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Article *in* Open Journal of Clinical Diagnostics · January 2021

DOI: 10.4236/ojcd.2021.112005

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Open Journal of Clinical Diagnostics, 2021, 11, 59-75
https://www.scirp.org/journal/ojcd
ISSN Online: 2162-5824
ISSN Print: 2162-5816

Review of Photoacoustic Malaria Diagnostic Techniques

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How to cite this paper: Memeu, D.M., Sallorey, A.M., Maina, C. and Kinyua, D.M. (2021) Review of Photoacoustic Malaria Diagnostic Techniques. *Open Journal of Clinical Diagnostics*, **11**, 59-75.
https://doi.org/10.4236/ojcd.2021.112005

Received: October 6, 2020
Accepted: June 26, 2021
Published: June 29, 2021

Abstract

Malaria is one of the leading causes of mortality and morbidity in developing countries. Accurate and complete diagnosis is key for effective treatment of the disease. However, mainstream malaria diagnostic techniques suffer from a number of shortcomings. There is therefore an urgent need for development of new and more efficient techniques for malaria diagnosis. *In vivo* Photoacoustic spectroscopy is an emerging technique, which has great potential of delivering a nearly ideal method for early diagnosis of the disease. The technique promises to be highly sensitive and specific. In this paper, a description of photoacoustic malaria sensing is given. This is followed by a review of photoacoustic-based malaria diagnostic techniques and suggestions for future improvements.

Keywords

Plasmodium parasites, *In Vivo*, Chromophores, Hemoglobin, Hemozoin, Spectroscopic Inversion, Time Domain and Frequency Domain Photoacoustics

1. Introduction

1.1. Background of Malaria Disease

Malaria is a common disease in tropical countries that poses serious public health concerns. It is one of the leading causes of mortality globally. Nearly 0.5 million deaths were attributed to the disease in the year 2015 according to the world malaria report [1]. The majority of these deaths comprise of children less than

five years old from sub-Saharan Africa. Besides, it is estimated that approximately 200 million people are infected with the disease and 3.2 billion people are at risk of contracting the disease annually according to the report. This has created a heavy social-economic burden to the affected countries due to huge treatment costs and absenteeism from work and schools [2].

The disease is caused by protozoan parasites of the genus *Plasmodium* which lives in peripheral blood, spleen or liver of humans. The parasites are introduced to the human host through bites by infected mosquitoes—malaria vectors. Once in the bloodstream, the parasites move to the liver for incubation and multiplication. They later move back to the bloodstream and attack the Red Blood Cells (RBCs). The parasites feed on the cells' hemoglobin reducing or completely inhibiting the cells' ability to bind oxygen. Hemoglobin is a complex bio-molecule made of two simpler molecules; the iron compound called heme and a protein molecule globin. The parasite digests globin to meet its nutritional needs but converts heme (which is toxic to the parasite [3]) into a by-product called hemozoin. Hemozoin, an inert insoluble crystal composed of iron nano-rods [4], is then deposited in parasites' food vacuoles. Presence of hemozoin in RBCs in peripheral blood is, therefore, an indicator of malaria infection. Notably, hemozoin production is also associated with other blood-feeding parasites such as *Schistosoma mansoni* and *Rhodnius prolixus* [5] [6]. However, neither Schistosomamansoni nor Rhodniusprolixus resides in peripheral blood.

There are five species of *Plasmodium* parasites that infect humans [7], namely; *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodiumvivax*, *Plasmodium malariae*, and *Plasmodium knowlesi*. *Plasmodium falciparum* is the most fatal while other species cause mild to moderate illnesses. Each of the five species has distinct morphological features which are used for *Plasmodium* parasites species identification during optical microscopy diagnosis. The parasites undergo three main inter-erythrocyte life stages, namely; the early stage is also known as ring stage, intermediate stage also called trophozoite stage and Mature stage also called schizont stage (the reproductive stage). Upon maturity of the parasites, the infected cells' rapture and the merozoites (offspring of the parasites) are released into the bloodstream where they attack other health erythrocytes and the cycle starts afresh. However, some *Plasmodium* merozoites formed during asexual reproduction of the schizonts differentiate to become the sexual form of the parasites (gametocytes) that are taken up by mosquitoes during blood meals and aid in the transmission of the disease [8]. **Figure 1** [9] shows the life cycle of the *Plasmodium* parasite in the human host.

