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Optical clearing in photoacoustic flow cytometry

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Abstract: Clinical applications of photoacoustic (PA) flow cytometry (PAFC) for detection of circulating tumor cells in deep blood vessels are hindered by laser beam scattering, that result in loss of PAFC sensitivity and resolution. We demonstrate biocompatible and rapid optical clearing (OC) of skin to minimize light scattering and thus, increase optical resolution and sensitivity of PAFC. OC effect was achieved in 20 min by sequent skin cleaning, microdermabrasion, and glycerol application enhanced by massage and sonophoresis. Using ~0.8 mm mouse skin layer over a blood vessel *in vitro* phantom we demonstrated 1.6-fold decrease in laser spot blurring accompanied by 1.6-fold increase in PA signal amplitude from blood background. As a result, peak rate for B16F10 melanoma cells in blood flow increased 1.7-fold. By using OC we also demonstrated the feasibility of PA contrast improvement for human hand veins.

OCIS codes: (170.3660) Light propagation in tissues; (170.6935) Tissue characterization; (110.5125) Photoacoustics; (330.6130) Spatial resolution.

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1. Introduction

Photoacoustic (PA) flow cytometry (PAFC) is a clinically relevant tool for noninvasive enumeration of circulating tumor cells (CTCs), cancer stem cells, pathogens, clots, and abnormal blood cells [1–4]. Recently, PAFC entered clinical trials to demonstrate noninvasive label-free enumeration of pigmented circulating melanoma cells directly in human blood circulatory. *In vivo* PAFC dramatically increases chances of rare CTC detection by analyzing a large volume of blood (up to 1-2 L) flowing through a large deep vein. However, efficient delivery of laser energy into deep vessels is hindered by significant light scattering in skin. The use of near infrared lasers for PA detection of melanoma CTCs made it possible to minimize absorption and scattering background in skin [2] and to reduce blood background absorption of laser light as hemoglobin absorption of 1060 nm light is very low. Nevertheless, further reduction of light scattering is required to increase laser fluence inside

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blood vessel through improved laser beam focusing in deep tissues to enhance PAFC sensitivity, while maintaining or decreasing patient laser exposure.

During last thirty years various methods of tissue optical clearing (OC) based on changing of tissue scattering properties have been proposed [5–7]. Most of these techniques are based on topical application, injection or enhanced transepidermal delivery of clearing chemical agents [5–8], mechanical compression [5, 6, 9, 10], and photodynamic or photothermal clearing of the skin among many other [11]. Exogenous hyperosmotic chemicals, referred to as OC agents, penetrating into tissues replace water and improve matching of refractive index between scatterers, for example, collagen fibrils, cell organelles, and interstitial fluids or cytoplasm. Tissue dehydration also results in more regular and homogeneous packing of scatterers and less tissue layer thickness.

We demonstrated first applications of OC to enhance sensitivity of photothermal (PT) and PA detection methods for *in vitro* and *in vivo* flow cytometry and improve imaging of sentinel lymph nodes (SLNs) [12–16]. Specifically, OC efficiency was compared for 5 min administration of 40%-glucose, 100%-DMSO, and 80%-glycerol. It was demonstrated that glycerol significantly decreased laser beam blurring in skin and lymph node tissues. *In vitro* OC with 80%-glycerol allowed label-free imaging of a fresh node at the cellular level, localization of immune related, metastatic, and other cells (e.g., lymphocytes, macrophages, dendritic cells, and melanoma cells) and of surrounding microstructures (e.g., afferent lymph vessel, subcapsular and transverse sinuses, the medulla, a reticular meshwork, follicles, and the venous vessels). Recently, OC using glycerol solution was demonstrated as an efficient way to enhance sensitivity and resolution of PA microscopy [17].

