Applied Physics Research; Vol. 8, No. 2; 2016
ISSN 1916-9639 E-ISSN 1916-9647
Published by Canadian Center of Science and Education

Laser-Induced Autofluorescence Technique for *Plasmodium falciparum* Parasite Density Estimation

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Received: January 7, 2016 Accepted: January 29, 2016 Online Published: March 3, 2016

doi:10.5539/apr.v8n2p43 URL: http://dx.doi.org/10.5539/apr.v8n2p43

Abstract

Malaria parasites, *Plasmodium falciparum* (*P.falciparum*) infections are taking a great toll on the lives of people worldwide, especially in developing countries. Recently, haemozoin detection using optical techniques tends to provide comparable parasite densities (PDs) estimation. We conducted feasibility studies on *P.falciparum* infected blood (*i*-blood) and uninfected blood (*u*-blood) samples from volunteers employing laser-induced fluorescence technique for PDs estimation. Fluorescence results show high intensity in *u*-blood than *i*-blood. PeakFit analysis with Loess smoothing under Lorentzian curve shows that fluorescence peak of *i*-blood appears red-shifted with increasing PDs. The Lorentzian curves depict that fluorescence peak intensity ratio increases with increasing PDs in *i*-blood samples. This technique may be potentially applied in PDs estimation to improve malaria diagnosis.

Keywords: autofluorescence, *Plasmodium falciparum*, parasite density, malaria diagnosis.

1. Introduction

Malaria diagnosis and treatment are of great interest due to the high death rate caused by malaria, especially in the developing countries (Sachs & Melaney, 2002; Hay et al., 2004; Amexo et al., 2004, WHO, 2013). The causative malaria parasites, *P.falciparum* digests haemoglobin (Hb) for nutrient in host blood stream (Moore et al., 2006). To improve the cure rates of *P.falciparum* infections, a diagnostic tool, which can detect the parasites at any stage, is greatly needed. A major decision factor in the *P.falciparum* diagnosis is evaluation of the degree of infections termed as parasite densities (PDs). The accepted technique commonly used for PDs estimation is manual assessment of the number of parasites from blood smears slides in relation to microscopic high power fields (Pammenter, 1988; WHO, 1988; Bloland & Ettling, 1999). *P.falciparum* infects red blood cells (RBCs) of any age and has the ability to develop high-grade PDs (Molla et al., 2001). Due to the rapid growth of this parasite, the PDs can double over a 2-day period without treatment (Rowe et al., 2002). In clinical settings, the stage of the PDs is essential as one of the criteria in establishing severity in *P. falciparum* infections and to study the effect of anti-malarial therapy (Dubey et al., 1999). The manual assessment technique is, however, prolonged, grueling and requires an expert microscopist (Payne, 1988; Coleman et al., 2002; Mitiku et al., 2003; Bates et al., 2004). Moreover, the accuracy of the final assessment ultimately depends on the expertise and experience of the microscopist (Pammenter, 1988; Sio et al., 2006; Opoku-Ansah et al., 2013), for which the agreement rates for assessing the same sample is remarkably low (Mitiku et al., 2003).

Development of malaria antigen test, rapid diagnostic tests (RDTs), which has threshold for detecting parasites more than 100 parasites/μl, within 15 to 20 minutes, is the technique mostly used presently(Moody, 2002; Wongsrichanalai et al., 2007). These immunochromatographic tests are commercially available in kit with all the necessary reagents, they are user-friendly, easier to perform and the associated procedure does not require extensive training or equipment to perform or to interpret the results (Peyron et al., 1994; Singh et al., 1997). Unskilled clinicians can be trained in RDTs techniques within a day (Premji et al., 1994). However, RDTs has

accompanied challenges (WHO, 2000); they give contrasting information regarding its sensitivity and specificity (Guerin et al., 2002; Murray, 2008). The agreement rate between RDTs and microscopy is comparatively low (Forney et al., 2001; Fernando et al., 2004; Wilson et al., 2011).