1.2. Malaria Diagnostic Techniques

Malaria diagnosis entails detection of the presence of the *Plasmodium* parasites in the patient blood and determination of the following parameters; species, life stages, and parasitemia estimation—an indicator of disease severity given by either the number of *Plasmodium* parasites or the number of infected erythrocytes

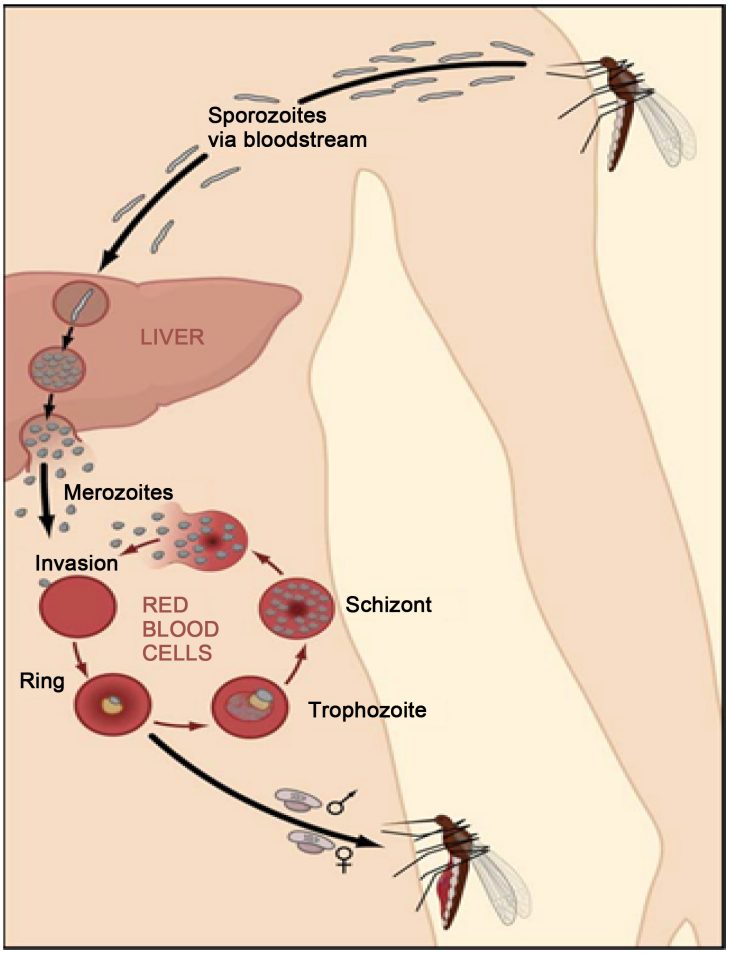

Figure 1. *Plasmodium* life cycle in the human host. Sporozoites are released into the blood by the mosquito, they are delivered to the liver for incubation and multiplication. After multiplication, the merozoites are released into the blood to attach red blood cells. While in the red blood cells they feed on hemoglobin, undergo cell division and eventually make the cell rapture for the merozoite to be released and attach more cells. Some merozoites differentiate to be the sexual form and these are the ones that the mosquito takes during blood meal to perpetuate transmission. The diagram is adopted from ref [9].

per unit volume of blood. A number of malaria diagnostic techniques exist and more are under development. The gold standard method is optical microscopy [10] [11]. The technique involves examination of stained blood smear samples using a light microscope to aid visualization of *Plasmodium* parasites in erythrocytes. Labeling (staining) of blood smear samples with Giemsa is undertaken prior to examination of the slides. Staining helps to highlight the parasites' pigment (hemozoin) and hence makes the parasites conspicuous. This technique is time-consuming and labor-intensive due to the need for sample preparation and the enumeration of detected parasites in the microscope field of view. A single malaria test takes between 30 to 60 minutes. In addition, reliability of the results from this method depends on the experience of the microscope operator.

