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High-throughput and label-free parasitemia quantification and stage differentiation for malaria-infected red blood cells

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Abstract

This work reports a high throughput and label-free microfluidic cell deformability sensor for quantitative parasitemia measurement and stage determination for *Plasmodium falciparum*-infected red blood cells (*Pf*iRBCs). The sensor relies on differentiating the RBC deformability (a mechanical biomarker) that is highly correlated with the infection status. The cell deformability is measured by evaluating the transit time when each individual RBC squeezes through a microscale constriction (cross-section $\sim 5\mu\text{m} \times 5\mu\text{m}$). More than 30,000 RBCs can be analyzed for parasitemia quantification in under 1 min with a throughput ~ 500 cells/s. Moreover, the device can also differentiate various malaria stages (ring, trophozoite, and schizont stage) due to their varied deformability. Using *Pf*iRBCs at 0.1% parasitemia as a testing sample, the microfluidic deformability sensor achieved an excellent sensitivity (94.29%), specificity (86.67%) and accuracy (92.00%) in a blind test, comparable to the gold standard of the blood smear microscopy. As a supplement technology to the microscopy and flow cytometry, the microfluidic deformability sensor would possibly allow for label-free, rapid and cost-effective parasitemia quantification and stage determination for malaria in remote regions.

Keywords

cell deformability; single cell; malaria; microfluidics; parasitemia quantification

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1 Introduction

Malaria is one of the most prevalent infectious diseases for which almost half of the global population is at risk^{1–4}. According to the world malaria report, more than 212 million people suffer from malaria infections, and over 400 thousand people died from this preventable disease each year¹. Parasitemia is a critical parameter for quantifying the parasite load in the organism and indicates the degree of an active parasitic infection^{5, 6}. Quantitative measurement of parasitemia and stage determination is important in many phases of malaria assessment, such as the diagnosis and the therapy follow-up, particularly in the chronic phase⁷. The microscopy based morphology analysis still remains the gold standard for parasitemia quantification. The advantages of microscopy are several folds: it is possible to differentiate between species, quantify parasitemia with high sensitivity (0.001%) and observe asexual stages of the parasites^{8,9}. Nevertheless, the microscopy method has several drawbacks, including the reliance of an expert reading Giemsa-stained blood smears, subjectivity, low reproducibility and relatively low speed (>5 mins/slide)¹⁰. The flow cytometry could offer higher sensitivity and overcome the speed limitation of the microscopy. However, the high cost of the instrumentation and the labeling process limits its widespread usage^{11, 12}. In addition, both microscopy and flow cytometry require time-consuming staining or labeling processes. It is highly desirable to develop a label-free technology for parasitemia quantification. To that end, Peng *et al.* recently reported a micromagnetic resonance relaxometry (MRR) based label-free platform for parasitemia quantification in whole blood^{13, 14}. This method detects the variation in spin-spin relaxation time of MRR signals from paramagnetic hemozoin particles with a bulk measurement.

It is well known that *Plasmodium falciparum*-infected red blood cells (*Pf*iRBCs) become increasingly rigid (less deformable) as they mature¹⁵, thus the deformability has the potential to be used as a mechanical biomarker to distinguish the healthy and the infected RBCs at various stages¹⁶. To date, various microfluidic-based methods have been developed to interrogate the cell deformability. For example, hydrodynamic pressure-based interrogation (e.g., inertial microfluidics^{17–20}, and real-time deformability cytometry²¹), and physical constraint-based interrogation (e.g., single micropore^{22, 23} and microchannel^{24–27}, and arrayed microchannels^{28–34}). Despite success in the qualitative characterization of *Pf*iRBCs deformability properties, a high throughput quantitative parasitemia measurement at the single cell level has yet to be developed.

In this work, we demonstrated a label-free microfluidic cell deformability sensor for quantitative and high-throughput parasitemia measurement and stage determination for *Pf*iRBCs. The deformability sensor is capable of analyzing more than 30,000 RBCs within 1 min (throughput ~ 500 cells/s). Moreover, the device is able to differentiate various malaria stages among ring, trophozoite, and schizont stage. With 0.1% *Pf*iRBCs as a testing sample, the microfluidic deformability sensor achieved an excellent sensitivity (94.29%), specificity (86.67%) and accuracy (92.00%) in a blind test, comparable to the gold standard of the blood smear microscopy. With minimal manual work, the microfluidic deformability sensor could deliver sensitive and accurate parasitemia measurement directly from a small volume of blood samples within a few minutes, which is highly promising for sensitive malaria diagnosis in field settings.