A number of optical techniques developed for assessing malaria parasites are based on haemozoin detection as a characteristic for *P.falciparum* infection and antimalarial activity. The haemozoin is formed from detoxified and crystallized haeme together with iron derived from partially metabolized Hb (Loyevsky et al., 2001). Several techniques such as wide-field confocal polarization microscopy (Campbell et al., 2007), laser desorption mass spectroscopy (Scholl et al., 2004), third harmonic generation imaging (Belisle et al., 2008), magneto-optical testing (Mens et al., 2010), photo migration in the field of tissue optics (Friebel et al., 2006), have been applied to whole blood in the parasite detection. These techniques are not easily applicable in malaria endemic areas because they involve expensive equipment and well-equipped laboratories (Makler et al., 1998).

In many decades, laser-induced fluorescence (LIF) diagnostic methods have been used to measure the fluorescence spectra of healthy and pathological tissue samples to find out structural difference between the tissue samples and reflect the structural characteristics of endogenous spectra about fluorophores inside the tissues (Karadaglic et al., 2009; De Goes Rocha et al., 2010; De Oliveira Silva et al., 2010; Kalnina et al., 2010; Al-Salhi et al., 2011; Masilamani et al., 2011). LIF, is therefore, an adequate analytical technique with advantages of high sensitivity, safety, non-invasiveness, low sample consumption, short testing time, and suitable for *in situ* testing. Therefore, it has become one of the most widely used spectroscopic methods for *in vivo* diagnosis in recent years. LIF on human blood have shown the regions where fluorescence occurs. A number of studies have been done on human blood samples using LIF (Gao et al., 2004; Peng & Liu, 2013). These studies have shown that fluorescence spectra of the whole human blood are within 500-900 nm and that the fluorescent peak apparently shifts with change in blood concentration. Masilamani et al., (2014) used fluorescence spectra of *P.falciparum* infected human blood plasma to diagnose malaria (Masilamani et al., 2014). In spite of this development, no work has been done to assess the whole human blood sample for the presence of *P.falciparum* infections and PDs using LIF.

In this work, we present feasibility studies done on *P.falciparum* infected whole human blood samples from volunteers utilizing laser-induced fluorescence technique for PDs estimation.

2. Experimental Procedure and Methods

2.1 Sample Preparation

Blood samples from 80 volunteers were collected in test tubes containing ethylenediamine tetra acetic acid (EDTA) as an anticoagulant. 5 ml of blood samples was collected from each of the volunteers after being tested positive for *P.falciparum* malaria parasites (infected). These samples were obtained before treatment. Following antimalarial treatment and being tested negative (uninfected), another 5 ml of blood sample, was collected from each of the volunteers. Thus, a total of 160 blood samples were collected from the 80 volunteers. The blood samples collection was handled by a phlebotomist. The infected blood samples were grouped by a pathologist into four categories (+, ++, +++, ++++) based on PDs, which indicates the level of infection. For each blood sample, 2 ml was used for fluorescence spectral determination. The volunteers were informed about the investigation, and proper consents were obtained. The blood samples and volunteers data collection procedures were approved by Ghana Health Service Ethical Review Committee (GHS-ERC-09/05/14). The ages of the volunteers range from 1-90 years with mean age of 16 years. 40 % of volunteers were males and 60 % of them females.