The list of typical agents used for OC of skin and other tissues includes: glycerol, glucose, dextrose, fructose, sucrose, sorbitol, xylitol, propylene glycol, butylene glycol, and polyethylene glycol (PEG) [5–7, 18–22]. The substances are often used together with chemical or physical enhancers to increase diffusion across stratum corneum and other skin layers. Among chemical enhancers there are dimethylsulfoxide (DMSO), ethanol, oleic acid, sodium lauryl sulfate, azone, and thiazone (benzisothiazol-3(2H)-one-2-butyl-1,1-dioxide). Some agents, such as DMSO and PEG, can be used both as OC agents and enhancers. Glycerol is one of the most effective and safe agents broadly used for OC of skin [5–8, 13–20, 23, 24]. However, its slow diffusion in skin is its major disadvantage. Typically OC takes more than an hour [5,6], but it can be accelerated with chemical or physical enhancers. Another popular agent, DMSO, has extremely high permeability and high refraction index making it efficient both as OC agent and penetration enhancer [14, 16 ,18, 24–26]. However, despite a long history of its use in different medications and ointments, its use was significantly restricted [26]. Also, ethanol is commonly used as skin cleaner and enhancer of OC agents' permeability. At high concentration it creates pores in epidermal membrane and, thus, enhance skin permeability [27, 28].

Penetration of OC agents through skin barrier may be enhanced by physical or chemical removing of the upper layer of stratum corneum. Such protocols are widely used in cosmetology, tattoo removal, topical drug delivery, and research [29–56]. This includes microdermabrasion, chemical peeling, cosmetic emery boards, the use of wax strip, different sticky tapes, and salts. Microdermabrasion is more often used prior to laser treatment [29–35]. There are more than thirty chemical peels for dermatology and cosmetology, and more than ten of them are applied together with lasers [36–40]. Diffusion through skin may be enhanced by sonophoresis (phonophoresis), electrophoresis (iontophoresis), external pressure, and massage [20, 41–46]. The major advantage of these methods is considerable reduction of treatment time from hours down to several ten minutes [41, 47–56]. Skin optoporation based on fractional tissue ablation with pulsed lamp or Er:YAG laser radiation was reported to considerably enhance epidermal permeability for OC agents and nanoparticles [57–59]. Similar effects were observed for preliminary skin laser treatment (laser-phoresis) [60].

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In this paper we study the efficiency of OC technique using a combination of various approaches to enhance detection sensitivity of PAFC. Specifically, we applied following sequent procedures: alcohol skin cleaning, microdermabrasion removal of topical skin level, and glycerol application enhanced by massage and sonophoresis. The reduction in light scattering and improvements in laser beam propagation were demonstrated that resulted in improvement of PA detection of melanoma cells using *in vitro* phantom of a blood vessel. We also demonstrate clinical perspectives of OC technique for PA assessment of veins in human hand.

2. Materials and methods

2.1. Experimental setup

PAFC setup was built on the basis of Yb-fiber laser YLPM-0.3-A1-60-18 (IPG Photonics Corp.) having 1060 nm wavelength, pulse repetition rate of 10-600 kHz, and pulse duration of 0.8-10 ns, (Fig. 1(d)). A red 635 nm pilot laser CPS180 (Thorlabs, Inc.) was added through 757 nm dichroic mirror (Semrock, Inc). Laser radiation was focused into the sample by an assembly of aspheric (C560TME-C) and cylindrical (LJ1310-L1-C) lenses (Thorlabs Inc.) resulting in a tight focal spot of 7 × 680 μm at a working distance of 4 mm in air. Laser power was controlled in real time by power meter PM100USB with S302C head (Thorlabs, Inc.). A mechanical chopper MC2000 (Thorlabs, Inc.) was introduced into the system to provide pulse repetition rates below 10 kHz, in particular 1 kHz. 1060 nm laser provided energy of 240 μJ/pulse after focusing optics. Fast photodetector PDA10A (Thorlabs, Inc.) with 150 MHz bandwidth was used to trigger data acquisition hardware. Dimensions of the laser spot were controlled using Xli DX-2M camera (Brunel Microscopes, Ltd, UK).