2 Materials and methods

2.1 *P. falciparum* culture and sample preparation

The *P. falciparum* 3D7 was cultured in type O⁺ human red blood cells in RPMI 1640 medium supplemented with 25 mM HEPES, 50 mg/L hypoxanthine, 25 mM NaHCO₃, and 0.5% AlbuMix (Thomas Scientific, U.S.A). The parasite culture was synchronized by treatment of ring-stage parasites with 5% D-sorbitol. The parasites were taken at 2 h, 18 h, and 32 h post synchronization to represent the ring, trophozoite, and schizont as determined by microscopy. The cultured *P. falciparum* sample prior to electrical sensing measurement consisted of a mixture of *Pf*-iRBCs and uninfected RBCs at a parasitemia of ~3%. In all electrical measurements, 30 μ L blood was diluted in 1 mL buffer solution consists of phosphate buffer saline (PBS; Thomas Scientific, U.S.A) and 1% w/v Bovine Serum Albumin (BSA; Sigma-Aldrich, Canada).

2.2 Thin smear microscopy

Parasitemia was examined by Giemsa staining. A drop of parasite culture was smeared on a glass slide, dried at room temperature, fixed with methanol for 10 sec, and stained with Giemsa working solution for 10 min. The slide was then rinsed briefly in tap water, dried and examined under a microscope. Parasitemia was determined by counting at least 1000 RBCs under a microscope, equipped with a 100 $\times$ oil-immersion objective.

2.3 Microfluidic device fabrication

The cell deformability sensor was designed in a layout editor and printed on a transparent mask. The casting mold was fabricated by a standard double layer lithography process on a 4-inch silicon wafer³⁵. The microfluidic chips were made of polydimethylsiloxane (PDMS) by casting onto the SU8 mold. It consists of two layers with different thicknesses: micropore area with ~5 μ m and loading channel area with ~25 μ m, respectively. The loading channel's cross-sectional area (1000 $\times$ 25 μ m²) is much larger than that of the micropore to reduce the hydrodynamic and the electrical resistance. The micropore size needs to be small enough for improved time resolution but also large enough for avoiding the micropore clogging by cells. We found 5 μ m is the optimized size for the experiments. The length of the micropore (~45 μ m) also needs to be optimized. A longer channel makes the cell transit time longer, amplifying the differences between cells and making the device more sensitive. On the other hand, a longer channel increases the risk of clogging. To avoid the micropore clogging by cell aggregates and debris, we have incorporated two stages of filters on the microfluidic chip (filter 1 in the loading channel area and filter 2 in the micropore area, Figure 1a).

The Au/Cr electrodes (20 nm adhesive Cr layer and 80 nm Au layer) were evaporated on a cover glass (thickness ~ 130 μ m, Ted Pella) through a laser machined Polymethylmethacrylate (PMMA) shadow mask. To enhance the signal-to-noise ratio, the microelectrodes were positioned close to the micropore area. The PDMS replica was permanently bonded to the cover glass through oxygen plasma treatment.

2.4 Electrical measurement and data analysis

After device fabrication, the microfluidic chip was housed inside a home-made Faraday cage to shield the environment noise. A constant voltage (~ 600 mV) was applied across the micropore constriction. The polarity of the applied voltage was switched periodically to avoid severe electrode damage due to possible electrochemical reactions. The ionic current was monitored as each single individual RBC translocating through the micropore. An adjustable pressure source was used to drive the cells on the microfluidic chip. Since the buffer solution ($1\times$ PBS) were constantly flowing during the experiments, the accumulation of the electroactive species near the electrode is very unlikely to interfere with the ionic current measurement. The ionic current traces were recorded by an amplifier (Axopatch 200B, Molecular Devices, U.S.A). The analog output of the amplifier was sampled with 16-bit DAQ card (NI PCI-6363, National Instruments, U.S.A) and a data acquisition software (LabVIEW). The sampling rate for the measurement was 100 kHz. A custom-built MATLAB (MathWorks) program was developed to analyze the data off-line. The transit time was extracted from each individual cell when translocating the micropore.

3 Results and Discussion

3.1 Sensing principle

Figure 1a shows the schematic and the principle of the microfluidic deformability sensor. The corresponding equivalent circuit model is presented in Supplementary Information (Figure S1). The electrical resistance of the deformability sensor is mainly dominated by the micropore constriction due to the cross-sectional dimension ratio between the micropore and the loading channel. When an RBC squeezes through the micropore, an ionic current dip occurs as a result of the reduced conduction cross-section. In a typical experiment, a pressure of 1–1.5 psi is applied, corresponding to a flow rate of ~ 180 $\mu\text{m}/\text{ms}$.

