Diagnosis of malarial infection using change in properties of optically trapped red blood cells

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ABSTRACT

Background: In previous work studying the properties of red blood cells (RBCs) held in an optical tweezers trap, we observed an increase in the spectrum of Brownian fluctuations for RBCs from a *Plasmodium falciparum* culture—due to increased rigidity of the cells—compared to normal RBCs. We wanted to extend the study to patient samples, since the earlier work was done with cultures grown in the lab.

Methods: Individual RBCs were held in an optical-tweezers trap. Its position fluctuations were measured and the power spectrum determined. The corner frequency ($f_c$) of the spectrum gave a quantitative measurement of the spectrum.

Results: The value of $f_c$ was 25 Hz for normal cells, which increased to 29 Hz for infected cells—both for *P. falciparum* and *Plasmodium vivax* infections.

Conclusion: The technique of measuring $f_c$ can be used as a screening tool for malaria in patients with fever, since RBCs not carrying the parasite will also show the change due to the bystander effect, irrespective of whether it is caused by *P. falciparum* or *P. vivax*.

Early and accurate diagnosis of malaria is essential to manage this deadly disease because it continues to remain a global public health problem [1,2]. In earlier work [3], we had studied the properties of red blood cells (RBCs) trapped in an optical-tweezers trap, and found an increase in the spectrum of Brownian fluctuations from nRBCs (normal cells) to iRBCs (infected cells). The change was primarily due to increased rigidity of iRBCs, which, though slightly controversial, is fairly well established [4–6], and what prevents diseased cells from behaving normally. Interestingly, we found a bystander effect [7], in which hosting and non-hosting RBCs showed the same change in their properties. Further experiments with various inhibitors have confirmed that the substance responsible for the bystander effect is mediated by ATP or cAMP [8,9]. The bystander effect is consistent with several recent reports which discuss the role of extracellular vesicles in influencing cell-to-cell communication among RBCs [10–12].

However, the use of the tweezers method for disease diagnosis remained questionable, partly because the earlier studies were done with cultures grown in the lab. The cultures only studied *Plasmodium falciparum* infection, but could not be applied to *Plasmodium vivax* infection. This was because *P. vivax* predominantly infects reticulocytes, and it is quite difficult to maintain long-term cultures of such cells in the lab.

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Scientific background on the subject

Optically trapped red blood cells (RBCs) could be potentially used for diagnosis of malaria, particularly because it takes advantage of the bystander effect. However, its use in actual patients suffering from malaria remained questionable because earlier studies were done with cultures grown in the lab.

What this study adds to the field?

This study extended our previous work by extending it to patients suffering from malaria and admitted to nearby hospitals. It also helped us to study *P. vivax* infection, which are difficult to culture in the lab (because they predominantly infect reticulocytes) even though they form the majority of patient infections.

In this work, we extend the use of this method to blood samples drawn from patients suffering from malaria, obtained from hospitals in and around Bangalore. This has now allowed us to study *P. vivax* infection, since they form the majority of samples. Consistent with our earlier work using cultures, the corner frequency—which is a measure of the Brownian spectrum—increased from nRBCs to iRBCs for both kinds of infections. The results give a direct confirmation of the bystander effect because:

(a) For *P. falciparum* samples, no attempt was made to see if the cell actually hosted the parasite or not. Given the low parasitemia count in patients, it is likely that most of the RBCs being studied are non-hosting, and therefore made rigid by the substance released into the blood stream.

(b) For *P. vivax* samples, which predominantly infect reticulocytes and since our study only looks at mature RBCs, there is negligible chance that the parasite is inside the RBC. Therefore, the change in rigidity can only be caused by some substance other than the parasite.

The bystander effect has been studied in detail in our previous work with cultures—e.g. by looking exclusively at non-hosting RBCs, or by incubating nRBCs in a spent medium.

The above results show that the tweezers technique can be used as a general screening test for all kinds of malarial infection. It has the following advantages over other existing methods.

(i) It is easily automated.
(ii) It is statistical, and hence does not require trained personnel.
(iii) It is independent of the stage of development of the parasite, and hence does not require the blood sample to be drawn at a particular time—during a febrile episode, for example.
(iv) It can be used during the earliest stage of the disease when the parasitemia count is extremely low and the only symptom is high fever, because it takes advantage of the bystander effect and hence does not require the RBC to host the parasite.

Methods

Optical tweezers

The set up for the optical-tweezers trap has been described in detail in our earlier work [3], and is reviewed here for completeness. As shown in Fig. 1, it consisted of a 100× oil-immersion objective coupled to a Zeiss inverted microscope. The trapping laser was formed from an infrared laser operating at 1064 nm. The output of the laser was imaged on to the 5 mm back plane of the objective using a pair of lenses. The incident power at the back plane of the lens was 300 mW, which got reduced to 10% of this value at the sample plane due to transmission of the objective at the trapping wavelength of 1064 nm.

A small amount of red laser beam (from a HeNe laser operating at 633 nm) was mixed with the trapping laser. The back-scattered light from this beam was used to monitor the position of the trapped particle—its position was measured using a quadrant photo-detector (QPD). The corner frequency $f_c$ of Brownian fluctuations for each trapped RBC was measured as follows:

(i) Its position along the x direction was measured using the QPD.
(ii) The position was measured as a discrete time series at a sampling rate of 16 kHz for a total sample length of 100,000 points.
(iii) The data were fast Fourier transformed (FFT) to yield the spectrum of Brownian fluctuations.
(iv) The spectrum was fitted to a Lorentzian function to get $f_c$.

