Preclinical photoacoustic models: application for ultrasensitive single cell malaria diagnosis in large vein and artery

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Abstract: In vivo photoacoustic flow cytometry (PAFC) has demonstrated potential for early diagnosis of deadly diseases through detection of rare circulating tumor cells, pathogens, and clots in nearly the entire blood volume. Before clinical application, this promising diagnostic platform requires verification and optimization using adequate preclinical models. We show here that this can be addressed by examination of large mouse blood vessels which are similar in size, depth and flow velocity to human vessels used in PAFC. Using this model, we verified the capability of PAFC for ultrasensitive, noninvasive, label-free, rapid malaria diagnosis. The time-resolved detection of delayed PA signals from deep vessels provided complete elimination of background from strongly pigmented skin. We discovered that PAFC’s sensitivity is higher during examination of infected cells in arteries compared to veins at similar flow rate. Our advanced PAFC platform integrating a 1060 nm laser with tunable pulse rate and width, a wearable probe with a focused transducer, and linear and nonlinear nanobubble-amplified signal processing demonstrated detection of parasitemia at the unprecedented level of 0.00000001% within 20 seconds and the potential to further improve the sensitivity 100-fold in humans, that is approximately one million times better than in existing malaria tests.

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1. Introduction

Conventional flow cytometry using scattering and fluorescent detection methods has been for many years a fundamental tool of discoveries in cell biology and disease diagnosis [1]. Invasive extraction of cells from a living organism, however, may lead to changes in cell properties, prevent the long-term study of cells in their native environment, limit sensitivity due to a small blood sample volume, and require multiple time-consuming sampling. Most of these problems can be solved by the use of in vivo flow cytometry, which provides noninvasive, continuous examination of nearly the entire blood volume circulating in the peripheral blood vessels [2]. In particular, in vivo photoacoustic (PA) flow cytometry (PAFC) is based on the irradiation of circulating targets with short laser pulses followed by time-resolved detection of laser-induced acoustic waves (referred to as PA signals) with an ultrasound transducer gently placed on the skin [3–5]. In vivo PAFC combines sensitivity and spectral specificity of optical spectroscopy with spatial resolution and depth penetration of ultrasound techniques. Since its first development in 2006, in vivo PAFC has demonstrated enormous potential for detection and enumeration of individual circulating normal and abnormal cells, including circulating tumor cells (CTCs), cancer stem cells, clots, sickle cells,
bacteria, and infected cells using linear and nonlinear nanobubble-based detection modes [3–5]. A PAFC clinical prototype with hand-worn PA probe, demonstrated detection of CTCs in 1-2 mm blood vessels at depth of 1-3 mm with the sensitivity of 100 CTC/mL in melanoma patients [6] that was approximately 100-fold better than that seen with existing CTC assays ex vivo [7].

Nevertheless, before routine use in clinical conditions, especially in new applications, this promising diagnostic platform requires multiple verification, optimization, and calibration using preclinical animal models with vessels that are comparable to human vessel parameters [6,8,9]. Various animal models were already used with various optical and PA methods including mice, rats, rabbits, dogs, and sheep [2,3,9]. In particular, mice were previously used with the PA technique focusing on assessment in small vessels in the ear or abdominal wall [2], because routine use of large animals in standard research laboratories is difficult due to high cost, complex equipment required and regulatory issues. Therefore, it is essential to develop a small animal model to simplify testing procedures, reduce financial burden, streamline a research protocol, and eventually, verify high sensitivity of PAFC. Here, we show that mice can serve as an adequate animal model for some important clinical applications of PAFC due to similarities in the large mouse vein and artery parameters (e.g., size, depth and flow velocity) to selected human vessels. Using this preclinical model, in the current work we verified the unprecedented capability of the PAFC platform for early malaria diagnosis. In spite of global efforts, around 0.6 million people die each year from malaria [10–12]. The sensitivity of existing detection methods is not adequate for early malaria diagnosis before disease symptoms manifest and when treatment is more effective (see [12–17] and references there). Multiple theoretical and experimental studies (e.g., see the references in [18]) revealed that malaria pigment hemozoin more strongly absorbs light in the infected red blood cells (iRBCs) compared to normal RBCs (nRBCs). Thus, hemozoin can be used as a PA high contrast agent to generate PA signals from iRBCs above the background of nRBCs. Using a PAFC platform and small mouse ear vessels, we have recently demonstrated dramatic improvements in noninvasive, label-free, malaria parasite detection at an extremely low parasitemia of 0.0000001%, which is ~1000 times better than the level of detection in existing malaria detection methods [18]. In current work using large vessels in our mouse model, we provided comprehensive verification of our previous results [18]. Moreover, here we demonstrate further improvement of the sensitivity threshold ~10 times while simultaneously reducing testing time to 20-40 seconds.

