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Vyacheslav Kalchenko
Yuri Kuznetsov
Dina Preise
Igor Meglinski
Alon Harmelin

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Vyacheslav Kalchenko,[a,*] Yuri Kuznetsov,[a] Dina Preise,[b] Igor Meglinski,[c] and Alon Harmelin[a,*]
[a]Weizmann Institute of Science, Department of Veterinary Resources, Rehovot 76100, Israel
[b]Weizmann Institute of Science, Department of Plant Sciences, Rehovot 76100, Israel
[c]University of Otago, Department of Physics, PO Box 56, Dunedin 9054, New Zealand

Abstract. Laser speckle imaging with long exposure time has been applied noninvasively to visualize the immediate reaction of cutaneous vessels in mice in response to a known primary irritant and potential allergen—methyl salicylate. The compound has been used topically on the surface of the pinna and the reaction of the vascular network was examined. We demonstrate that irritant-induced acute vascular reaction can be effectively and accurately detected by laser speckle imaging technique. The current approach holds a great promise for application in routine screening of the cutaneous vascular response induced by contact agents, screenings of mouse ear swelling test, and testing the allergenic potential of new synthetic materials and healthcare pharmaceutical products.
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Keywords: laser speckle imaging; long exposure time; vascular network; mouse ear swelling test; contact irritant; allergens.

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With the recent advances in biomedical technologies and extensive implementation of new drugs, the need for accurate evaluation and prediction of possible immune reactions, such as irritations and allergies, is clearly recognized and ultimately required.[1] Current practices of identification of the contact allergens rely on a panel of conventional tests, such as the mouse ear swelling test (MEST),[2] guinea pig maximization test,[3] or local lymph node assay.[4] Various modifications of noninvasive ear swelling assays are used in rodents in order to study both primary irritants and delayed-contact hypersensitivity reactions to sensitizers. MEST arguably is the most popular of the noninvasive allergy tests and it is widely used in various studies associated with the development of new antiallergic drugs.[2,5] During MEST, a material with a potential propensity of allergy induction is repeatedly applied on the skin of the mouse ear to locally stimulate an immune reaction. After the rest period, the challenge is done by applying it in a maximum nonirritating dose on a mouse ear. The reaction of skin is identified as a swelling that is often observed as a change of thickness of the mouse ear in response to contact with the allergen. The presence and level of swelling in the place of application are associated with the sensitization potency or irritation of the applied material. In a similar way, the allergic reaction of skin (if any) induced by the application of new healthcare pharmaceutical products can be assessed. In fact, the routine implementation of this test in day-to-day clinical practice is struggling due to strong limitations, specifically (1) reliability in the detection of weak or moderate sensitizers or their low concentration and (2) uncertainty in the quantitative assessment of the degree of allergic reaction quantitatively.[5]

Recently we reported the development of a highly sensitive laser speckle imaging (LSI) system capable of detecting very small (down to 1 to 5 μm/s, and postmortem) variations in blood microcirculation in tissues.[6] The developed LSI technique combined with the fluorescent intravital microscopy (FIM) has been extensively used for visualization of skin vascular network[7] and tumor surroundings,[8] as well as for observation of blood and lymph microflow *in vivo*.[9,10]

In the current letter, we report the application of the developed LSI system for visualization and feasibility of quantitative assessment of an acute vascular reaction in immediate response to topical application (without prior sensitization) of a known mild irritant. The latter is recognized as the most challenging for identification by currently available diagnostic practices.

The developed LSI is a part of the dual-mode imaging system schematically presented in Fig. 1. The dual-mode imaging system utilizes the LSI and FIM modes, which provide an access for simultaneous imaging of the same area of external mouse ear skin surface *in vivo*.[7–10]

LSI utilizes a diode laser module (LDM808/3LJ, 808 nm, 3 mW, Roithner Lasertechnik, Vienna, Austria). The laser beam 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 a charge-coupled device (CCD) camera (Pixelfly QE, PCO, Kelheim, Germany). The high-grade CCD camera has been used to acquire a laser speckle pattern at various exposure times in a range of 33 to 650 ms. This approach allows noninvasive visualization of the mouse ear vascular network and observation of blood flow and blood microcirculation in real time with a high dynamic range. The camera control and image acquisition are performed by utilizing CamWare (PCO, Germany). A special macrocode for Fiji/ImageJ (image processing package[11]) is used for the image processing and analysis of acquired image sequences, typically 300 frames.