Laser-induced acoustic waves were detected using a custom cylindrical 28-μm polyvinylidene fluoride (PVDF) ultrasound (US) focused transducer with broadband frequency response, 0.2-32 MHz. The transducer was mounted on an independent XYZ-stage to allow micrometer-precision adjustment of its position. Cylindrical geometry of the transducer surface was custom designed to provide PA signal acquisition across the capillary from a minimal blood volume (acoustic resolution PAFC). At focal distance of 8 mm its acoustic resolution was 40 × 1100 μm along the short and long axes, respectively. Axial resolution was estimated as 20 μm. The transducer signals were pre-amplified using 20 dB amplifier (0.05-100 MHz bandwidth, AH-2010-100, Onda Corp.) attached to the transducer and amplified by a second amplifier (40dB, 0.2-40 MHz, 5678, Olympus-NDT Corp.). The signals were recorded using a high-speed digitizer (ATS9350, Alazar Technologies, Inc.) on a Precision T3500 workstation (Dell, Inc.) under control of MATLAB (MathWorks, Inc.) based software.

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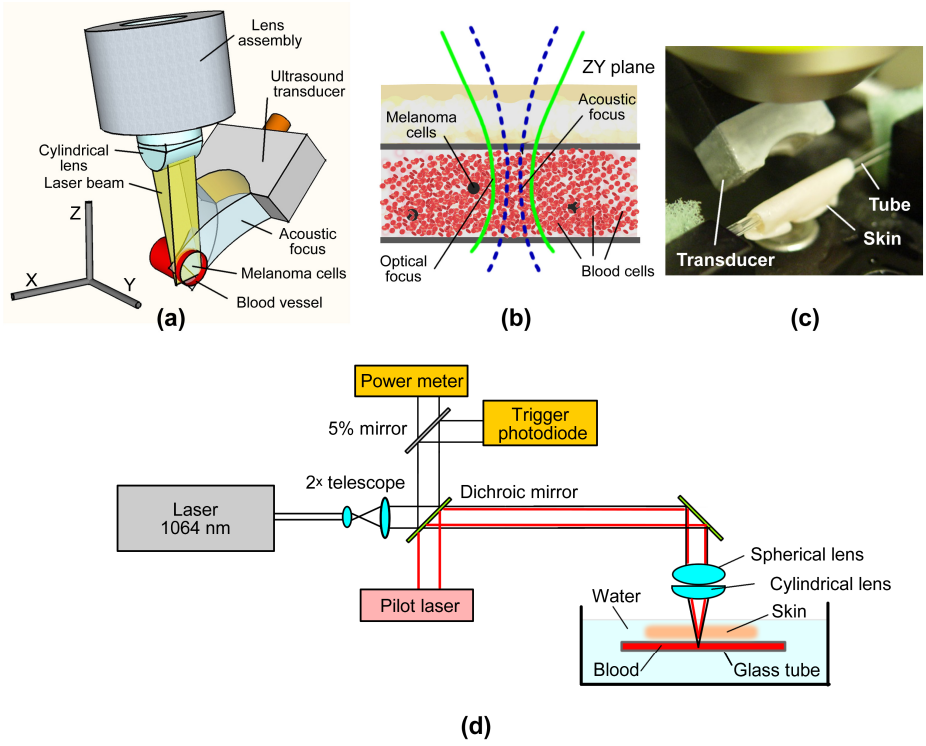

Fig. 1. (a) Schematic representation of PAFC principles of melanoma cell detection in deep blood vessels with a focused US transducer. (b) Schematics and (c) photo of *in vitro* phantom of blood vessel with mouse skin covering the tube. (d) Optical scheme.

2.2. In vitro phantom

In vitro phantom (Fig. 1) was developed for PAFC study of OC agents. The system included a blood vessel phantom (i.d. 0.8 mm, glass tube, Corning, Inc) under a layer of mouse skin (~830 μm thick). Fresh whole mouse blood (~1.7mL total volume, 3.2% sodium citrate) was pumped through the tube by a variable-flow peristaltic pump, model 13-876-1 (Fisher Scientific, Inc.) at linear flow velocity of 10.6 mm/s. US gel (Aquasonic Clear, Parker Labs, Inc.) was used for acoustic coupling between PAFC system and the phantom, while both the focusing optics and transducer immersed into the gel. To reduce the chance of air bubbles being trapped in the gel, it was centrifuged at 500 rpm for 5 min. Acoustic mismatch between the glass tube and medium around it was minimized by selecting tube with thin (100 μm) walls. Moreover, this mismatch was reducing PA signal from both blood and CTCs, thus marginally affecting PAFC sensitivity.