2. Materials and methods

2.1 Principles and features of PAFC

In general, PA techniques can assess in the circulatory system (Fig. 1) both small and large vessels of different locations (Fig. 2) with diameters from 5 to 10 μm (superficial capillary) to 0.9-1.5 cm (jugular vein (JV) or carotid artery (CA), respectively) with the depth in a few studies of up to 7 cm [9]. For this PAFC platform, larger vessels must be used because they have a higher flow rate allowing examination of whole blood volume during shorter time periods (Fig. 1(a) and Table 1) as the flow dynamics of blood in the circulatory system differ between vessel types and sizes [19,20]. For clinical applications of PAFC, near-infrared (NIR) spectral range (700-1100 nm) with deeper penetration of laser light in most bio-tissues (so called tissue transparency window) is preferred, especially if there are appropriate strongly absorbing PA positive contrast agents. Hemoglobin (Hb) in nRBCs, melanin in melanoma cells, and hemozoin crystals in iRBCs are examples of intrinsic PA positive contrast (Fig. 3). On the contrary, low absorbing objects (e.g., white platelet-rich clots) can be indirectly detected through negative PA contrast agents in blood background [3].

Most in vivo PAFC preclinical studies have been done using thin (250-300 μm), relatively transparent mouse ears with well-distinguished veins and arteries. Rat or mouse mesenteries are almost ideal models, consisting of very thin (7-15 μm) transparent connective tissue with
a single layer of blood and lymph microvessels [3]. PAFC was also applied to 200-300 μm diameter blood vessels in the nude mouse abdominal area at a depth of 300-500 μm either through skin or by using a window chamber model [3]. All of these superficial vessel models offer high PA contrast and good optical resolution (OR), but due to low flow rate it may take hours if not days (Table 1) to monitor a significant blood fraction.

Fig. 1. (A) Schematic of blood circulatory system and PAFC detection points. The insets show the differences in the artery (left) and vein (right) associated with larger vessel wall thickness and smaller diameter of the lumen (internal space) in the artery. (B) Flow velocity, total internal area of vessels of this type, and monitoring time (“circulation cycle”) in small and large vessels.

Fig. 2. Detection points in human (left) and rodent (right) for wearable PAFC devices. Top (from left): human ear [21], ventral surface of human wrist with wearable PAFC [3,4], and mouse ear [21]. Middle: wearable (human hand-worn) PAFC [6], and mouse neck area with JV. Bottom: ultrasound images of human vein in dorsal area (left) and mouse JV (right).
Fig. 3. Absorption spectra of hemozoin [17,18], melanin [4], magnetic beads [3], arterial (oxygenation ~96%) and venous (oxygenation ~70%) blood [18]. Similarity in absorption spectra of hemozoin and magnetic beads indicate a potential to use magnetic beads as hemozoin phantom for calibration of PAFC. Excitation (Ex) and emission (Em) spectra of green fluorescent protein (GFP) expressed by parasites [18]. Arrows indicate laser wavelengths used: 488 nm (for continuous wave fluorescence excitation), and 671 nm and 1060 nm (for pulsed generation of PA signals).