Since the micropore has a slightly smaller size than that of the RBCs (diameter ~ 7 μm), RBCs have to squeeze through the micropore. The cell transit time is a complex function of various parameters: the applied pressure, the cell size, the cell deformability, the micropore size, and the PDMS and cell surface properties *etc.*^{36, 37}. These parameters except the cell deformability were controlled to remain the same for a given experimental setup. Therefore, the transit time is an indirect measurement for the cell deformability alone. In other words, the rigid *Pf*iRBCs will spend more time (on average) squeezing through the micro-constriction than the more deformable healthy RBCs (hRBCs) (Figure 1b).

In the intraerythrocytic cycle, the *P. falciparum* parasites evolve from ring stage to the trophozoite and finally to the schizont stage (Figure 1c). During this process, the stiffness of the cells increases monotonically from 8 $\mu\text{N}/\text{m}$ to 16 $\mu\text{N}/\text{m}$ at ring stage and finally to 53 $\mu\text{N}/\text{m}$ at schizont stage^{15, 38, 39}. Therefore, the transit time of the *Pf*iRBCs is also expected to increase as parasites develop into late stages (Figure 1c). The *Pf*iRBCs deformability variations among various stages could thus be utilized for malaria stages differentiation.

We can model the total time distribution data as a mixture consisting of maximal k populations (healthy, ring, trophozoites and schizonts). Each of these populations can be assumed as a Gaussian distribution $N(\mu, \sigma)$. As a result, the mixture distribution of the

transit time can be represented by summing the Gaussian distribution function

$t = \sum_{i=1}^k \alpha_i N(\mu_i, \sigma_i)$ with the constraint of $\sum_{i=1}^k \alpha_i = 1$. The weight α_i represents the population percentage for each population, which can be fitted by using expectation maximization (EM) algorithm.

3.2 Differentiate ring stage Pf-iRBCs and hRBCs

Before testing with the RBCs, the microfluidic deformability sensor and the testing apparatus was validated by the measurements of a mixture of polystyrene microbeads with diameters of 5 μm and 10 μm (with a concentration of $1 \times 10^5/\text{mL}$ and $3 \times 10^4/\text{mL}$, respectively. Figure S2). After that, the ring stage *Pf*-iRBCs at 12% parasitemia and the control hRBC samples were tested. Figure 2a shows a typical current trace, where the transit time and the current dip for each single cell can be extracted.

Figure 2b shows the scattering plot of the transit time and ionic current dip for the 100% hRBC sample, while Figure 2c shows the case for the sample at 12% parasitemia (*i.e.*, ~12% *Pf*-iRBCs and ~88% hRBCs). Both hRBCs and *Pf*-iRBCs deformed when squeezing through the micropore constriction under the same experimental conditions (*i.e.*, same flow rate, voltage bias, sampling rate and the device conditions). As the cells passing through the micropore constriction, we tracked their size and transit time by evaluating the amplitude and width of the ionic current dip, respectively. As shown in Figure 2c, a clear separate population appears for the 12% parasitemia sample as compared to the hRBC sample shown in Figure 2b. This additional population has a longer cell transit time than the hRBC population.

Figure 2d and Figure 2e plot the transit time histogram for the control hRBC sample and the 12% *Pf*-iRBCs sample. The transit time for the control hRBC sample shows a normal distribution (Figure 2d). The correlation coefficient of the Gaussian fitting curve is $R^2=99.1\%$, indicating that the normal distributions provide a good description of the measured data. The 12% *Pf*-iRBCs sample shows two peaks in the normal plot on the cell transit time distribution, with mean values of $78 \pm 20.4 \mu\text{s}$ (hRBC population) and $155 \pm 24.8 \mu\text{s}$ (*Pf*-iRBC population) (Figure 2e). This is consistent with the hypothesis that cell deformability is a determining factor for cell transit time across the micropore constrict. The differentiating ability of the microfluidic deformability sensor can be evaluated based on a

parameter given by³⁵, $R_s = (\mu_1 - \mu_2) / \sqrt{\sigma_1^2 + \sigma_2^2}$, where μ_1, μ_2 , are the mean value and σ_1, σ_2 denote the standard deviation of transit time for two groups. It is noteworthy that different applied pressure will lead to an overall transit time shift for each population (μ_1 and μ_2). Nevertheless, the difference of the mean transit time ($\mu_1 - \mu_2$) is independent of the flow rate.

