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Combined laser speckle imaging and fluorescent intravital microscopy for monitoring acute vascular permeability reaction

Vyacheslav Kalchenko,[a,*] Igor Meglinski,[b,c,d] Anton Sdobnov,[b] Yuri Kuznetsov,[a] and Alon Harmelin[a]

[a]Weizmann Institute of Science, Department of Veterinary Resources, Rehovot, Israel
[b]University of Oulu, Optoelectronics and Measurement Techniques Laboratory, Oulu, Finland
[c]National Research Nuclear University MEPhI, Institute of Engineering Physics for Biomedicine (PhysBio), Moscow, Russia
[d]National Research Tomsk State University, Interdisciplinary Laboratory of Biophotonics, Tomsk, Russia

Abstract. Optical clearing agents (OCAs) and many chemicals are widely used in functional diagnosis of skin tissues. Numerous studies are associated with the transcutaneous diffusion of OCA in epidermal, dermal, and hypodermal tissues, which results in changing their optical properties. In addition, an objective approach that is suitable for screening the influence of utilized OCA, as well as various chemical agents, synthetics, and nanomaterials, on blood and lymph flows is highly desirable. In our study, a highly sensitive laser speckle imaging (LSI) system and fluorescent intravital microscopy (FIM) were used team-wise to inspect the acute skin vascular permeability reaction in mouse ear during the local application of OCA on the skin surface. Fluorescent contrast material administrated intravenously was used for quantitatively assessing the intensity of vascular permeability reaction and the strength of skin irritation. The obtained results suggest that a combined use of LSI and FIM is highly effective for monitoring the cutaneous vascular permeability reaction, with great potential for assessment of allergic reactions of skin in response to interactions with chemical substances. [DOI: 10.1117/1.JBO.24.6.060501]

Keywords: acute vascular reaction; skin; optical clearing; laser speckle imaging; fluorescence; vascular permeability; contact irritant; allergens.

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Many optical methods and advanced imaging modalities, such as laser speckle imaging (LSI),[1] optical coherence tomography (OCT),[2] confocal Raman microscopy,[3,4] and multiphoton tomography,[5] are successfully implemented for noninvasive diagnosis and imaging of skin. However, a strong scattering of light in the "stratum corneum" and living epidermis dramatically reduces the spatial resolution for the above-mentioned techniques. To deal with this limitation, to increase the depth of imaging and/or to obtain more enhanced image contrast, the so-called optical clearing technique has been implemented.[6] Various optical clearing agents (OCAs), including water solutions of glucose and glycerol, trazograph, polyethylene glycol, and iohexol, have been extensively used in biomedical applications.[6] Diffusing from the surface into the skin, topically applied OCA temporarily substitutes for the interstitial fluids in tissue. Due to the matching of refractive indices of skin components, skin dehydration, and reversible dissociation of collagen fibers, OCA diffusion produces a significant reduction of light scattering that increases the probing depth[7,8] and significantly improves the image contrast.[9,10] The impact of various chemical substances and their components on the epidermal, dermal, and hypodermal tissues results in changes of their properties. Many cosmetic and healthcare products contain OCA-based compounds, including glycerol, dimethyl sulfoxide (DMSO), and iohexol. In this regard, numerous studies associated with the quantitative assessment of skin optics utilize the evaluation of transcutaneous diffusion of OCA.[3–10] In fact, an objective approach suitable for screening the influence of these reagents on blood and lymph flows is required. This is also highly desirable for further developments of transcutaneous drug delivery, as well as for assessment of optimal applications of medical preparations.