2.3. Cell models

B16F10 melanoma cells (ATCC, CRL 6475) were grown in 5% CO_2, at 37 °C, as a monolayer in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Invitrogen) on T75 flasks (Fisher Scientific, Pittsburgh, PA). Media were replaced every 3-4 days. Cells rinsed with phosphate buffered saline (PBS) were harvested using Versene (Invitrogen). Cells were centrifuged (10 min, 300 × g) and washed three times with PBS. Final cell concentration in suspension was ~3 × 10^6 cells/mL. Cell viability, in (96%) was ensured using Trypan Blue staining.

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2.4. Animal and human studies

PAFC experiments were performed whole blood samples (total volume of around 1.7 mL) withdrawn from donor mice right before the experiment (terminal blood collection) and stabilized with 3.2% Sodium Citrate solution. Animals in this study were used in accordance with protocols approved by the UAMS Institutional Animal Care and Use Committee. Nude nu/nu mice weighing 20–25 g, was purchased from Harlan Sprague-Dawley (Indianapolis, IN). Blood samples were spiked with B16F10 melanoma cells at a final concentration of 200 cells/mL. For a 0.8mm i.d. of tube and linear flow velocity of 10.6 mm/s the blood volume rate was ~0.32mL/min. Thus, total number of melanoma cells under the laser beam was ~64 cells/min.

OC efficiency was tested in healthy human volunteers in accordance with protocols approved by UAMS Institutional Review Board. PA signals were acquired from dorsum of the left hand before and after OC treatment. Photo and US images (M7, Mindray DS USA, Inc.) of the treated area were also acquired to further characterize changes in skin parameters.

2.5. Skin optical cleaning procedure

The OC technique consisted of the following. First, skin was wiped using medical 95% ethyl alcohol (190 proof) for 2 min. Second, top skin layer was removed by using a professional grade Diamond Microdermabrasion Machine for 4 min (NV-07D, HealthAndMed, Corp.). Third, 99% glycerol (Sigma-Aldrich, Inc.) was topically applied to the skin followed by a manual massage for 4 min, and then sonophoresis (Sonicator 740, Metter Electronics, Corp.) for 10 min. Total duration of skin OC was about 20 min.

2.6. Data processing

Each measurement and procedure was performed three times, and the average for all three experiments was used. Peak (cell) count data (M counts) presented as $M \pm \sqrt{M}$. MATLAB (MathWorks, Inc.) was used for all the statistical calculations.

3. Results

At the first stage, we measured total light transmission of a skin before and after OC procedure. In this case only the photons being able to penetrate the skin were accounted for. On average, light transmission for 1060 nm laser beam through 830 μm skin layer was 38 ± 4%, and 25 ± 5% for a double layer of skin. After OC procedure, light transmission increased to 42 ± 4% and 28 ± 5% levels for single and double skin layers, respectively. Such marginal increase in transmittance is consistent with the data reported in literature [6, 15] and we expected a small influence of laser light transmission on PA contrast of the vessels. A decrease in total absorption is most probably due to lower loss of energy through scattering, but other issues like skin thinning and dehydration may also have an effect [5–7]. We also note that selected high quality US gel used for acoustic coupling in PAFC was not producing any beam blurring if there were no trapped air bubbles in the laser path.