2.2 Fluorescence Spectral Acquisitions

The 2 ml each of infected (*i*-blood) and uninfected blood (*u*-blood) samples was put into a quartz cuvette and fluorescence spectra were recorded using a laser source with 30 mW power, and excitation wavelength of 405 nm (O-Like). Figure 1 shows a schematic diagram of the set-up for fluorescence spectra recordings. The excitations from the blood samples were cut-off with the aid of a long-pass filter (Edmund Optics Inc.). The fluorescence scattered light was fiber-coupled via a 2.0 m SMA-905 optical fibre (Ocean Optics BIF600-UV-VIS) to a USB 4000 spectrometer (Ocean Optics-UV-VIS) connected to a Toshiba Laptop (3.0 GHz 8.0 GB, AMD A10-4600M). For each *i*-blood and *u*- blood samples, a total of 1420 spectra were recorded for each sample at a room temperature of 23 ^{o}C and the average of each set used for analysis. The power of the laser at illumination position was attenuated with a 1.0 neutral density (Thorlabs Inc., Absorptive filter) filter to avoid photo-bleaching. This was verified by repeating the experiment three times for each sample for an optimal duration of 120 seconds and observing no inter-replicate spectral differences. The fluorescence data was extracted with our own developed Matlab codes.

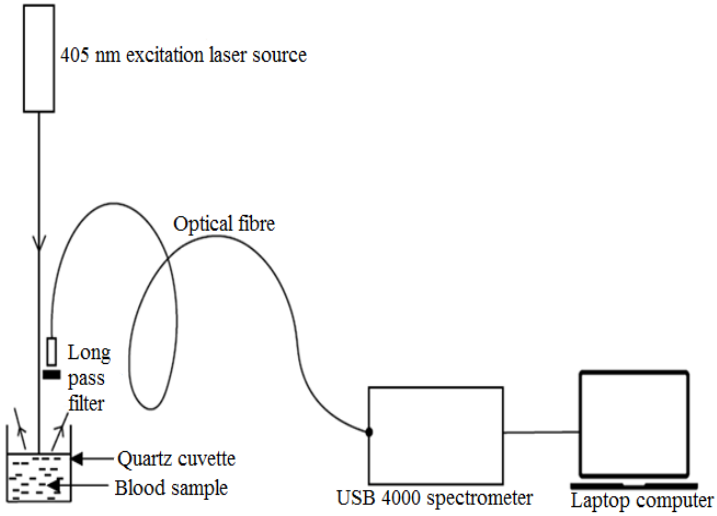

Figure 1. Experimental set-up for recording fluorescence spectra of blood samples from 405 nm excitation source

3. Results and Analysis

The mean fluorescence spectra for *i*-blood and *u*-blood samples were determined via our developed Matlab code (R2010a Matlab 7.10.0, Mathworks Inc.) the normalized spectra are shown in Figure 2. The mean fluorescence spectrum of *u*-blood samples is higher in intensity and broader in width than the *i*-blood samples. Within the *i*-blood samples, as the PDs increases the fluorescence spectrum decreases in intensity and in width. Each spectrum peaks around 612±1 nm and shows a pronounced shoulder around 685±2 nm. This reduction in fluorescence intensities in *i*-blood samples may be attributed to the presence of haemozoin in the blood, acting as a fluorescence quencher. It may, also, be attributed to the reduction in the Hb concentration, as a result of the presence of the parasites feeding on the Hb in the blood.