Double correlation OCT approach has been successfully used for assessing vaccine delivery and molecular diffusion into the skin.[11,12] However, this approach is limited to assessing the influence of blood flow on OCA concentrations and spatial variations in reticular dermis. LSI is a dynamic light scattering-based technique.[13] Recently, we introduced an effective highly sensitive LSI system, capable of detecting very small (down to 1 to 5 μm/s, and postmortem) variations in blood microcirculation in tissues.[14] Combined with fluorescent intravital microscopy (FIM), this LSI system was successfully used for visualization of skin vascular network,[14] imaging of tumor surroundings,[15] evaluation of cerebral blood flow,[16] and observation of blood and lymph microflows.[17–19] It was also demonstrated that such a dual-mode LSI–FIM imaging approach enables *in vivo* visualization and quantitative assessment of vascular permeability reaction in an immediate response to chemical agents and potential allergens' interaction with the skin.[20] LSI–FIM approach has been extensively used in the routine mouse ear swelling test screenings.[21]

In this letter, we report the results of LSI–FIM application for the monitoring of acute skin vascular permeability reaction in the mouse ear during topical administration of OCA on the skin surface. In our study, the fluorescent contrast agent was injected intravenously and possibly can be used as an inflammation marker.[22] The acute vascular permeability reaction was determined by the relative changes in the fluorescence intensity measured with the FIM. The LSI and FIM are applied simultaneously for the visualization of vascular permeability reaction in response to three different OCAs.

The developed in-house dual-mode LSI–FIM imaging system, specially designed to obtain the images of the same area of the skin surface with both LSI and FIM modes, is schematically presented in Fig. 1. A high-grade CCD camera was used to acquire laser speckle pattern at the exposure time in 45 ms.

*Address all correspondence to Vyacheslav Kalchenko, E-mail: a.kalchenko@weizmann.ac.il

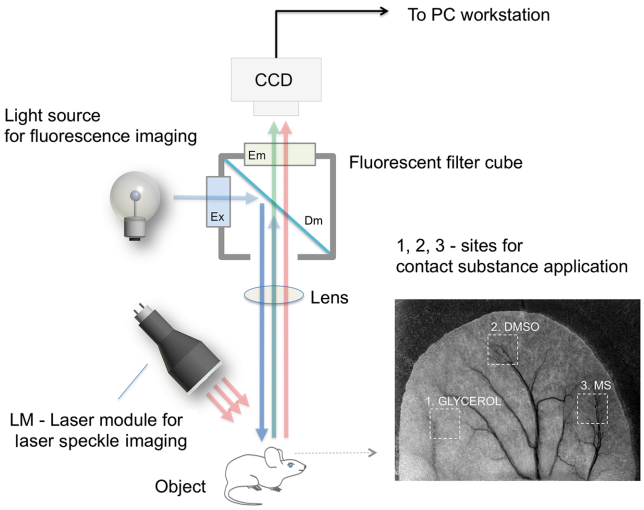

Fig. 1 Dual-mode LSI/FIM imaging system. LSI utilizes a diode laser module (LDM808/3LJ, 808 nm, 3 mW, Roithner Lasertechnik, Austria). The laser light passes through a ground glass diffuser (Thorlabs, Newton, New Jersey) and illuminates the mouse ear. The laser speckles, produced by diffusively reflected laser light, are registered by monochrome CCD camera (Pixelfly QE, PCO, Germany; 1392×1024, pixel size—6.4 μm) mounted by C-mount adaptor on top of the standard fluorescent zoom stereomicroscope SZX12 RFL2 (Olympus, Japan) used in the FIM mode. Mercury discharge lamp is utilized as a light source for fluorescence imaging. Light from the light source passes through the excitation optical filter (Ex) and is projected by dichroic mirror (Dm) onto the same area of mouse ear as visualized by LSI. The fluorescence signal filtered by the emission bandpass filter (Em) is detected by CCD. CCD is connected to a PC-based workstation used for LSI and FIM image processing. The areas of topical application of glycerol, DMSO, and MS are marked by white squares. Scale bar is equal to 1 mm.