#198586 - $15.00 USD Received 30 Sep 2013; revised 1 Nov 2013; accepted 25 Nov 2013; published 27 Nov 2013

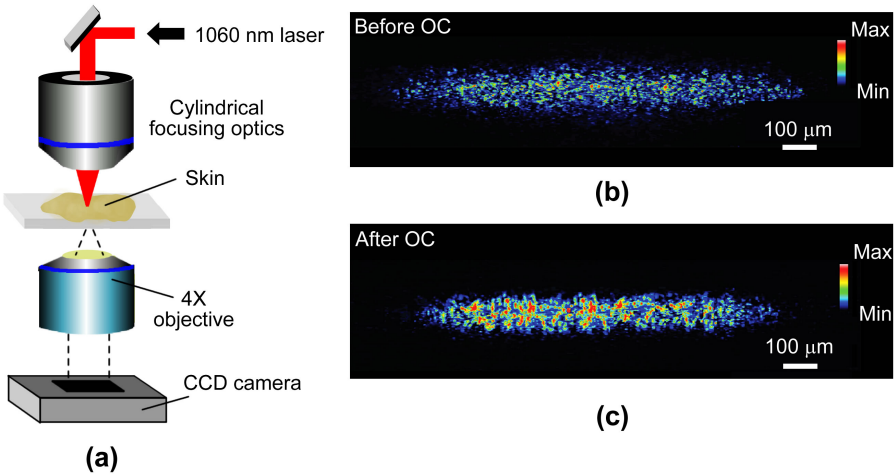

Fig. 2. (a) Monitoring of optical skin clearing using transmission imaging of the laser beam passing 830 μm skin layer. Typical images (b) and (c) of the laser beam spot before and after OC of the skin presented as a heatmap of irradiation intensity.

Second, the influence of skin layer on laser beam spot size (i.e. beam blurring) was analyzed by direct imaging of the focal spot after 830 μm layer of skin (Fig. 2(a)). Typical images of the focal spots before and after OC are presented in Figs. 2(b) and 2(c). As in the case of sample transmission (Fig. 3(a)), laser beam spot size decreased from 96 × 850 μm down to 68 × 760 μm, before and after OC, respectively (Fig. 3(b)). Similar effects were observed for single and double layers of skin. Thus, OC of 830 μm layer of skin resulted in spot width decrease by 30%, or ~1.4-fold; and length decrease by 11%, or ~1.1-fold. We associate this improvement with better matching of the refractive index inside the skin, and with certain tissue dehydration leading to better ordering scatterers resulting in increased mean free path for the photons in the tissues [15]. The total decrease of laser beam spot area was around 37%, or ~1.6-fold. Hence, one can expect an increase in beam fluence of ~1.6-fold as all the energy is concentrated in a smaller area. To prove that reduced light attenuation and minimized beam blurring are indeed increasing PAFC sensitivity, we performed in flow measurements using our phantom model. First, PA signals were acquired for whole blood in the phantom vessel before and after OC treatment of the skin. We observed an increase in PA signal amplitude of the blood in the vessel in the range of 1.2-1.8 folds with an average of ~1.6-fold or 60%. However, we should note that efficiency of skin clearing slightly varied across the sample. Typical PAFC traces for blood samples before and after OC are presented in Fig. 3(c).

#198586 - $15.00 USD Received 30 Sep 2013; revised 1 Nov 2013; accepted 25 Nov 2013; published 27 Nov 2013

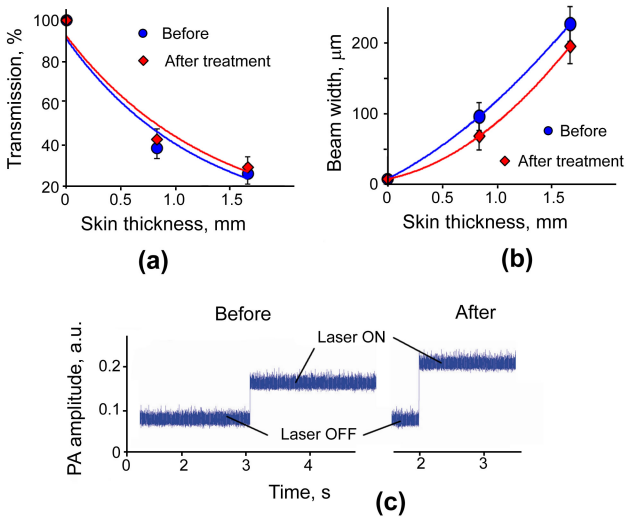

Fig. 3. Optical properties of skin layer after clearing: (a) transmission and (b) width of laser focal spot before and after OC. Solid curves represent best fit trends: power for (a) and polynomial for (b). (c) The increase in PA signal amplitude of whole blood in a phantom model before and after OC of the skin layer.