Table 1. The parameters of selected vessels in mice and humans

|                | Large Vessels | Small Vessels |
|----------------|--------------|---------------|
|                | Vein (JV)    | Artery (CA)   | Vein (ear) | Artery (ear) |
| Diameter (mm)  | 0.9 – 1.3    | 0.6 – 0.8     | 0.05 – 0.07 | 0.03 – 0.05  |
| Depth (mm)     | 0.8 – 1.2    | 1.5 – 2.1     | 0.1 – 0.2  | 0.1 – 0.2    |
| Velocity (cm/s)| 3.5 – 6.5    | 15.5 – 18     | 0.3 – 0.5  | 0.5 – 0.8    |
| Monitoring time (min) | 0.9-1.2 | 0.7 – 0.8 | 1860-8240 | 1000-2500 |

The transition from superficial to deep vessels demands one to consider light scattering by skin and tissues. The OR-PAFC (Fig. 4(a)) can be achieved in shallow 30-50 µm diameter vessels at a low depth of 50-100 µm only when the influence of light scattering is minimal [2,3]. The spatial resolution is determined by a minimal width of a linear laser beam with the typical range of 5-10 µm. Optical focusing makes it possible to use unfocused ultrasound transducers simplifying system schematics and signal analysis. In deep tissues, absorption and scattering effects lead to light attenuation and blurring, respectively that dramatically reduce OR. Under such conditions, to assess larger vessels (e.g. 0.5-1 mm) in deeper tissue (1-3 mm), acoustic resolution (AR) must be applied. In order to achieve this, we developed AR-PAFC with the focused ultrasound transducers (Fig. 4(b)). At these depths, the influence of light scattering is greater, while scattering of ultrasound waves is relatively low (e.g. ~0.6 dB cm⁻¹ MHz⁻¹), which provides their effective penetration to depths of 2-4 mm for 50-70 MHz [3,9]. The resolution of AR-PAFC depends solely on the ultrasound parameters (e.g. lateral resolution of 50-100 µm at a frequency of 30-60 MHz). The spherical focused transducer increases signal-to-noise ratio (SNR) by minimizing detection volume (blood background), but cells floating outside of this volume can be missed. The cylindrical focused transducer
provides an optimal detection window covering the full width of a vessel, while minimizing the detection volume in another direction. The OR and AR resolutions were initially estimated by theoretical modeling and eventually tested experimentally (Fig. 4, insets).

Fig. 4. Principles of in vivo PAFC. (A) Schematic of OR-PAFC for examination of superficial microvessels. (B) Schematic of AR-PAFC for assessing deep large vessels. The insets illustrate examples of experimental estimation of optical (A) and acoustic (B) resolution. Laser parameters: beam shape, linear; beam size, 6.5 µm x 780 µm; wavelength, 1060 nm. Lateral resolution in B (~90 µm) of the spherical focused transducer is represented as a PA signal distribution from black tape scanned with a focused laser beam with diameter of 2 µm at 532 nm and 10 ns pulse width.

2.2 PAFC setup

The OR-PAFC setup was built on the platform of a Nikon Eclipse E400 microscope (Nikon Instruments, Inc.) with a high pulse repetition rate (10 kHz), 10 ns pulsed 671 nm laser (QL671-500, CrystaLaser, LLC) for PA detection of hemozoin (henceforth referred to as PA detection) and 488 nm continuous wave laser for fluorescent excitation. In vitro laser beams were focused into cylindrical quartz capillary tubes with a diameter of 50 µm. In vivo, the beams were focused into a mouse ear using a 40x objective (Plan Fluor, NA 0.65; Nikon Instruments, Inc.) The same objective collected fluorescence emission in the spectral range of 510-518 nm from genetically engineered parasites expressing green fluorescent protein (GFP). PA signals were acquired by an unfocused transducer (5.5 mm diameter, 3.5 MHz, model 6528101, Imasonic, Inc. France), and amplified (0.05-4 MHz pre-amplifier 5662B, Olympus-NDT Corp.). PA and fluorescence data were acquired by a digitizer (PCI-5152, National Instruments Inc., USA).