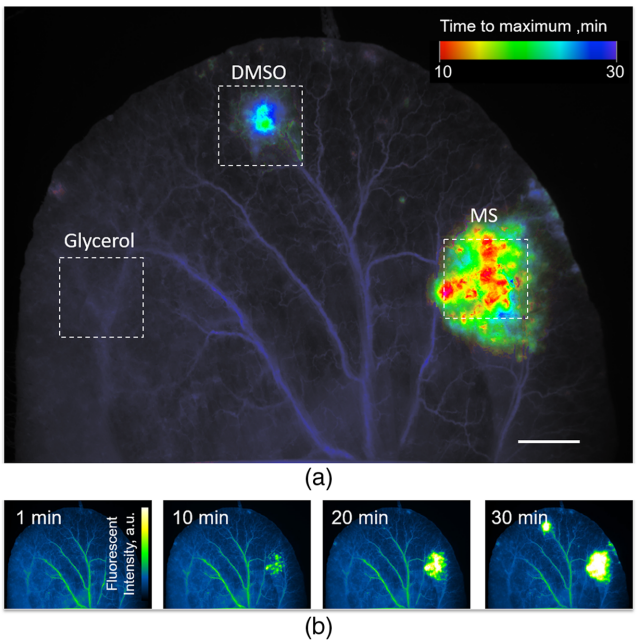

Fig. 2 (a) Fluorescent pseudocolor images of the external mouse ear *in vivo*. Squared dashed line highlights the places of the contact substance applications: 1—glycerol, 2—DMSO, and 3—MS. Fluorescence intensity value has been measured within each squared dashed line every minute. Color-coded bar represents time to maximum intensity (in minutes). (b) Sequence of fluorescent pseudocolor images of the same external mouse ear *in vivo* at a different time points (1, 10, 20, and 30 min) after the chemical agent application. Color bar shows fluorescence intensity of FITC fluorescence. Scale bar is equal to 1 mm.

The camera control and image acquisition were performed by utilizing CamWare (PCO, Germany). A special macrocode for Fiji/ImageJ (image processing package)[23] was used for the image processing and analysis of acquired image sequences, 400 frames. In the FIM mode, a mercury short arch lamp was used as a light source. The excitation light was adjusted by optical filter at 460 to 490 nm and 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 was detected by the same CCD camera. To avoid the photobleaching effect, 1 FIM frame was captured each minute from the beginning of the experiment. The LSI imaging mode was mainly used for mapping spatial distribution of blood vessels for selection of the most appropriate areas for OCA application, whereas, the FIM mode was utilized for visualization of acute vascular permeability reaction of the mouse ear skin in response to the selected OCA, applied simultaneously.

Five CD1 nude female mice aged 6 to 8 weeks from Envigo Laboratories were used in the experiments. Each animal was anesthetized with a mixture of ketamine (Fort Dodge, Iowa) (10 mg/kg) and xylazine (Kepro, Deventer, Holland) (100 mg/kg) by intraperitoneal injection and placed on a thermally controlled stage. To achieve stable images, the external ear of the mouse was gently attached (using double-sided glue tape) to the plastic platform.

Three square-sized pieces of blotting paper (1 mm^2) were placed on the surface of the mouse ear, as shown in Fig. 1 by squared dashed lines. A 2 μL of contact substances was applied on each paper patch in the following order: (1) ≥99.5% glycerol (Sigma Aldrich, Israel) (v/v), (2) ≥99.9% DMSO (Sigma Aldrich, Israel) (v/v), and (3) ≥99% methyl salicylate (MS) (Sigma Aldrich, Israel) (v/v). The vascular permeability reaction provoked by the application of OCA substances on the surface of the mouse ear skin was examined during 30 min. Glycerol and DMSO were selected as effective OCAs, which are typically used in skin studies,[6] whereas the MS was chosen as a known mild irritant.[21] For fluorescent imaging of blood vessels in FIM mode, a 50 μL of commonly used fluorescent agent dextran–fluorescein isothiocyanate (FITC) (0.5 M, 1 mg/mL) (Sigma Aldrich, Israel)[24] was injected intravenously. The excitation maximum of FITC–dextran was 490 nm. The emission maximum of FITC–dextran was 520 nm. Moreover, the high molecular weight of FITC–dextran allowed the agent to remain in blood circulation for at least 2 h.[25] Thus, no washout effect can be observed during the experiment.