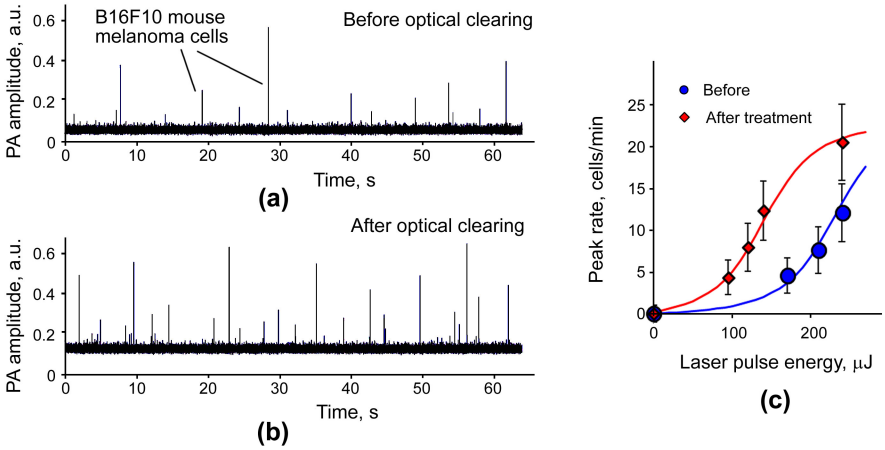

Fig. 4. OC improvement of PAFC detection sensitivity for B16F10 melanoma cells enumeration in skin covered flow *in vitro* phantom. Typical PAFC traces for melanoma cells in flow (a) before and (b) after OC of skin. (c) Dependence of peak rate on the laser pulse energy before and after OC of skin. Data points were fitted using a sigmoid curve.

The effect of OC on sensitivity of CTC counting was demonstrated by label-free PA enumeration of B16F10 mouse melanoma cells in flow vessel phantom. Peak rate (amount of cells detected per one minute) and PA peak amplitudes were analyzed. As some melanoma cells have very low pigmentation and cannot be detected using PAFC even using very high laser energy, we used relative cell count and discussed a relative peak rate increase after OC compared to initial findings. OC of the skin layer increased the peak rate (Figs. 4(a) and 4(b)) from 12 cells/min up to 21 cells/min after the treatment, i.e. an increase of ~1.7-fold. OC also resulted in appearance of much higher number of PA signals with high signal-to-noise ratio (SNR) compared to experiments with unconditioned skin. Hence, with the use of a simple OC skin conditioning it is possible to increase either PAFC detection sensitivity or reduce laser

#198586 - $15.00 USD Received 30 Sep 2013; revised 1 Nov 2013; accepted 25 Nov 2013; published 27 Nov 2013

exposure of the patient, as beam blurring in light scattering tissues is reduced. According to our data (Fig. 4(c)), around 1.6-fold decrease in laser pulse energy is possible without affecting the sensitivity of PAFC melanoma detection. Peak rate curve reaches maximum that indicates that almost every pigmented cell can be detected after OC.