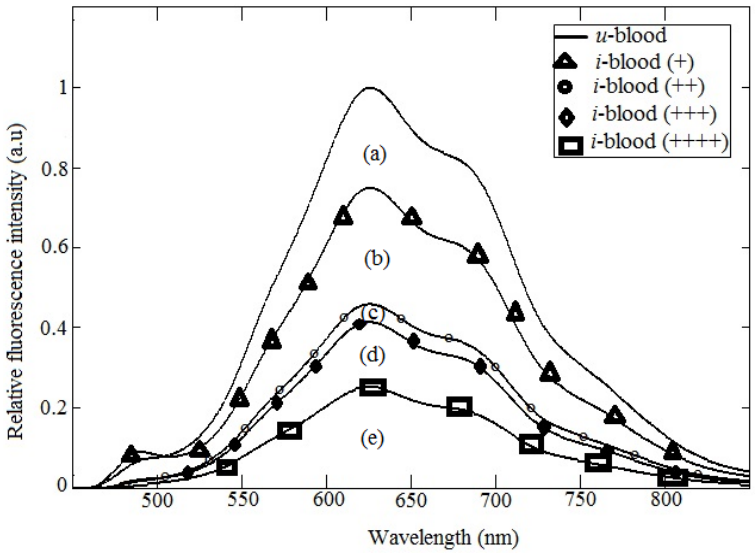

Figure 2. Normalized fluorescence spectra of uninfected blood (a), and *Plasmodium falciparum* infected blood with parasite densities: (b) (+), (c) (++), (d) (+++) and (e) (++++) at $\lambda = 405$ nm excitation

These observations may be attributed to variations in the energy distribution between fluorophores resulting to fluorescence quenching in *i*-blood samples. Increasing concentrations of the haemozoin or a reduction in Hb concentration with increasing PDs may have accounted for the decreasing intensities of the *i*-blood spectra. The hemozoin formation is preceded by formation of metalloprotein hemoglobin, which binds to an erythrocytic cell

membrane to form hemichrome. The hemichrome has a unique spectral signature which is different from haemoglobin (Peng et al., 2013; Masilamani et al., 2014).

In order to bring out the latent shoulder peaks within the fluorescence spectra, PeakFit software (4.11 version, Jandel Scientific, Germany) was used to analyze the fluorescence spectra from the blood samples. The PeakFit software, applied somewhere (Anderson et al., 2004), combines Loess smoothing function, Marquardt-Levenberg and Lorentzian spectral functions for analyzing the fluorescence spectra. It determines the accurate total minimum value of the sum of the squared deviations. The value of the relationship coefficient and the pattern of residue determine the quality of the fit. The Lorentzian spectral function aided with the choosing of a reasonable corresponding fit of the spectral. This enabled the determination of a high-quality *t*-test, standard errors for peak amplitude, peak centre and Full Width at Half Maximum (FWHM) for further analysis.

The characteristics of the Lorentzian bands obtained from the curve fit analysis of the fluorescence spectra for *u*-blood and *i*-blood samples with different PDs are shown in Figures 3 and 4 respectively. In the figures the ordinate scales are different across the sets. This was done as a visual aid to make the underlying peaks more conspicuous. The decision for the Lorentzian band was based on precise fit of the measured spectra with high-quality values. The figures show broader spectra bands for *u*-blood samples (Figure 3) as compared to *i*-blood samples (Figure 4). This can be attributed to the interaction between neighbouring molecules and the large number of vibration levels in *u*-blood samples with endogenous fluorophores in *i*-blood samples undergoing transformation.

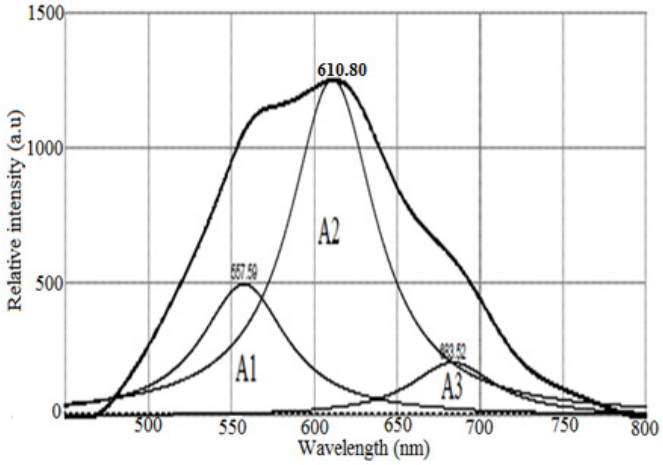

Figure 3. Fluorescence intensity distribution of uninfected blood sample fitted with Lorentzian function