The results of the experimental studies for the one of the mice are presented in Fig. 2. Temporal color coding [see Fig. 2(a)] allows for the representation of dynamic information relating to the process of fluorescent contrast material extravasation in a convenient manner. In addition to a conventional imaging, a sequence of images shown in Fig. 2(b) shows the progression of irritation.

The acute vascular permeability reaction in response to the topical application of each agent/OCA has been determined by changes in the fluorescence signal. Thus, the mean intensity value has been calculated inside the area of each agent/OCA application (indicated with squared dashed lines in Fig. 2) from each FIM frame. The quantitative results of these

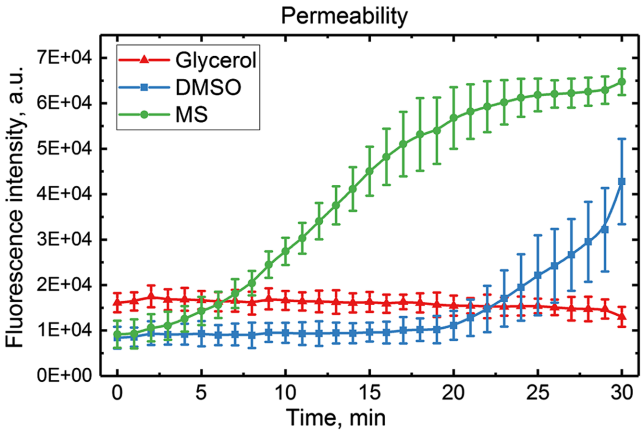

Fig. 3 Evolution of fluorescence intensity during the application of selected contact substances to the external mouse ear *in vivo*.

measurements are summarized in Fig. 3. It is clearly seen that the fastest and the most "severe" vascular permeability reaction was observed after the MS application. The fluorescence intensity started growing significantly after 2 min of the agent application. The most intense fluorescence signal was observed between 6 and 16 min after the beginning of the experiment with the succeeding saturation after 23 min. The acute vascular permeability reaction in response to the topical application of OCA/irritant resulted in an increase in vascular permeability that subsequently induced a massive plasma leakage from capillaries into nearby interstitial space, vasodilatation, and edema,[26] followed by significant blood flow reduction in small vessels, such as venules and arterioles, as well as in capillary loops.

DMSO application caused a mild vascular permeability reaction, compared to MS. Fluorescence intensity value started to increase after 20 min of agent application. Finally, the application of glycerol solution caused minimal (or even no) vascular permeability reaction, possibly due to the extreme high viscosity of pure glycerol solution (1410 cP at 20°C[5]), which has ≈705-fold difference, compared to the viscosity of DMSO and MS (1.9 cP[27] and 2.3 cP[28] at 20°C, respectively). This suggestion is in a good agreement with experimental results presented in Ref. 29, where it was shown that 80% glycerol solution has much less penetration ability than 50% DMSO solution. Thus, it could be possible that even 30-min application of pure glycerol is not sufficient to cause a significant vascular permeability reaction in skin.

To sum up, our current study mainly focuses on the development of a protocol for quantitative assessment of acute vascular permeability reaction in response to the simultaneous application of various OCAs on the skin of mouse ear *in vivo*. We utilize a combination of LSI and FIM techniques for simultaneous visualization of mouse ear vascular network, blood flows, and blood microcirculation in real time with a high dynamic range, and quantitative assessment of the intensity of vascular permeability reaction and strength of skin irritation. The obtained results suggest that such a combination of LSI and FIM is extremely useful for monitoring the cutaneous vascular permeability reaction induced by OCA and have great potential for express screenings of allergic reactions of skin in response to the application of new medical and pharmaceutical products. The results demonstrate a proof of concept for routine use of LSI for screening of skin vascular response induced by known or unknown irritants or allergens. The proposed methodology still requires further development to become a real clinical laboratory tool for noninvasive assessment of pharmaceutical products in terms of their potential skin allergic liability.

Disclosure

The authors have no financial interests in the manuscript and do not have any other potential conflicts of interest to disclose.

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