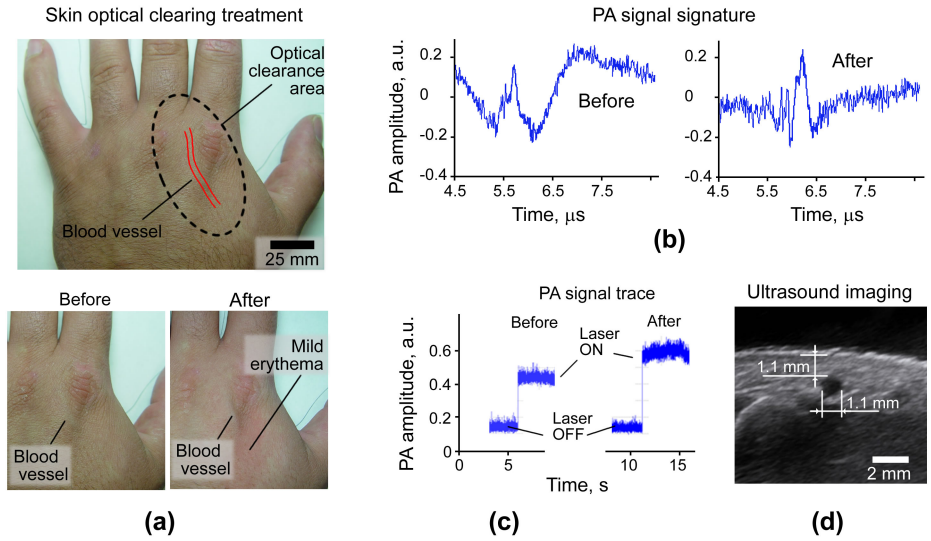

Fig. 5. OC for human skin. (a) Visual contrast of the vein. (b) Typical changes in PA signal waveform. (c) Typical PAFC traces for a vein in human hand before and after OC. (d) US characterization of the selected vein.

To demonstrate that the same OC techniques may be readily translated into clinical practice, we compared PA signals from hand veins of 4 healthy human volunteers before and after local OC. For monitoring we selected a conveniently located branch of Cephalic vein on the dorsum of a left hand. PA signals were acquired prior to OC and right after it. To minimize influence of the PAFC probe positioning error we acquired PA signals from several locations along the vessels and compared the average PA signal, before and after the treatment. On average PA signal amplitude was increased by 20-40% depending on the location of tested area (Figs. 5(b) and 5(c)). PA traces were acquired at 0, 30, and 60 min after OC procedure. In the time frame considered we observed no significant decrease in PA signal amplitude, i.e. no increase in the light scattering of the tissues. We hypothesize here that the US gel used during PAFC procedures may have a certain OC effect as was demonstrated earlier [5, 6, 15], and it maintains the efficacy of OC procedure. We should note that OC not only directly increased PA signal amplitude, but it also reduced the low frequency component of PA waveforms that we associated with PA and pyroelectric effects of the scattered light, (Fig. 5(b)). Local skin conditioning resulted in a slightly higher visibility of the selected vessel, simplifying initial PAFC system positioning, (Fig. 5(a)). A mild erythema (skin redness) was observed on skin after the OC procedure and most probably was caused by microdermabrasion and massage. Topical application of glycerol alone did not cause any skin redness. However, erythema did not affect PA measurements and usually disappeared 3-10 min after treatment. US imaging was used to identify parameters of the selected vein and of the skin layer over it. US imaging did not reveal any significant (>100 μm) differences in the size of the vein or in the thickness of skin after OC procedure, that previously were reported for some OC techniques [5, 6]. Average skin thickness for this location on a human hand was in the range of 0.95-1.3 mm that confirms adequacy of the selected phantom model.

#198586 - $15.00 USD Received 30 Sep 2013; revised 1 Nov 2013; accepted 25 Nov 2013; published 27 Nov 2013

4. Discussion

PAFC enumeration of rare circulating objects in blood flow, including tumor cells, blood clots and other objects requires analysis of large blood volumes to increase chances of detection. However, light delivery into deep large vessels with high flow rate allowing examination of large blood volume at shorter time poses a problem that is not significant for superficial capillaries: light attenuation and scattering by skin and tissues. Here, we tested clinically relevant procedures for optical skin conditioning designed to decrease laser beam scattering and blurring by skin to increase sensitivity of PAFC without increasing laser exposure. We show here that it is better laser focusing in deep tissues that increases PAFC sensitivity.

Taking into account the feature of PAFC's optical and acoustic schematics, PAFC can be defined as optical-resolution PAFC (OR-PAFC) and acoustic-resolution PAFC (AR-PAFC) [14]. In OR-PAFC, resolution is determined by optical parameters, in particular, the minimal width of a focused linear laser beam. Due to strong light scattering in tissue, the high spatial resolution at the level of 5-10 μm can be achieved in superficial 30-50 μm in diameter vessels at a low depth of 0.1-0.2 mm only. In AR-PAFC, in deeper tissue with strong light scattering, the resolution depends upon US focal parameters (e.g., 20-100-μm at a frequency of 10-50 MHz, respectively). We emphasize here that OC effects preferentially improve resolution of OR-PAFC and sensitivity of AR-PAFC.