Three constituent bands, A1, A2 and A3 were extracted for *u*-blood and *i*-blood samples. The A2 band, reported elsewhere (Opoku-Ansah et al., 2014), originated from the fluorescence spectra of Hb whilst A3 band originated from the fluorophores of the collapsed feeble bonds (Gao et al., 2004). The reduction in the relative fluorescence intensities of the A2 bands as the PDs increases may be attributed to the reduction in the concentration of Hb (Moore et al., 2006). Increase in PDs decreases the relative fluorescence intensities of the A3 bands. These observations may be attributed to self-absorption phenomenon and structural rigidity of the fluorophores. The *P.falciparum* parasite growing in the *i*-blood secretes enzymes to digest the proteins in the RBCs. The RBCs membrane becomes enlarged and inelastic. The deformability therefore reduces in the RBCs of *i*-blood. The higher the PDs in *i*-blood, the weaker the absorbed energy and the fluorescence peaks are shifted to longer wavelength (Boulnois, 1986).

For meaningful results in most LIF applications, the fluorescence intensities ought to be compared due to the relative nature of the spectra. In that vein, fluorescence peak intensity ratio (PIR) was derived from the fluorescence spectral bands A2 and A3 using the relation in Equation (1):

$$PIR = \frac{I_2 - \beta I_3}{I_3} \tag{1}$$

where β is a constant, I_2 and I_3 are the peak intensities of $A2$ and $A3$. The peak fit analysis of the fluorescence spectra from the blood samples had accompanied uncertainties. Using these uncertainties, error margins for the PIR was calculated for the blood samples. Table 1 shows the variations in peak wavelengths of *u*-blood and *i*-blood samples for the different PDs under the Lorentzian curves and the PIR values. The PIR values for *i*-blood ranges from 0.4 to 1.2, which is above that of *u*-blood. The mean PIR for *u*-blood samples was calculated to be less than 0.3.

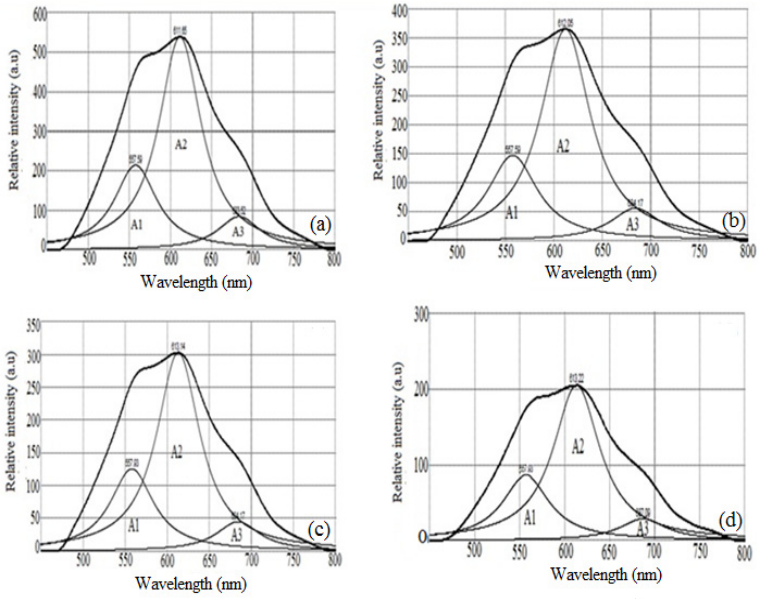

Figure 4. Fluorescence intensity distribution of *P.falciparum* infected blood sample with parasite densities: (a) (+), (b) (++), (c) (+++) and (d) (++++) fitted with Lorentzian function

Table 1. Peak wavelengths of uninfected blood and *P.falciparum* infected blood samples with different parasite densities under the Lorentzian curve