The AR-PAFC setup was based on 1060 nm Yb-fiber laser YLPM-0.3-A1-60-18 (IPG Photonics Corp.), pulse repetition rate of 1-10 kHz, and 800 ps, 5 ns and 10 ns pulse width. Laser energy was controlled by a power meter (PM100USB, S314C sensor, Thorlabs, Inc.). A fast photodetector (PDA10A, 150 MHz, Thorlabs, Inc.) triggered data acquisition. Two types of custom made focused ultrasound transducers were used: 1) a cylindrical focused transducer using a 28-µm polyvinylidene fluoride (PVDF) element (broadband frequency response of 0.2-32 MHz, focal distance of 8 mm); and 2) a spherical focused transducer (25-75 MHz; the
focal distance of 3.2 mm). The transducers were mounted on an XYZ-stage coupled to a goniometer stage (GNL20, Thorlabs, Inc). PA signals were pre-amplified (model AH-2010-100, Onda Corp; 20 dB, 0.05-100 MHz bandwidth) and further amplified (model 5678, Olympus-NDT Corp.; 40 dB, 0.2-40 MHz bandwidth) before being recorded by a digitizer (ATS9350, Alazar Technologies, Inc.) on a Precision T3500 workstation (Dell, Inc.), using custom software developed in MATLAB (MathWorks, Inc.). The laser beam was focused into a narrow line (6.5 μm × 780 μm) crossing the blood vessel by a combination of aspheric and cylindrical lenses (C560TME-C and LJ1598-L1-C, respectively, Thorlabs, Inc.).

2.3 Animal models

Animals were used in accordance with a protocol approved by the University of Arkansas for Medical Sciences (UAMS) Institutional Animal Care and Use Committee. The female C57BL/6J (The Jackson Laboratory, Inc.) mice were infected with 10^5 GFP+ Plasmodium yoelii 17XNL iRBCs via intraperitoneal injection. Uninfected C57BL/6J mice were taken as negative controls. The mouse model selected, parasitic strain and infection procedure are broadly used for malaria studies [15]. During experiments, the mice were anesthetized by inhalation of 1.2% isoflurane and placed on heated stage at 37 °C for in vivo monitoring. For this study we selected 40-70 μm (depth of 100-200 µm) mouse ear vessels, and ~1 mm vessels in neck area at a depth of 1-2 mm.

The transducers and optical components were placed above skin and adjusted to maximize PA signal from the vessel. Optically transparent ultrasound gel (Aquasonic Clear, Parker Labs, Inc.) was used for acoustic coupling between transducer and skin. Vessel anatomy was assessed by a medical ultrasound system (M7, Mindray DS USA, Inc.). Mouse blood was collected from tail ventral artery and lateral vein for in vitro testing with Giemsa staining, fluorescence microscopy, and PAFC.

In vitro PAFC-based studies were performed in static and flow condition in a capillary tube using a pulsed 671 nm laser with pulse widths of 10 ns and a 1060 nm laser with pulse widths of 800 ps, 5 ns, and 10 ns. In static condition 8.6 µL blood sample was placed in a Secure-Seal spacer (diameter of 9 mm, 120 µm spacer thickness, Cat. No. S24737, Invitrogen Corp.).

2.4 Data processing

Mice underwent in vivo monitoring every 1-3 days depending on the level of parasitemia, from 10 min to 3 hours. All measurements were performed at least three times, and the average for these data points was used in the figures. Peak-to-peak amplitudes of PA waveforms were traced, and points at least 3σ above the background were taken as PA signals. PA signal rate is described as number of pulses, i.e. PA signals per minute. Counted data (M counts) were presented as M ± SD (standard deviation). Signal analyses and statistical analysis were performed using MATLAB (MathWorks, Inc.) software.

3. Results

3.1 Mouse model of human hand vessels and possible artifacts

Most mouse vessels are much smaller than respective superficial human vessels for PAFC detection [3,4,22]. Only the mouse JV and CA are very close in size and blood flow velocity (Table 1) to human hand vessels as identified by ultrasound imaging (Fig. 2, bottom). However, assessment of these relatively deep vessels with PAFC may be complicated by artifacts that are not typically present or negligible in superficial vessels (Fig. 5). Most important are the artifacts associated with animal motion, breathing, and heart beating.

Unpredictable shifts in mouse body position due to non-optimal anesthetic dosage may cause major changes in PA signal regardless of the transducer’s location (Fig. 5(a)). Both OR and AR-PAFC detection schematics may be affected as the vessel shifts away from the focal
optical and/or acoustic focal areas. Mostly, the changes in PA signals related to animal movement are relatively slow, while transient PA signals from circulating objects are much shorter with high frequency spectrum. Breathing-related artifacts (Fig. 5(b)) are generated by contractions of the diaphragm causing periodic vessel displacement. They have a well-defined oscillation period that corresponds to mouse respiratory and heart beat rates [19,20]. The artifacts created by heart contractions (Fig. 5(c)) may be stronger (higher oscillation amplitude than for breathing) due to artery displacement, and vessel expansion and contraction during systolic-diastolic cycles. The breathing and heart-related artifacts appeared during monitoring of the aorta, vena cava and celiac vessels with the transducer’s location near thorax and abdomen. For mouse ear vessels only artifacts related to whole body motion were observed.