In the FIM mode, the mercury short arc lamp is used as a light source. The excitation light is 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 band-pass filter at 510 to 550 nm is detected by the same CCD camera (see Fig. 1). The FIM imaging mode has complementarily been used to verify and confirm the sensitivity of the LSI mode applied for the visualization of a mild reaction of the mouse ear skin vasculature in response to the applied material. Fluorescent imaging of blood vessels of the external mouse ear in FIM mode[7,8,10]

*Address all correspondence to: Vyacheslav Kalchenko, E-mail: a.kalchenko@weizmann.ac.il; Alon Harmelin, E-mail: alon.harmelin@weizmann.ac.il

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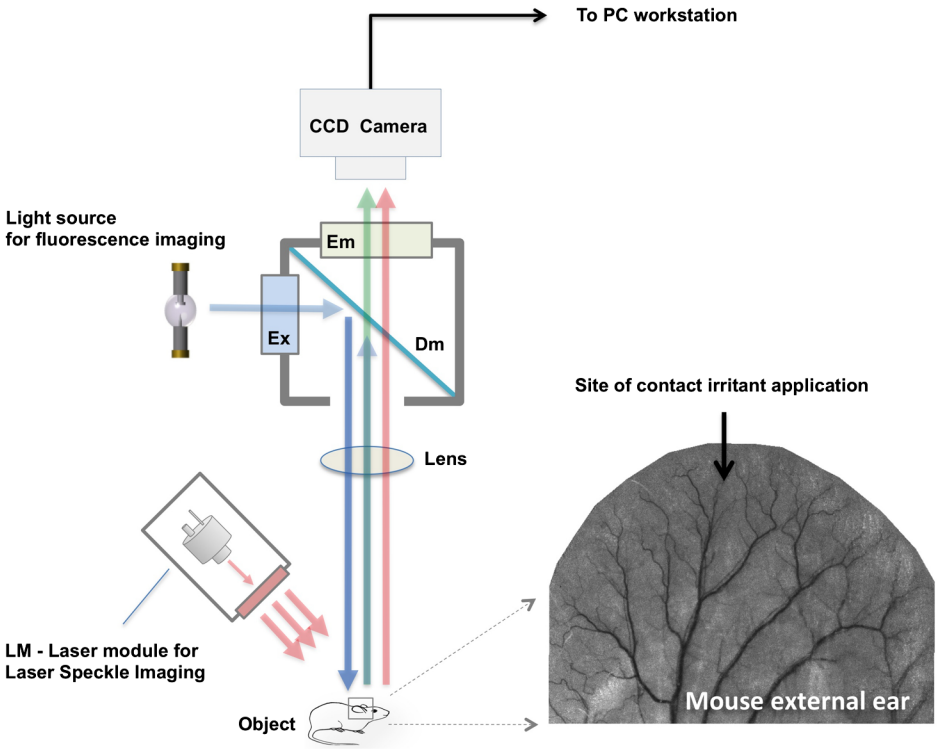

Fig. 1 Schematic presentation of the dual-mode imaging system used in the experiment. The image of external mouse ear obtained by using laser speckle imaging (LSI) mode with long (650 ms) exposure time. Site of topical application of contact irritant is marked by black arrow. In the LSI mode, the laser module with optical diffuser (LM) and digital CCD camera are used as light source and detector, respectively. CCD is mounted by C-mount adaptor on top of the standard fluorescent microscope used in the fluorescent intravital microscopy (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 band-pass filter (Em) is detected by CCD. CCD is connected to a PC-based workstation used for LSI and FIM image processing.

has been obtained by intravenous injection of 50 μl of dextran-fluorescein isothiocyanate (FITC) (0.5 M 1 mg/ml).