Other malaria diagnostic methods include; Polymerase Chain Reaction (PCR) based technique [12], *Plasmodium* parasite antigen detection based technique also referred as Rapid Diagnostic Test (RDT) [13] and Fluorescence microscopy-based techniques [11]. Antigen-based technique is widely used and boasts of a relatively fast detection speed of about fifteen minutes. However, the technique has low parasite detection sensitivity and specificity especially when the parasite load is low in the blood. The test is also relatively expensive with a single test costing about 1US$. In contrast, PCR technique has high detection sensitivity and specificity. It probes and amplifies genetic material of the *Plasmodium* parasites present in the blood. However, the technique demands the use of sophisticated equipments and expensive reagents. Besides, a highly skilled operator is also required to perform the test. The technique is therefore used in research laboratories and confirmation of malaria tests. Fluorescent based technique relies on staining of blood smears with a fluorescent dye that would highlight the parasite nucleus. A fluorescent microscope is then used to observe the stained blood sample. The technique has also not yet found wide clinical adoption in malaria screening due to high cost of equipment, reagents and demand for highly skilled expertise.

Some of the malaria diagnostic techniques reported by literature as being under development include; Third Harmonic Generation (THG) technique [14], Magneto-optic based technique [15], and computer vision assisted optical microscopy technique [16] [17]. Label-free optical microscopy using multispectral imaging and chemometrics techniques has also been explored [18] [19]. Recently, a few publications have reported on the development of Photo-Thermal (PT) and Photo-Acoustic (PA) based techniques for *in vivo* malaria diagnosis [4] [20] [21] [22] [23]. Preliminary results regarding the techniques' performance are impressive but more work is needed before the method can be clinically accepted for malaria screening.

Generally, the mainstream malaria diagnostic techniques suffer from a number of shortcomings [10] [11] which include low detection sensitivity and specificity, labor and time intensiveness and demand for reagents and sophisticated equipments which are often expensive and inaccessible to people in resource scarce settings where often malaria is endemic. There is therefore an urgent need for development of new and more efficient techniques for malaria diagnosis. The remaining part of this paper explains in detail the principle behind photoacoustic-based malaria diagnostic technique and the reported progress.

2. Photoacoustic Effect and Its Application in Biomedical Imaging and Diagnostics

Photoacoustic effect is a process in which acoustic waves in the Ultrasound (US) frequency range are emitted upon absorption of optical radiation by chromophores (light absorbing molecules). The phenomenon was first reported in 1880 by Alexander Graham Bell [24]. Photoacoustic effect involves conversion of the

absorbed optical energy to thermal energy which leads to temperature rise and thermo-elastic expansion in the irradiated region. Consequently, a transient pressure wave is generated and propagates through the sample. Reported biomedical applications of the technique include; anatomical and functional imaging of biological tissue [25]-[32], disease diagnostics [33]-[43] as well as guidance and monitoring of therapeutic interventions [44] [45].

Efficient generation of PA signals demands two conditions to be satisfied; thermal and stress confinement [46]. This means that both heat conduction and thermo-elastic expansion are confined within the illuminated tissue region during optical excitation time span. Generation of PA waves therefore requires use of either Time Domain (TD) techniques (where short optical pulses, typically in nanosecond range are used to excite the sample) [46] [47] [48] [49] or Frequency Domain (FD) techniques where intensity modulated Continuous Wave (CW) laser light is used to generate ultrasonic pressure waves in tissue [50] [51] [52] [53] [54]. In the case of FD techniques, either fixed frequency or linearly varying frequency (Chirped) optical signals are used to excite the sample. Use of pulsed optical beam produces stronger PA signals compared to fixed frequency intensity modulated optical excitation [55]. However, Chirped optical excitation combined with lock-in detection have been reported to yield PA signals whose Signal to Noise Ratio (SNR) is comparable to that of pulsed excitation [50]. They also have other salient advantages such as; wide dynamic range which implies low energy optical sources can be used and this has a congruent advantage of minimizing the risk of tissue damage due to excessive optical exposure, depth tenability, and capability to use continuous wave diode lasers as opposed to bulky and expensive pulsed lasers. Both TD and chirped FD techniques can be used for tissue depth profilometry as they carry time of flight information.