Fig. 5. Artifacts and their removal in PAFC traces in mouse model. (A) Animal motions during anesthesia. (B) Breathing-related vessel motion. (C) Heartbeat-related artifacts in large arteries. Traces were filtered by a high-pass filter to eliminate low frequency artifacts. Cutoff frequency was 10 Hz (A,B) or 50 Hz (C). PA signal amplitudes and corresponding amplitude spectra of both unfiltered (blue) and filtered (red) traces are presented in left and right columns respectively.
The artifacts in PA data traces can be significantly minimized or even completely eliminated by using a high-pass digital filter. The typical duration of transient PA signals from circulating objects (e.g. iRBCs) in the detection volume of large vessels is very short (0.5-2 ms at flow velocity of 5-10 cm/s). At the same time artifacts usually lie in the low-spectral-frequency range of <100 Hz. PA data trace filtration reduced (5-8-fold) all the observed phenomena (Fig. 5) and provided a reliable base for monitoring large vessels with PA background stable over a ½-1 hour period. Manual vessel tracking with minor adjustments for transducer position using PA signal from the vessel provided minimization of the artifacts caused by vessel displacement.

The appropriate positioning of the focal point of the laser in OR-PAFC (Fig. 4(a)) and acoustic transducer in AR-PAFC (Fig. 4(b)) in combination with time-resolved signal detection (below Fig. 6(a)) can further reduce the influence of artifacts and PA signals originating from “undesired” arteriovenous plexi in the skin (these structures were omitted from the Fig. 4 for simplicity).

Fig. 6. PA and fluorescent signal traces from different vessels. (A) Time-resolved detection of PA signals from deep JV and CA coming to transducer with a delay compared to background PA signals from pigmented skin. (B) PA signal traces from uninfected mouse (top), JV (middle) and CA (bottom) in infected mice. (C) Fluorescence (top, FL) and PA (bottom) signal traces from ~70 µm ear vein of infected mice. (D) Fluorescence (top, FL) and PA (bottom) signal traces ~50 µm ear artery of infected mice.
3.2 PA flow cytometry (PAFC) in malaria mouse model

Time delay between PA signals from topical skin layers with melanin pigmentation and deeper vessels is sufficient for discrimination of background skin signals from those of JV and the more distal CA. This delay (0.5-2 µs) between PA signals is directly proportional to the actual distances between skin and vessels (Fig. 6(a)). In control (uninfected) mice, there were no transient PA signals significantly different from blood background (Fig. 6(b), top). In contrast, the data from malaria infected mice (Fig. 6(b), middle, bottom) show multiple transient PA signals having high amplitude and short duration. These peaks have been directly associated with the presence of iRBCs [18]. Combined PA and fluorescence detection confirmed coincidence of GFP+-parasite-related fluorescent signals (Fig. 6(c), top) with hemozoin-related PA signals (Fig. 6(c), bottom). The observation of some peaks in fluorescence and PA traces having no statistically significant temporal matches suggested the presence of individual parasites and hemozoin crystals (released from iRBCs) in plasma, respectively. The numbers of both fluorescent (Fig. 6(d), top) and PA signals (Fig. 6(d), bottom) in small arteries were much higher than the number of detected signals in small veins (Fig. 6(c)) for a similar blood flow rate and experiment duration. The artery lumen is slightly smaller than the vein (Fig. 1, top) and thus creates a smaller PA background allowing achieving high SNR for detected PA peaks. Smaller blood volume also provides better fluorescence detection conditions because of minimal autofluorescence background. Moreover, flow velocity is higher in arteries, than in veins, while volume flow rate has to be the same to equalize blood inflow and outflow. Thus, the notable difference in numbers of detected peaks is most probably related to slightly better detection conditions in arteries. Similar results were obtained for deep vessels, where the number of peaks from CA (Fig. 6(b), bottom) was higher than in JV (Fig. 6(b), middle). Compared to ear vessels, both JV and CA, provide a much larger level of background PA signal.