Compared to our previous studies [12–16], where various OC agents: 40%-glucose, 100%-DMSO, 80%-glycerol, were used to enhance optical imaging of cells and tissues and sensitivity of laser based PT and PA detection techniques, in the current work a novel OC procedure is designed specifically for PAFC monitoring of CTCs in superficial blood vessels of human patients. We demonstrated for the first time that a combination of several OC techniques (ethanol cleaning, microdermabrasion, glycerol application enhanced by massage and sonophoresis) allows several-folds improvement in the sensitivity of PAFC in a reasonable time convenient for cancer patients. Moreover, both laser fluence and beam quality inside blood vessel were significantly improved to allow label-free noninvasive PA detection of circulating melanoma cells throughout the whole vessel. We also note here, that OC opens a way for more efficient theranostics of circulating melanoma cells when cell detection is supplemented by killing it using high energy laser pulses [1, 15].

Our data indicated that the increase in sensitivity was related not to the overall increase in skin transmission (~2%) but to a dramatic laser beam blurring reduction that resulted in almost 40% laser spot area decrease for 830 μm skin layer. Our data correlated well with previously published results for OC using glycerol and mechanical compression [23] (2.5-fold improvement for 2 mm skin). Approximation of our data to a 2 mm skin layer (Fig. 3(b)), results in a similar 2.6-folds value. Notably, a combination of several techniques for skin conditioning including cleaning, top layer removal and glycerol penetration enhancement by massage and phonophoresis made it possible for us to achieve such results in only 20 min compared to more than 1 h reported for applying glycerol only, and 65-80 min for glycerol and 830 μm mouse skin (our preliminary data not reported here). Previously a 4h long glycerol exposure of mouse skin was reported to increase PA signal amplitude 3-folds [17]. Thus, rapid procedure demonstrated here has perspectives for future clinical use in PAFC as well as in other laser based modalities including PA microscopy, tomography, and imaging [61].

According to our data, the reduced blurring of laser beam increased laser fluence by ~60%. Given that acoustic resolution of the PAFC setup is better than optical, the increase in fluence was expected to proportionally increase PA signal of blood. Indeed, 1.6-folds PA signal increase perfectly matched calculated fluence increase. Thus, two opportunities arise: one can use PA signal increase to control the efficiency of OC procedures in non-transparent

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samples (bulk tissue samples), or to calculate laser fluence distribution deep inside the skin after OC.

PAFC monitoring is a non-invasive technique, thus power of excitation laser should remain safe and produce no adverse effects to human skin [62]. Our data presented in this paper shows that high efficiency of OC allows either to decrease (1.6-folds) laser energy while having the same sensitivity (Fig. 4(c)), or to increase detection sensitivity (1.7-folds) without increasing laser exposure. However, any further increase in PAFC sensitivity may be achieved only either through monitoring smaller sized shallow vessels (low total blood volume will limit PAFC sensitivity toward rare cells) or through OC procedures proposed here to improve laser delivery. We achieved this main goal of this work and demonstrated an increase in the sensitivity of PAFC label-free melanoma detection in a phantom of a human hand vein using a clinically relevant OC technique. For circulating B16F10 mouse melanoma cells we demonstrated higher detection sensitivity resulted in 1.7-fold cell count increase. This improvement is directly related to laser fluence increase as was described earlier [2]. We successfully tested the developed OC protocols with human volunteers and observed improvements in PA signal amplitude up to 40%. The OC effect remained in place for whole 1 h long PAFC procedure. Elimination of low frequency PA background was associated with scattered laser light reaching US transducer and generating both PA (high frequency) and pyroelectric (low frequency) signal components. In the absence of such background components, vessel identification using PA signature and PAFC alignment on the vessel were simplified.

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