Intensity of the generated PA waves is a measure of the tissue's optical absorption coefficient μ_a—a parameter which infers the molecular composition and molar concentration of the constituent tissue chromophores (see Equation (1)) as well as the tissues mechanical and thermal properties [56]. To perform quantitative photoacoustic spectroscopy, the tissue should be excited by multispectral optical radiation (pulsed or intensity modulated) and the acoustic waves induced by each optical wavelength detected by either a focused ultrasonic transducer or transducer arrays placed in close proximity to the sample being probed. The signal is then amplified and processed to compensate for signal attenuation in the medium and the detector response. Spectroscopic inversion of the recovered initial photoacoustic pressure intensity $P_0^{\lambda_i}$ (see Equation (2)) is then performed to determine sample properties such as optical absorption coefficient spatial distribution map, molecular composition, or concentration of chromophores in the sample. Some of the reported literature on these subjects can be found in references [57]-[63].

Photoacoustic imaging and sensing boasts of a number of advantages over other imaging modalities [56]; first is high spatial resolution and sharp image

contrast due to low scattering of the generated ultrasonic signal and high optical absorption sensitivity by bio-molecules. Second is the ability to perform multi-scale imaging hence yielding images of varying spatial resolution (from sub-micrometer to centimeter scale) and therefore enabling imaging of cell organelles, cells, tissues and organs of the same specimen using a single imaging modality. This has the potential to enable simultaneous studies of biological phenomena at varying spatial and time scales with minimal intra-subject variability. Since non-ionizing optical radiation is used to excite the tissue, the technique is safe. It is also relatively cheap compared to other medical imaging modalities such as Nuclear Magnetic Resonance Imaging (NMRI) and X-Ray Computed Tomography (X-Ray CT). Recently, Photoacoustic Flow Cytometry (PAFC) has been proposed for detection of Circulating Tumor Cells (CTC) [64] [65] [66] [67]. The technique has also been explored for malaria diagnosis [21] [22] [23].

2.1. Malaria Diagnosis Using Photoacoustics

The major chromophores in malaria infected blood are total hemoglobin (Hbt) and hemozoin [20]. Hbt is the sum of oxy-hemoglobin (HbO_2) and deoxy-hemoglobin (Hb) molecules in the blood. **Figure 2** is an excerpt of the spectral plots of hemozoin (Hz) and Hemoglobin (Hb) extracted from the work of saha *et al.* [16], and Cai *et al.* [18]. **Table 1** gives values of the optical absorption coefficients and molar concentrations of Hemozoin (Hz) in an infected erythrocyte

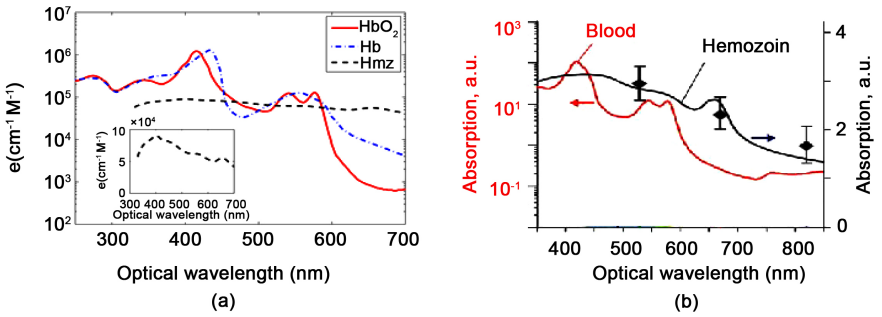

Figure 2. (a) Molar extinction coefficients of oxyhemoglobin, deoxyhemoglobin, and hemozoin [20], the inset is a zoomed up view of the hemozoin spectrum showing its characteristic peaks. (b) 70% oxygenated blood and hemozoin [22].