3.3 Parasitemia staging in deep vessels

PA detection of circulating iRBCs with hemozoin crystals from the 1st to the 41st day after infection demonstrated the unique capability of PAFC diagnostic platform to monitor all the stages of the disease from a few PA signals at earliest stage to the day GFP+-parasites were completely eliminated from blood. Figure 7 represents the average PA signal rate (number of PA signals from hemozoin crystals per minute) both in small and large vessels. As previously reported for this mouse model [18], the peak of parasitemia was represented by the maximal number of PA signals in the period from 14th to 22th day, followed by a slow decline and disappearance of all the disease signatures.

Here, we demonstrated that for both superficial (Fig. 7(a), 7(c)) and deep (Fig. 7(b)) vessels the PA data provided consistent information of the disease stage. As noted earlier, PA signal rates were higher in arteries than in veins, which is in line with recent data for malaria obtained ex vivo [23]. However, PAFC can provide these data in vivo in real time. These findings were validated in vitro to exclude possible differences in flow velocity (i.e. increased iRBC rate through the same detection volume). Arterial and venous blood was collected from the same animals (see Methods) and monitored by PAFC in vitro in a quartz flow tube (Fig. 7(d)). The same difference in the number of iRBCs was found as under in vivo conditions: the rate of PA signals was higher for arterial blood than for venous blood. We hypothesized here, that this is attributed to iRBCs being larger, more rigid, and, possibly, aggregating more than nRBCs. This can result in passive capturing of iRBCs in the capillary bed between the arterial and venous systems (Fig. 1, top). This capturing can be enhanced because iRBCs can express adhesion proteins increasing interactions with endothelial cells lining the vessel walls. Indeed, scanning of the vessel walls with a focused laser beam revealed multiple PA signals originating from static objects, most likely non-moving iRBCs, but validation of these findings would require additional studies.
3.4 PAFC sensitivity in deep vessels

The comparison of PA signal amplitudes and numbers in superficial and deep vessels may be used to prove the hypothesis of higher PAFC sensitivity using larger vessels with higher blood flow rate. This measurement was done at peak parasitemia (16th day after infection), at the beginning of infection (2nd day) and just prior to clearance of parasitemia (32nd day) initially in vein and then in artery. We obtained similar results for both vessels and included only results from the arteries to simplify the presentation. In CA, the PA signal amplitudes are smaller, and the PA signal rate is higher (Figs. 8(a)-8(c), left) compared to ear artery (Figs. 8(a)-8(c), right). Thus, strong light attenuation and large blood background in deep tissues lead to the decrease in SNR for iRBCs. At the same time, higher flow rate present in CA than in small superficial vessels provide the opportunity to assess a much larger blood volume circulating in large vessels for shorter time. Indeed, at low parasitemia (2nd and 32nd day) only one (32nd day) or no signals (2nd day) could be detected in small ear artery (Fig. 8(b), 8(c) right bottom), while a significantly higher number of PA signals were detected in the larger CA (Fig. 8(a)-8(c), left). Thus, the large blood volume analyzed in CA dramatically improves PAFC sensitivity, as opposed to superficial vessels. Whole mouse blood volume (2 ml) passes through CA (0.9 mm in diameter, flow velocity of ~5 cm/s) in mice during approximately 40-60 seconds (Table 1). This corresponds to a PAFC ultimate detection limit
of only one iRBC in 2 ml or 0.00000001% parasitemia (the percentage of infected cells among uninfected cells). This is approximately 10-fold greater than we achieved previously using small ear vein [18] and 10^4 - 10^5 times better than threshold of the current gold standard for malaria diagnosis – Giemsa-stained blood smear (0.001%) [11–13].