Blood samples		Peak wavelength (nm)			Peak intensity ratio
		A1	A2	A3	(PIR)
u-blood		557.51±0.04	610.80±0.03	683.52±0.05	0.23±0.07
i-blood	+	557.59±0.15	611.65±0.05	683.52±0.04	0.43±0.04
(PDs)	++	557.59±0.09	612.05±0.08	684.17±0.09	0.56±0.05
	+++	557.93±0.07	613.14±0.05	684.17±0.18	0.78±0.08
	++++	557.94±0.04	613.22±0.10	687.09±0.17	1.21±0.13

Energy transfer between endogenous fluorophores in *i*-blood with different PDs triggered the blood absorption by ground state molecules, resulting in fluorescence quenching in *i*-blood. When blood sample with different PDs was excited by the same light source, peak wavelength fluorescence spectra are red-shifted with the increment of PDs in *i*-blood. This red-shifted phenomenon with the increase of PDs in *i*-blood could be due to the environmental changes of the fluorophores as a result of haemozoins in *i*-blood.

Figure 5 depicts the graphical variations in PIR values (Table 1) for *i*-blood with different PDs. It can be observed from Figure 5 that PIR increases with increasing PDs. These observations suggest that within the range of fluorescence spectra of Hb, fluorescence peak intensity for the Lorentzian curve under A2 increases whilst fluorescence peak intensity for Lorentzian curve under A3 decreases. This suggests that Hb degradation in *i*-blood results in high PIR for the spectra region representing A2 than in A3.

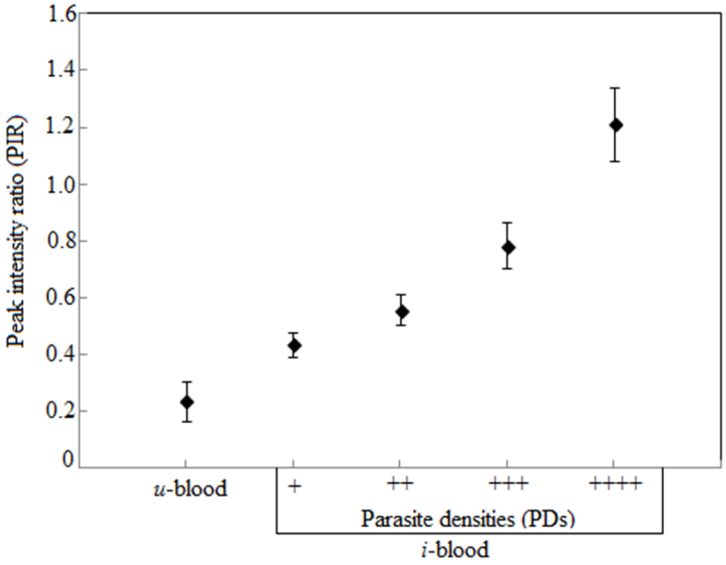

Figure 5. Variations in peak intensity ratio of *P. falciparum* infected blood samples with different parasite densities

5. Conclusions

In this work, we have shown that it is feasible to use laser-induced fluorescence (LIF) technique for *P. falciparum* parasites density (PDs) estimation. Employing LIF technique, *u*-blood shows high fluorescence intensity than *i*-blood. Fluorescence peak intensity of *i*-blood exhibits red-shifted phenomenon with increasing PDs. This research has shown that any PIR values greater than 0.3 has *P.falciparum* parasite. And, the PIR values of the various sample PDs has been found to range from 0.4 to 1.2. This spectroscopic technique may be potentially applied in PDs estimation for improved malaria diagnosis.

Acknowledgements

We wish to express our profound gratitude to International Programme for Physical Sciences (IPPS), International Sciences Programme (ISP), Uppsala University, Sweden for funding. We wish to express our appreciation to the Office of External Activities and Associate scheme of Abdus Salam ICTP, Trieste, Italy for funding and stay at ICTP. Immense thanks to Aboma Merdasa of Department of Chemical Physics, Lund University, Sweden for assistance. We also thank members of African Spectral Imaging Network (AFSIN). Finally, to all members of Laser and Fibre Optics (LAFOC) Research Group for their motivation and contributions.

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