Table 1. Optical absorption coefficients and molar concentration of hemozoin in both healthy and *Plasmodium* infected erythrocytes. Values of μ_a are extracted from [20].

	Optical Absorption Coefficients μ_a (cm^{-1})		Hemozoin Molar concentration (M)
	λ = 434 nm	λ = 700 nm	
Healthy erythrocyte	6351.59	20.47	0
Ring stage infected erythrocyte	6075.31	40.22	5.3×10^{-13}
Trophozoite infected erythrocyte	4239.99	171.41	6.2×10^{-12}
Schizont infected erythrocyte	2858.57	270.15	1.7×10^{-11}

as extracted from the work of Saha *et al.* [20]. Based on the values of molar concentration of Hz present in the cell, it is possible to differentiate various stages of infection. Previous studies have demonstrated the possibility of *in vivo* imaging of oxygen saturation and malignant tumor hypoxia [50] [68]-[73], oxygen metabolic rate [74], methemoglobin [75] and oxygen release rate in blood vessels and individual erythrocytes using sub-wavelength Optical Resolution Photoacoustic Microscope (OR-PAM) [76]. This suggests that a similar approach can be used to detect the presence of other dominant optical absorbers in individual erythrocytes such as hemozoin in case of malaria.

The following is a brief analysis of the problem. Optical absorption coefficient due to a malaria infected erythrocyte can be described by the following equation.

$$\mu_a^{\lambda_i} = \varepsilon_{Hz}^{\lambda_i}\left[c_{Hz}\right] + \varepsilon_{tHb}^{\lambda_i}\left[c_{tHb}\right] \tag{1}$$

$\varepsilon_i^{\lambda_i}$ is the molar extinction coefficient of the i^{th} chromophore present in the cell. Both μ_a and ε are wavelength dependent hence the superscript λ_i. $\left[c_i\right]$ is the molar concentration of the i^{th} bio-molecule in the cell. The initial photoacoustic signal generated due to absorption of an optical pulse of wavelength λ_i is the product of the deposited optical energy $A_e = \mu_a^{\lambda_i}\Phi^{\lambda_i}$ and a factor called gruneisen parameter (Γ). Φ is the local optical fluence distribution in the blood which depends on the intensity of the incident light as well as blood optical properties (mainly, optical absorption and scattering coefficients). In case the chromophore relaxation after photon absorption is not 100% non-radiative, a down scaling factor is included in the product to account for the fraction of photon energy converted to heat energy. Therefore, the initial PA pressure is given by the following equation [63].

$$P_0^{\lambda_i} = \Gamma n_{th}\mu_a^{\lambda_i}\Phi^{\lambda_i} \tag{2}$$

With the transformed thermal energy density due to non-radiative relaxation of chromophores given by

$$H = n_{th}\mu_a^{\lambda_i}\Phi^{\lambda_i} \tag{3}$$

n_{th} is the fraction of optical energy converted to heat energy ($n_{th} = 1$ for non-fluorescent and non-photoluminescent chromophores). The grueneisen parameter is a thermodynamic quantity which gives the photoacoustic efficiency of the tissue, *i.e.*, the fraction of the thermal energy converted to acoustic waves.

The generated acoustic signal P_o (also referred as initial PA pressure) propagates through the tissue and is detected by an ultrasonic transducer placed at the tissue surface, a distance r from the target absorber (in this case a skin subsurface blood vessel). The detected acoustic signal, P_d differs from the initial PA signal due to; acoustic attenuation through the tissue, partial detection geometry of the traducer and the transducer's detection efficiency $\eta(f)$. The transducer output signal, P_d is therefore a product of the initial signal, P_o and a scaling factor, T_d which accounts for the described signal attenuation *i.e.*,

$$P_d(f) = T_d(f)P_o \tag{4}$$

with

$$T_d(f) = \sqrt{\eta(f)}\frac{NA}{2}\mathrm{e}^{-\alpha_o f^2 l} \tag{5}$$

where; NA is the traducers' numerical aperture, l is its focal length and α_o is the tissue attenuation coefficient. Numerical values for the parameters in Equation (4) for some commercially available ultrasonic transducers can be found in reference [77].