![Fig. 8. PA signal traces for superficial ear artery (30-40 µm in diameter) and deep CA (~0.9 mm in diameter) at (A) the peak of infection (16th day of infection) and (B,C) at low parasitemia levels (32nd and 2nd day) at energy fluence of 200 mJ/cm²](image)

3.5 Selection of laser pulse width

Generation of PA signals from small nano-sized particles could be more optimal with the use of shorter laser pulses due to fulfillment of requirements of acoustic confinement [3]. Thus, picosecond laser sources may provide additional advantages compared to commonly used nanosecond excitation sources in PA techniques. However, picosecond lasers have lower cost-efficiency, in general are larger compared to nanosecond laser sources and require more durable optical components. For the translation of the PAFC system into clinics it is essential to compare nanosecond and picosecond generation of PA signals in iRBCs. This was performed ex vivo for healthy and infected blood using a 1060 nm laser operating at 800 ps, 5 ns and 10 ns pulse widths (Fig. 9). Blood samples were collected at the 6th and 16th days of infection corresponding to low and high parasitemia, respectively. Our study revealed that PA signal amplitudes can be increased up to 2-2.5 times at shorter (800 ps) laser pulses at the same laser energy fluence.
With increased energy fluence PA signals non-linearly increase too due to a previously discovered PA signal amplification due to laser generation of nanobubbles around overheated...
absorbing objects [3-5]. However, the use of high energy is not optimal because it can lead to exceeding the ANSI (American National Standards Institute) laser safety limits and to PA signal saturation likely due to the object being damaged at the beginning of the laser pulses [18]. PA signals in normal blood also increase at shorter laser pulses (Fig. 9(a)). However, this increase was almost negligible (20-30%), suggesting better acoustic confinement of relatively large nRBCs with nanosecond laser pulses compared to smaller hemozoin crystals (50-1000 nm) [18]. Even slight dependence of PA signals on pulse width indicates that individual nRBCs in blood at a typical hematocrit can be considered as at least partly acoustically independent targets.

The increase in PA signals in iRBCs was more profound with decreased laser pulses than in nRBCs, but much less than expected for small hemozoin (~11-fold) as a ratio of the longest and shortest laser pulses used. This discrepancy can be explained either by the real size of hemozoin and their clusters in iRBCs which are larger than 200-300 nm, or nonlinear nanobubble-based PA signal amplifications are less sensitive to the classical acoustic confinement and depend more on thermal confinement as we recently discovered [3] (and [33] in the reference list there).

4. Discussion

In this work we for the first time demonstrated a prospective mouse model of human vessels that would allow cost-effective validation of the PAFC diagnostic platform. Using this model, we confirmed our previous finding [18] that the PAFC platform currently provides the best sensitivity for malaria detection. The current gold standard for malaria diagnosis is the Giemsa-stained blood smear. Current sensitivity threshold of this technique is about 10 parasites/µL in a standardized research environment and approximately 50-100 parasites/µL outside of that setting and even higher in inferior resourced facilities [11–15]. Moreover, complete suppression of background from pigmented skin through time-resolved detection of PA signals from deep vessels (Fig. 6(a)) provided an additional sensitivity improvement in parasitemia detection at the level of 0.00000001% that is approximately 10^4-fold better than in the existing malaria detection methods [12–17]. The circulation of whole blood volume (~2 ml) in large ~1 mm mouse blood vessels takes just around 40-60 seconds (Table 1) that allows quick detection of just one iRBC among billions of nRBCs.

We have already demonstrated 100-fold sensitivity improvements in the detection of CTCs in melanoma patients. Because the parameters of vessels in melanoma patients and in the preclinical mouse model presented here are relatively comparable (1-1.5 mm size, 1-2 mm depth and ~5 cm/s flow velocity), and the absorption spectra of melanin and hemozoin as PA contrast agents are similar too (Fig. 3), we have sufficient evidence to support a potential increase in the sensitivity of malaria detection in humans. By the examination of a larger blood volume in humans than in mice, parasitemia detection threshold may be improved by two orders of magnitude to an unprecedented level of 0.0000000001% or a few infected cells in almost a trillion nRBCs, which is a million times better than in existing malaria tests. Comprehensive clinical validation of unique PAFC platform in humans will be published soon.

Our current data suggest that there may be some advantages to the use of arterial monitoring as opposed to venous monitoring. Figure 3 shows notable differences in absorption spectra of arteries and veins due to different oxygenation levels. Thus, these vessels can be identified by taking advantage of this property and irradiating it with lasers of differing wavelengths (e.g. 671 nm for vein and 1060 nm for artery), as they have stronger absorbance at their respective wavelength. These features can be exploited when searching for the optimal point of detection in a real-world scenario as cases may differ. Veins have thinner walls (Fig. 1(a), insets) and therefore less laser light scattering, and they are found more proximal to the surface of the skin compared to their arterial counterpart, they appear to be a better candidate for PAFC diagnosis of pathological conditions such as malaria.
However, the greater PA signal rates in the arteries show benefit in providing more cells in the detection window within an equal monitoring time, and hence better detection limit.

