of emission fluorescence light is carried out through filter F2 in the range of wavelengths 510–550 nm). In the DLS regime the illumination of the sample is performed using the laser source S2. For the both regimes the images are obtained by using the same optical setup of the microscope. The fluorescence filter cube is used only in the FIM regime, and it is automatically switched off when the DLS regime is activated. This allows the laser light to unreservedly enter the matrix of a CCD camera, which is controlled by a special Camware software, PCO (Germany). The camera exposure time in both regimes is 50 ms. ImageJ NIH (US) software is used to analyse and process images.

We use the Dextran-FITC 500.000 Sigma (Germany) marker to implement the FIM regime. The mechanism of fluorophore injection for visualisation of lymphatic and blood vascular systems is schematically presented in Fig. 3. By conventional hydrodynamic injection of Dextran-FITC the fluorophore is delivered through the tail vein of mice to visualise blood vessels (see Fig. 3) in an amount of 30 mg kg^{-1} body weight. Thus, one-two seconds later the

fluorescent marker goes to the arteries and arterioles of external mouse ear. Colouring of lymphatic vessels is carried out by intradermal microinjection of a fluorophore directly into external mouse ear; as a result, a fluorescent contrast agent enters the lymphatic system through the capillary network.

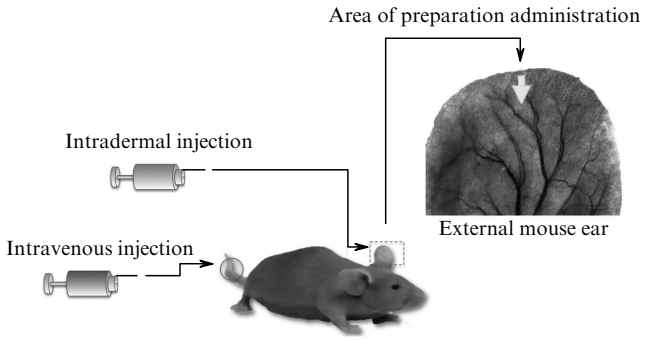

Figure 3. Schematic presentation of mechanisms of contrast material injections.

All the experiments have been performed in accordance with the international standards and approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

5. Results and discussion

The image of a microvascular bed of the blood circulatory system obtained by FIM is shown in Fig. 4. The blood vessels are bright (white) branched lines of different sizes and shapes.

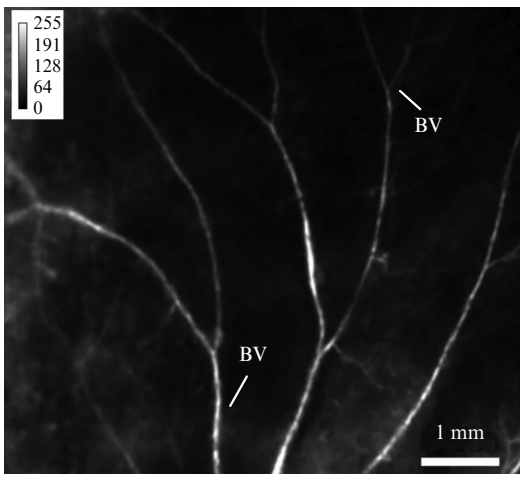

Figure 4. Monochromatic image of blood vessels (BVs) of the external mouse ear obtained by FIM.

It should be emphasised that most of the fluorescent contrast agents used to visualise blood vessels by FIM are extremely toxic that imposes significant restrictions and limitations on the practical application of this method. Presently, an accurate systematic assessment of the toxicity effects of fluorescent dyes on the animal during and/or immediately after irradiation of biological tissues does not exist. In the scientific literature the toxic effects associated directly with a fluorescent dye or with its conjugate, as well as the toxic effects caused by the formation in the body cells of the photolysis products are typically considered [61, 62]. The phototoxicity is associated with the violations of leukocyte-endothelial interactions that occur with fluorescently labelled leukocytes [63]. Negative reactions on the fluorescein injection are observed in 5 % of cases, resulting in violations of the cardiovascular system and gastrointestinal tract, in formation of thrombophlebitis at the injection area and allergic reactions. There are known cases of cardiac arrest and anaphylaxis with fatal outcome after intravenous injection of fluorescent markers [64]. Thus, the safe use of FIM requires a significant decrease in the concentration of fluorescent markers used in experiments *in vivo*, and a more detailed clinical and laboratory tests.

Figure 5a presents the image of raw speckle patterns obtained by illuminating the external mouse ear with a laser diode. Figure 5b shows the same section of the mouse external ear applying image processing. Lower contrast values correspond to the higher velocity of red blood cells in blood vessels. Comparing the images obtained separately by FIM and DLS (see Figs 4 and 5b) we can see that both techniques provide equivalent images of the same vascular bed (Fig. 6).