Five CD1 nude female mice aged six to eight weeks from Harlan Laboratories were used in the experiments. Each animal was anesthetized with 10 mg/100 mg/kg ketamine (Fort Dodge, Iowa) and xylazin (Kepro, Deventer, Holland) 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 (see Fig. 1). The vascular reaction has been provoked locally by application of methyl salicylate (MS) at a dose of 5 μl (inside the paper of 2-mm diameter) on the surface of the mouse ear skin for 30 min. The irritant was applied on an experimental group of mice, while the control group underwent an application of saline solution.

In the current study, we have mainly focused on the development of a protocol for quantitative assessment of acute vascular reaction in response to the topical application of MS on the skin of mouse ear without prior phase induction. At the early stages, an acute vascular reaction in response to the topical application of an allergen/irritant is mediated by increasing the vascular permeability that subsequently induces a massive plasma leakage from the capillaries into the nearby interstitial space, vasodilatation, and edema,[12] followed by significant blood flow reduction in small vessels, such as venules and arterioles, as well as in capillary loops.

Importantly, the increase in blood vessel permeability is not the only factor responsible for the swelling reaction. Most inflammatory processes, including allergic reactions, cause local lymphatic dysfunction, involving a slowdown of the lymph flow, lymphedema, etc.[12,13]

It has been shown earlier that slow blood flow can be effectively detected with an LSI approach.[6] It has also been demonstrated that lymph flow can be observed by an LSI approach with a long exposure time, similar to the blood flow reported in the current letter.[9,10]

Thus, the LSI approach utilizing a long exposure time ($T \sim 650$ ms) has been applied for the evaluation of the acute vascular reaction induced by MS (Fig. 2).

The FIM mode has been used for verification of the immediate vascular reaction induced by MS. For this purpose, a fluorescently labeled high molecular weight dextran was injected into the tail vein during external MS application, and the vascular permeability has been monitored in the FIM mode. The permeability was clearly observed after 30 s of FITC-dextran administration, manifested as a red- to purple-colored cloud around blood vessels [Fig. 2(d)].

To sum up, we demonstrate that LSI modality with a long exposure time is sensitive enough for the observation of an acute vascular reaction of skin induced by MS. The results demonstrate a proof of concept and the great potential of the experimental technique for routine preclinical screening of

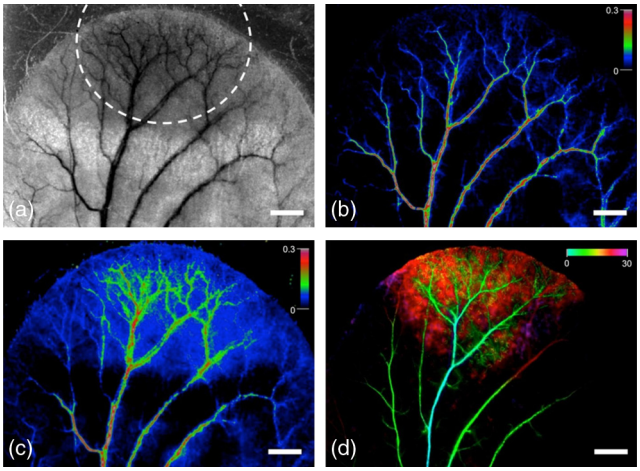

Fig. 2 The images of the external mouse ear *in vivo*. (a) The monochrome image of the area of methyl salicylate (MS) agent topical application (highlighted by the dashed line), obtained by LSI mode with the long (650 ms) exposure time. (b) and (c) show, respectively, the color-coded images before and after 30 min of MS agent application. Color bar shows speckle contrast in arbitrary units. (d) Temporal color-coded image of the same area of mouse ear observed in the FIM mode after injection of FITC-Dextran. The image shows temporal color-coded FITC contrast filling of the mouse ear vasculature and tissue during 30 s as shown in the color bar. White bar is 1 mm.

skin vascular response induced by known allergens. The proposed methodology is not yet mature and still requires further development to become a real quantitative tool for noninvasive assessment of allergic reactions. Further development of the proposed methodology might substitute for the classic MEST and concomitantly reduce animal suffering. We also anticipate translation of the technique to clinical practice for effective testing of allergens in human skin, and arguably for testing of new synthetic materials and healthcare pharmaceutical products in terms of their potential allergic liability.

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