The advances in PAFC technology makes it possible to develop an affordable wearable watch-like AR-PAFC device utilizing small nanosecond or picosecond (e.g. even sub-nanosecond) 1060 nm high pulse repetition rate laser with adjustable pulse width and hemozoin as an 'intrinsic' PA contrast agent for noninvasive malaria detection. We emphasize that the unprecedentedly high sensitivity of PAFC provides flexibility for development of a robust diagnostic device with a simplified schematic using a commercially available, cost-effective 1060 nm laser with 1-2 ns pulse width and 1-10 kHz pulse repetition rate, while reducing PA signal amplitudes insignificantly compared to lasers with shorter pulse widths and wavelengths (Fig. 3).

The possible issues that can be further addressed with PAFC include confirmation of the presence of hemozoin as a malaria marker and PA contrast agents in people infected with different parasite species and at different disease stages. We have described early detection of malaria in mouse models [18], but it would be interesting to analyze how quickly after initial infection we can identify iRBCs in humans. We will determine if parasites that do not produce hemozoin lead to PAFC false-negativity. On the other hand, hemozoin can remain in circulation for an extended period of time after the infection clears, which can lead to PAFC false positivity.

Another intriguing discovery in the current work is the higher iRBC counts in the arterial system rather than in the venous network as mentioned above. Further studies using PAFC and available literature data [23–25] will provide the insight on this finding, which may be due to the properties (e.g., rigidity and clustering), associated with iRBCs and artery-vein features.

Clinical scenarios may include in vivo rapid blood screening for early malaria diagnosis, testing for recurrence, studying the efficiency of new drugs, and individualized assessment of therapy efficiency. Indeed, the ability of PAFC to quantitatively count every single iRBC in circulation can provide a unique tool for monitoring vaccine efficiency through real-time counting of iRBCs before, during, and after treatment. PAFC platform has the potential to provide individualized characterization of disease progression and treatment efficiency as we have already demonstrated with PAFC for cancer, clots, sickle cells, and other infections [3, 26–28]. Potential applications may be different in personal usage, clinics, and large infectious disease centers using different device versions (e.g. wearable hand-worn and stationary). Since laser-induced high localized nanobubbles can provide spatially selective destruction of parasites in iRBCs without harmful effects on surrounding nRBCs, further studies may address the important question: could laser ablative therapy be effective for treatment of drug resistant parasite populations in circulation or must it be integrated with another treatment to achieve potentially synergistic therapeutic effects.

In a few experiments we slightly exceeded the ANSI laser safety limits, which is 100 mJ/cm² for 1060 nm at relatively low pulse repetition rate [29] to explore possible nonlinear effects as well as photothermal therapy at higher energy fluence. In the clinical trials in humans we plan to use lower energy fluences (e.g. 10-20 mJ/cm² or lower), which is sufficient for high sensitivity detection of circulating infected cells as was already proven in our previous studies [18]. Moreover, the unprecedentedly higher sensitivity of the PAFC technology compared to existing malaria tests allows us to use much lower laser energy, because despite some decreases in PA signal amplitudes, the sensitivity of the PAFC system is still high enough to provide early malaria diagnosis that is impossible with the existing methods.

Further improvement of the PAFC platform can be achieved by use of multicolor PAFC [4,18] for spectral identification of iRBCs among nRBCs and use of optical clearing of skin [22] to reduce laser energy losses due to light scattering phenomena several folds.
5. Conclusion
In our current work, we have demonstrated that in vivo PAFC has great potential for diagnosis of malaria. This technique achieves an increase in sensitivity that is in part due to its ability to monitor nearly the entire blood volume of a patient. For rodent models to become better indicators of clinical potential, we have discussed several parameters that must be considered and we have established strategies that allow for better correlation between rodent and human systems. We have presented techniques for elimination of background noise, laser wavelength selection and the potential concept for a wearable, cost-effective device that can improve current malaria diagnostic sensitivity. While further work must be done for optimization of this system for clinical utility, our data strongly suggests the potential for this use.

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