2.2. Reported Work

A limited number of studies have so far been carried out regarding *in vivo* photoacoustic diagnosis of malaria. Generally, the principle of *in vivo* Photoacoustic Flow Cytometry (PAFC) [78] has been adopted. The technique entails use of a focused ultrasonic transducer to detect PA signals emitted due to optical excitation of the flowing erythrocytes in blood vessels located below the skin surface. The signal peak intensity is monitored in time to keep track of the flowing erythrocytes (infected and non-infected). Healthy erythrocytes produce a nearly constant amplitude signal. Hemozoin – an endogenous malaria biomarker has either higher or lower optical absorption than hemoglobin depending on the optical wavelength used. This implies that the amplitude of *Plasmodium* infected blood would differ from that of healthy blood. The following is a brief review of the few reported literature.

Cai *et al*. [22] proposed an *in vivo* (PAFC) technique for early diagnosis of malaria. They used a focused US transducer for detection of PA signal from flowing erythrocytes irradiated by three pulsed laser lights of wavelengths 532 nm, 671 nm, and 820 nm. The signal was then amplified, digitized and loaded to a PC for processing. The system's spatial resolution was reported to be between 1 - 6 μm. Both *in vitro* and *in vivo* experiments using mice as the animal model were conducted to determine the sensitivity of the device. The authors reported that their technique was 1000 times more sensitive than optical microscopy method and could detect an infected erythrocyte 3 hours after inoculation of *Plasmodium* parasites into a healthy mouse compared to 3 days taken before detection using optical microscopy. They observed that when the optical energy used was increased beyond a certain threshold, hemozoin vapor nano-bubble formation occurred in the infected cells and this produced PA signal of increased intensity—a process termed as nonlinear photoacoustics. They also used the process to perform real time destruction of infected cells (photo-thermal malaria therapy) while monitoring the efficiency of the therapy. They observed that the PA signal ratio of infected cells to non-infected cells was highest at 671 nm optical excitation wavelength.

Lukianova-Hleb *et al*. (2014) [21] described a picosecond pulsed laser technique for generation and detection of Hemozoin induced Vapor Nano-Bubbles (H-VNB). The authors argued that when hemozoin crystals in infected RBCs absorbs the ultra-short pulsed radiation (having picoseconds range pulse width)

of sufficient energy, nano-volume liquid around the hemozoin crystals is overheated and vaporized resulting in generation of transient vapor bubbles which undergo explosive expansion and collapsing—a process that is accompanied by emission of high intensity acoustic waves. Both *in vitro* and *in vivo* sets of hemozoin positive and hemozoin negative samples consisting of blood smears, whole blood and live mice were studied. Laser pulses were shone on samples and the emitted acoustic waves detected by an ultrasonic transducer. The transducer output was coupled to a digital oscilloscope. The detected signal was then analyzed in the time domain. It was found that samples containing hemozoin produced acoustic signals of higher amplitudes than hemozoin negative samples. It was reported that the technique yielded high correlation in differentiating *Plasmodium* parasite life stages and parasitemia estimation in comparison with optical microscopy. The optical wavelength used for excitation of PA signals was 672 nm—an absorption peak for hemozoin. The optical energies per pulse used for *in vitro* and *in vivo* experiments were 10 μJ and 15 μJ respectively. Lukianova-Hleb *et al*. (2015) [23] used the technique for *in vivo* detection of *Plasmodium* parasites in humans and infected mosquitoes. Similar results were obtained.