3.3 Parasitemia quantification at ring and trophozoite stages

The microfluidic cell deformability sensor can not only differentiate ring stage *Pf*-iRBCs and hRBCs but can also quantify the parasitemia. Parasitemia ranging from 0.023% to 3% were prepared with $2 \times$ serial dilution of *Pf*-iRBCs samples of cultured asexual parasites with known parasitemia. These samples were tested under the same experimental conditions.

Figure 3a and Figure 3b show the scattering plot of the transit time and current dip for the ring and trophozoite *Pf*iRBCs samples, respectively. A decreasing cell population of larger transit time was clearly observed when sample parasitemia was reduced. This trend is clear for both the ring and the trophozoite samples. Figure 3c and Figure 3d show the parasitemia determined by the microfluidic deformability sensor versus the parasitemia determined by the thin smear microscopy for ring and trophozoite *Pf*iRBCs, respectively. Remarkably, there was an excellent linear relationship ($R^2 > 98\%$) between the parasitemia determined by deformability sensor and microscopy, both for ring and trophozoite stages. The agreement between these two methods validates the parasitemia quantification ability of the microfluidic deformability sensor. It is noteworthy that the microfluidic deformability sensor can analyze more than 30,000 RBCs within 1 min, a much higher throughput than that of the microscopy method (~5 mins/slide).

It is also interesting to compare the transit time of ring and trophozoite stages at the same parasitemia (Figure 3a and Figure 3b). Note that each of the two samples would have a common healthy RBC population and a stage-specific population. For the common healthy RBC population, we would expect the same transit time distribution while for the stage-specific population, a right shift of the trophozoite transit time would be expected since trophozoite parasites are stiffer than ring parasites. Although experiment-to-experiment variations (device size, and pressure etc.) can lead to an overall shift in the transit time distribution, the healthy population could be used as the reference to infer the relative population transit time difference for the two stages (Supplementary Information, Figure S3).

3.4 Parasite evolution in an intraerythrocytic cycle

In the intraerythrocytic cycle, the stiffness of the *Pf*iRBCs increases monotonically from the ring stage to the schizont stage. To evaluate the sensor's stability to monitor the stiffness change during an intraerythrocytic cycle, the hRBCs were firstly infected with 2% *P. falciparum* parasites at time zero. The infected RBC was then continuously monitored (interval of 5~8 hours) for a typical intraerythrocytic cycle (~48 hours). The deformability and the parasitemia evolution was analyzed by the microfluidic deformability sensor, benchmarked with the reference method of thin smear microscopy.

Figure 4a shows the scattering plot of the transit time and current dip of the monitored sample in a typical intraerythrocytic cycle. A clear right-shift of the transit time can be observed from 0 h to 48 h, corresponding to the parasite evolution from ring stage and to the late stage of trophozoite and schizont. This observation is consistent with the fact that *Pf*iRBCs become stiffer as parasites develop into late stages. The result suggests the deformability could be used as a potential mechanical biomarker to differentiate malaria at different stages. It is noteworthy that the parasite evolution in an intraerythrocytic cycle is asynchronous. Therefore, the infected population is of a mixed nature at a specific time spot. A clear turning pointing from one stage to the other does not exist.

Figure 4b shows the parasitemia quantified by the deformability sensor and the thin smear microscopy during the intraerythrocytic cycles. The parasitemia determined by the two methods agrees with each other very well. This can also be clearly seen from Figure 4c. A

linear relationship between the parasitemia determined by both methods ($R^2 > 96\%$) further confirms the reliability of deformability sensor in parasitemia quantification. It is very interesting to note that the parasitemia standard deviation is less in deformability sensors than that in the thin smear microscopy (Figure 4b). This is due to the fact that the deformability sensors can examine a lot more cells than the microscopy method. An increased sample size can reduce the statistical variations. In addition, both methods confirm a gradual parasitemia increase before reaching ~2% level at the time of 48 hours. This is attributed to the fact that parasites evolution during the intraerythrocytic cycles is asynchronous⁴⁰.

3.5 Blind test with Pf-iRBCs at 0.1% parasitemia

In order to evaluate the use of the microfluidic deformability sensor for qualitative positive/negative diagnosis, we performed a blind study on the deformability sensor with traditional microscopy method as the reference. We infected hRBCs with *P. falciparum* at a percentage of 0.1% for a total of 50 samples. The *P. falciparum* was at different stages ranging from the ring stage to the schizont stage. The infectious status of each sample was recorded but kept blind to the technicians. We asked three technicians with experienced microscopic skills and a technician well-trained with microfluidic deformability sensor to independently make a positive/negative call. The measured scattering plot of the transit time and current dip for these 50 samples from the deformability sensor were described in Figure 5. The sample was deemed as positive in the microfluidic deformability sensor when the cell number larger than the threshold transit time is over 100 (this process was automated by the software). Otherwise, it was regarded as negative. At the end of the blind experiment, the results were checked against the recorded information to determine the rate for true positive, true negative, false positive and false negative (Supplementary Table S1). Table 1 shows the statistical summary of the blind test. The microfluidic deformability sensor achieved an excellent sensitivity (94.29%), specificity (86.67%) and accuracy (92.00%) in a blind test. This is comparable to the gold standard of the blood smear microscopy (sensitivity of 95.21%, specificity of 89.17% and accuracy of 92%).