The trapped RBC was imaged with a video camera.

Fig. 1 Experimental schematic of the optical tweezers set up.

Abbreviations used: M: mirror; DM: dichroic mirror; L: lens; QPD: quadrant photo detector; IR: infrared.
Ethics

The study was approved both by the Bangalore Medical College and Research Institute Ethics Committee (reference number: BMCRI/PS/04/2015-16) and by the Indian Institute of Science Human Ethics Committee (reference number: 25/2014). Details of the study were explained in native language to the participants. Samples were taken after obtaining written consent from each participant.

Blood samples from patients

Blood samples from both in-patients and out-patients from hospitals coming under the purview of Bangalore Medical College and Research Institute (BMCRI) were included in the study. First, a full medical history and clinical examination was recorded on a standard form. Next, patients who exhibited febrile episodes and other symptoms of malaria were tested for the presence of parasites in their blood. For this, Giemsa staining of peripheral blood smear from a finger-prick blood sample was used. Malarial infection was confirmed using microscopic examination of the blood smear. Differential diagnosis for the two strains was done by examination of the smear for the presence of either ring stage parasites for the *P. falciparum* strain; or ring, trophozoite, and schizont stage for the *P. vivax* strain. Venous samples (volume 2 ml) from confirmed malaria patients were collected in anticoagulant-containing (EDTA) vacutainer tubes, and transported on ice for the optical-tweezers studies in the lab.

RBC preparation

The RBCs used in the study were prepared as follows:

(i) Blood sample was centrifuged at 2,000 rpm for 15 min, and plasma and buffy coat removed.

(ii) The RBCs were suspended in 1× PBS solution.

(iii) Individual cells were trapped in the optical-tweezers trap.

Results

For the $f_c$ measurements, 2 *P. falciparum* and 4 *P. vivax* samples were used. As control, a sample from a healthy volunteer in the lab was used. From each sample, an individual RBC was trapped and its corner frequency measured. This procedure was then repeated so that $N = 25$ independent values were obtained. The average and standard deviation for each data set were determined.

The results are summarized in Fig. 2. The point shown in the graph is the average value, while the error bar is the standard deviation in the mean (standard deviation in the set divided by $\sqrt{N}$). As seen, the value of corner frequency for nRBCs from the control sample is centered around 25 Hz. This value is similar to what we have measured in our earlier work in Ref. [7]. This ensures that the alignment of the laser is similar to what we had before, and is important because the optical-tweezers trap is highly sensitive to alignment. Such calibration also ensures that the value of $f_c$ is “absolute”—in the sense that the $f_c$ value for nRBCs is smaller than 25 Hz with poor alignment and reaches 25 Hz only with the best alignment.

The figure shows that iRBCs from both *P. falciparum* and *P. vivax* infections have a higher corner frequency centered around 29 Hz. This increase of about 15% is similar to what we have seen in our earlier studies with a culture—showing that our earlier results in Ref. [7] are equally valid for cultures and patient samples. In particular, the results are a direct confirmation of the bystander effect that we had observed earlier. This is because *P. vivax* infects mainly reticulocytes, whereas the RBCs used in the study are mature cells. In addition, the RBCs were chosen at random and no attempt was made to see if the cell hosted the parasite. Thus, it is likely that most of the RBCs from *P. falciparum* samples are non-hosting. This makes the tweezers technique advantageous for malarial diagnosis compared to other methods, which primarily rely on visual identification of the parasite.

It is also well known that a trapped RBC gets folded in the trap because the RBC is biconcave in shape. But the folding time—the time elapsed before complete folding in the trap—for nRBCs is different from iRBCs, mainly because their rigidities are different. The trapped RBC was imaged for several seconds using the video camera. This also tells us that the particle being trapped is an RBC, and not a reticulocyte which is roughly two times larger in size. Image graphs before and after folding for both kinds of RBCs are shown in Fig. 3. The folding time increases from 0.8 s for nRBCs to about 1.33 s for iRBCs (measured for all infected samples but shown only for one). Thus, consistent with other studies [13], the folding time in the trap can also be used to determine whether a cell is infected. However, using it as a diagnostic tool has potential problems because (i) it is not specific to malaria, and (ii) is not easily automated.
Conclusion

In summary, we have shown that an optical-tweezers trap can be used to measure morphological changes in RBCs due to malarial infection, and hence can be used for disease diagnosis. The power spectrum of Brownian position fluctuations in the trap was measured, and characterized by its corner frequency $f_c$. The value of $f_c$ showed an increase from normal cells to infected cells due to an increase in rigidity caused by the infection. In contrast to previous studies where we used $P. falciparum$ cultures grown in the lab, here we use patient blood samples—both due to $P. falciparum$ and $P. vivax$ infections. Studies with $P. vivax$ samples unequivocally demonstrate the bystander effect, because $P. vivax$ primarily resides in reticulocytes whereas only mature RBCs are used in the study. The bystander effect results in morphological changes in the RBC even though it does not host the parasite. This is useful for diagnosis of the disease over other existing methods, especially at an early stage when the parasitemia count is very low.

Conflicts of interest

The authors declare that they have no competing interests.

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Fig. 3 Images before and after folding in the trap. (A) Folding time for nRBCs is 0.8 s. (B) Folding time for iRBCs is 1.33 s.
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