In general, photoacoustic based malaria diagnosis boasts of several advantages over other diagnostic methods which includes; use of endogenous contrast agent (hemozoin) for parasite detection as opposed to exogenous contrast agents as is the case in optical microscopy, potential for *in vivo* diagnosis. Use of non-ionizing radiation, which makes it safe, real time monitoring and capability for early detection of the disease hence making treatment more effective. In addition, it is possible to use the technique for diagnosis of other blood related disease. However, the reported photoacoustic diagnostic systems suffer from a number of challenges which must be addressed before the technology can be clinically adopted. For example, the technique cannot perform quantitative measurement of chromophore molar concentration in absolute units. In most of the reported work [21] [22] [23], the distinction between healthy and diseased samples is made based on the relative amplitudes values of the induced PA signal between the two sets of samples. The limitation of this approach is wide signal amplitudes variability within subjects of the same class, which makes system calibration difficult. Secondly, the reported PA systems are bulky and expensive due to the demand for high-energy nanosecond pulsed multispectral laser sources, high frequency sensitive ultrasonic transducers and lock-in amplifiers. Besides, the use of fluence estimation models for quantitative PA spectroscopy makes the measurements time intensive and quasi real time. Their accuracy also depends on how well the tissue geometry is known a priori.

There is a promising trend in addressing these challenges going by the number of publications and research groups working on the problem. Simplification of PA hardware has been attempted by substitution of the bulky and expensive Optical Parametric Oscillator (OPO) tuned laser sources with much smaller and cheap diode laser sources [79] [80] [81] and Light Emitting Diodes (LEDs) [82]

that have a wide range of optical wavelengths and are commercially available. Notably, Quantellaser™ has developed a compact high energy multispectral pulsed diode laser module operating in four different wavelengths (808, 915, 940 or 980 nm) in the NIR region customized for PA applications. Its high optical pulse energy (of 1 mJ per emitted wavelength per 80 nS pulse width) is achieved by stacking multiple diode lasers of the same wavelength. Attempts to develop a compact hand held photoacoustic probe for *in vivo* imaging have also been reported [83] [84] [85] [86].

Use of chirped FD techniques in photoacoustic spectroscopy applications [50] [68] has the potential of simplifying the instrumentation complexity of the PA systems by enabling use of cheap and portable CW diode lasers as PA optical sources. Moreover, eliminate the need for computationally demanding numerical algorithms for fluence estimation. Development of a sensitive FD photoacoustic technique for hypoxia monitoring and early cancer detection has been reported [34] [35]. The technique is termed as Wavelength Modulated Differential Photoacoustic Technique (WM-DPAS). The technique uses two optical wavelengths (680 nm and 808 nm) to monitor variations in oxyhemoglobin concentration in tissue. One wavelength (680 nm in this case) should have maximum variation in extinction coefficients for oxyhemoglobin and deoxyhemoglobin while the other wavelength (808 nm which is the isosbestic wavelength) should ideally have the extinction coefficients coinciding. Two CW laser sources are intensity modulated out of phase (differentially) and their output coupled to the sample for photoacoustic excitation. The emitted PA signal is detected by an ultrasonic transducer. Upon signal preprocessing, the Fourier transform of the signal is performed to obtain both amplitude and phase signals of the differential PA time domain signal. The phase signal is said to be independent of the optical fluence and its variation is only due to variations in the molar concentration of the probed chromophore. Judging from the reported features and performance of WM-DPAS, this technique is likely to be very suitable for *in-vivo* detection of malaria.

3. Future Prospect

In order to make photoacoustic based malaria diagnostic techniques clinically viable and a method of choice, the technique has to process the following qualities; high diagnostic sensitivity and specificity, be fast, affordable, compact and portable. More work is needed in development of efficient PA techniques for detection of endogenous disease biomarkers such as hemozoin. Simplification of the instrumentation in PA systems will also be a significant contribution. As far as malaria diagnostics is concerned, it would be valuable to explore the possibility of using a PA based system to differentiate different species of *Plasmodium* in a malaria patient. This could be done by determining oxygen saturation levels in erythrocytes infected by different *Plasmodium* species. It would also be worthwhile to investigate the potential of WM-PAS in non-invasive malaria detection.

4. Conclusion

A review of reported literature on photoacoustic-based malaria diagnostic techniques and a discussion of the existing research gap have been presented in this paper. Suggestions for future refinements have also been offered.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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