4 Conclusion

Using the unique cell deformability property as the mechanical biomarker, a high throughput and label-free microfluidic deformability sensor was developed and demonstrated for potential cost-effective malaria diagnosis. We have shown the parasitemia measurement can be rapidly quantified (<1 min) by evaluating the deformability of each single cells in a large population (>30 k) passing through the microscale constrict. We have also shown that the malaria infectious stage information can be derived by using a Gaussian mixture model. In addition, the practical usage of the device is validated in a blind qualitative positive/negative test. The device has shown a high sensitivity (94.29%), specificity (86.67%) and accuracy (92.00%) in the blind test. For the current device, the removal of the white blood cells (WBCs) is essential. Future works to filter the WBCs on the chip and to integrate the peripheral electronics and the pressure sources would be important towards the final goal of clinical usage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- High throughput and label-free microfluidic cell deformability sensor
- Quantitative malaria parasitemia measurement at a throughput ~500 cells/s
- Infectious stage determination for *Plasmodium falciparum*-infected red blood cells
- Excellent sensitivity (94.29%), specificity (86.67%) and accuracy (92.00%) in a blind test

Figure 1.

Overview of the label-free microfluidic cell deformability sensor. (a) Schematic of the deformability sensor for highly-throughput and label-free parasitemia quantification. (b) The principle of the deformability sensor to differentiate hRBCs and *Pf*-iRBCs. When an RBC squeezes through the micropore, an ionic current dip occurs due to the reduced conduction cross-section. The cell translocation time is determined by the cell deformability. (c) The stiffness of the red blood cells increases monotonically as *P. falciparum* parasites evolving from ring stage to the trophozoite and finally to the schizont stage. The deformability sensor could thus differentiate malaria at different stages.

Figure 2.

(a) The current trace for *Pf*-iRBC sample at 12% parasitemia. The scattering plot of transit time and ionic current dip for (b) pure RBC sample and (c) *Pf*-iRBC sample at 12% parasitemia. Histogram plot of cell transit time for (d) pure RBC sample and (e) *Pf*-iRBC sample at 12% parasitemia.

Figure 3.

Scattering plot of transit time versus ionic current dip for the *Pf*-RBCs with various parasitemia for (a) ring and (b) trophozoite *Pf*-iRBC. Corresponding parasitemia determined by the microfluidic deformability sensor and the microscopy method for (c) ring and (d) trophozoite *Pf*-iRBC.

Figure 4.

The analysis for the *Pf*-iRBCs at different stages during the intraerythrocytic cycles. (a) Scattering plot of transit time versus ionic current dip for the *Pf*-iRBC during the intraerythrocytic cycle at various time spots. (b) The parasitemia quantified by the deformability sensor and microscope during the intraerythrocytic cycles. (c) Comparison of parasitemia between the deformability sensor and the microscope.

Figure 5.

The scattering plot of the transit time and current dip for the 50 samples in the blind test.

Table 1

A summary of the overall performance of deformability sensor as compared to the blood smear microscopy in the blind test. The variables measured were the number of true positives (TP), number of true negatives (TN), number of false positives (FP) and number of false negatives (FN). Sensitivity was calculated as $TP/(TP + FN)$, specificity was calculated as $TN/(TN + FP)$ and test accuracy is defined as $(TP + TN)/(TP + TN + FP + FN)$.

Test parameters	Microfluidic Sensor	Microscopy			
		T1	T2	T3	Mean Std. dev.
True positive, TP	33	34	33	32	33.0 1.00
True negative, TN	13	14	14	13	13.7 0.580
False positive, FP	2	1	2	2	1.67 0.580
False negative, FN	2	1	1	3	1.67 1.15
Sensitivity	94.3%	97.1%	97.1%	91.4%	95.2% 3.28%
Specificity	86.7%	93.3%	87.5%	86.7%	89.2% 3.63%
Test accuracy	92.0%	96.0%	94.0%	90.0%	93.3% 3.06%