Figure 6a shows a monochrome image of the external mouse ear, obtained by FIM *in vivo*. Figure 6b demonstrates the image obtained by DLS for the same area of the mouse

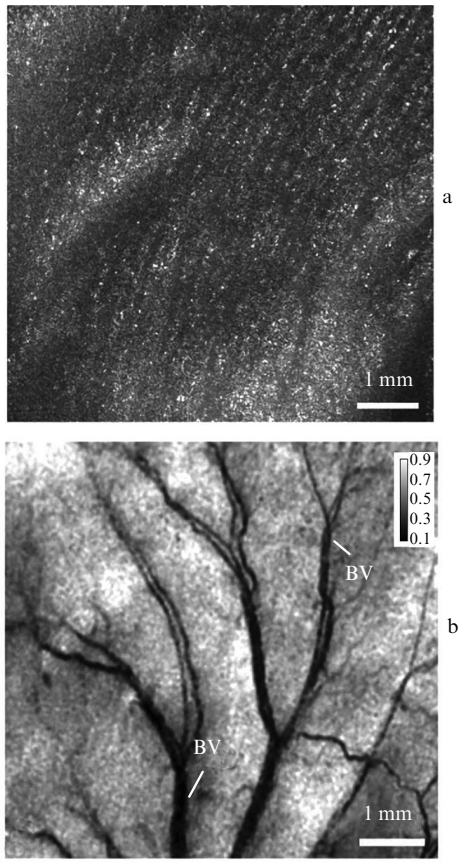

Figure 5. Image of the external mouse ear obtained by DLS: (a) raw speckle image; (b) blood vessels appearing after image processing.

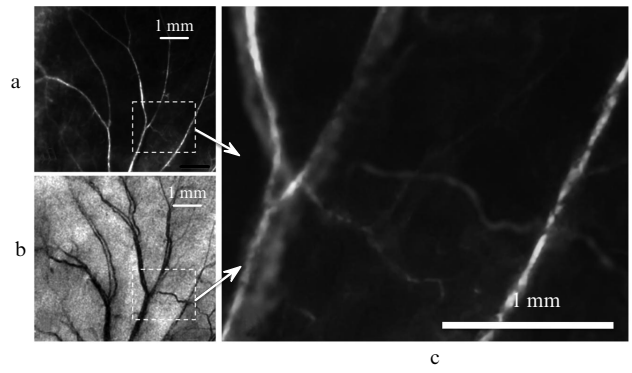

Figure 6. Image of blood vessels obtained by FIM (a) and DLS (b) at the same area of the external mouse ear; (c) combined image obtained by superimposing images (a) and (b).

ear. Dashed lines indicate the areas being framed. The image presented in Fig. 6c is the superimposition of images obtained by FIM and DLS. Comparing the images it is clearly seen that both DLS and FIM techniques allows one to observe equally the same group of blood vessels.

The results of the combined simultaneous use of FIM and DLS techniques for visualisation of blood and lymph vessels are shown in Fig. 7. The inverted monochrome image of the mouse external ear (see Fig. 7a) is obtained by DLS. Blood vessels are clearly distinguishable and represent areas of increased brightness. The monochrome image of the external mouse ear (see Fig. 7b) is obtained in the FIM regime after intradermal injection of a microdose of fluorescent marker. Injected intradermally the fluorescent agent comes exclusively in the lymphatic vessels; as a result, these vessels are clearly visible due to fluorescence (areas with high brightness).

Figure 7c shows the image of blood and lymph vessels, obtained by superimposing images presented in Figs 7a and b. One can see that the combined application of DLS and FIM techniques allows one to observe simultaneously the capillaries of circulatory and lymphatic systems. Herewith, it is possible significantly reduce the use of fluorescent markers, and, consequently, tenfold reduce the toxicity and minimise side effects caused by the use of fluorescent contrast agents.

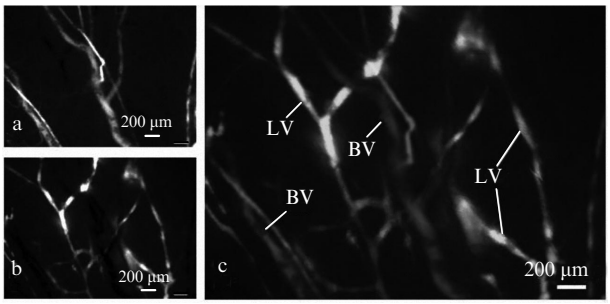

Figure 7. Image of blood (a) and lymphatic (b) vessels (LVs) of the external mouse ear, obtained simultaneously by DLS and FIM at the same area (c).

6. Conclusion

In the current report we have demonstrated the ability of simultaneous use of DLS and FIM in physiology studies of blood vessels. Both techniques are well known and actively applied in various biomedical applications, but their mutual use in vascular biology has not been implemented to date. The presented multimodal imaging approach allows one to integrate the strengths and capabilities of each imaging modality together and diminish their limitations that provides new advanced opportunities in the study of the peripheral cardiovascular system. This multimodal imaging approach seems to be particularly promising and useful for the visualisation of the vascular bed, blood and lymph flow *in vivo*, as well as for the studies of permeability of blood vessels, allowing the leakage of the contrast agent to be monitored beyond the vessels in real-time. In addition, the simultaneous use of FIM and DLS modalities allows one to renounce the use of fluorescent markers for visualisation of blood vessels and, therefore, significantly (tenfold) reduce the toxicity of the technique and minimise side effects caused by the use of contrast fluorescent agents.

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