Refractive index maps and membrane dynamics of human red blood cells parasitized by *Plasmodium falciparum*

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Parasitization by malaria-inducing *Plasmodium falciparum* leads to structural, biochemical, and mechanical modifications to the host red blood cells (RBCs). To study these modifications, we investigate two intrinsic indicators: the refractive index and membrane fluctuations in *P. falciparum*-invaded human RBCs (Pf-RBCs). We report experimental connections between these intrinsic indicators and pathological states. By employing two noninvasive optical techniques, tomographic phase microscopy and diffraction phase microscopy, we extract three-dimensional maps of refractive index and nanoscale cell membrane fluctuations in isolated RBCs. Our systematic experiments cover all intraerythrocytic stages of parasite development under physiological and febrile temperatures. These findings offer potential, and sufficiently general, avenues for identifying, through cell membrane dynamics, pathological states that cause or accompany human diseases.

erythrocyte | febrile temperature | malaria | mechanical properties | optical techniques

During the intraerythrocytic development, the malaria parasite *Plasmodium falciparum* causes structural, biochemical, and mechanical changes to host red blood cells (RBCs). Major structural changes include the formation of parasitophorous vacuoles that surround the growing parasite in their host RBCs, loss of cell volume, and the appearance of small, nanoscale protrusions or “knobs,” on the membrane surface (1). From the biochemical standpoint, a considerable amount of hemoglobin (Hb) is digested by parasites during intraerythrocytic development and converted into insoluble polymerized forms of heme, known as hemozoin (2, 3). Hemozoin appears as brown crystals in the vacuole of parasite in later maturation stages of *P. falciparum*-invaded human RBCs (Pf-RBCs).

Two major mechanical modifications are loss of RBC deformability (4–6) and increased cytoadherence of the invaded RBC membrane to vascular endothelium and other RBCs (7). These changes lead to sequestration of RBCs in microvasculature in the later stages of parasite development, which is linked to vital organ dysfunction in severe malaria. In the earlier stage, where some loss of deformability occurs, Pf-RBCs continue to circulate in the bloodstream.

Membrane dynamics of RBCs can be influenced by human disease states. Fluctuations in phospholipid bilayer and attached spectrin network are known to be altered by cytoskeletal defects, stress, and actin-spectrin dissociations arising from metabolic activity linked to adenosine 5'-triphosphate (ATP) concentration (8–12). Proteins transported from invading organisms, such as the virulent malaria-inducing parasite *P. falciparum*, to specific binding sites in the spectrin network are considered to introduce significant alterations to RBC membrane dynamics and mechanical response (13, 14). These changes could provide insights into possible mechanistic pathways in the pathogenesis of malaria, because the parasite alters biophysical properties of RBCs during its intraerythrocyte stage that lasts up to 48 h. Despite the broad realization that

membrane fluctuations provide information on critical interactions among subcellular structure, mechanical stress, and biochemical links between the cell interior and the external environment, systematic experiments of cell membrane dynamics, over the physiologically relevant temperature range, have not been performed.

A clinical feature of symptomatic *P. falciparum* malaria is the presence of periodic episodes of high fever. Previous studies report that fever influences *P. falciparum* survival rate and deformability of Pf-RBCs (14, 15). Specifically, loss of deformability at ring stage was found to be more severe at febrile temperature (41°C) compared with physiological temperature (37°C). However, *in vitro* experiments at febrile and physiological temperature only reveal a partial story of what is experienced *in vivo*. When physiological temperature is restored after a fever episode, infected RBCs in circulation might not fully recover properties typically observed at physiological temperature. If the changes to deformability observed at fever conditions are irreversible, prior effects of fever on Pf-RBCs would remain. As a consequence, Pf-RBCs in circulation at physiological temperature may actually display deformability closer to those measured at febrile temperature. To explore this possibility, we measure the deformability of Pf-RBCs at physiological temperature after a transient exposure to febrile temperature to simulate the *in vivo* behavior after a febrile episode. When combined with deformability measurements at static physiological and febrile temperatures, these results give useful insights into how Pf-RBCs behave *in vivo*.

In this article, we present two intrinsic indicators that quantitatively and noninvasively elucidate the consequences on cell biomechanics of *P. falciparum* malaria: three-dimensional distributions of refractive index and the membrane fluctuations in Pf-RBCs. The refractive index maps of Pf-RBCs show the morphological alterations of host RBCs and the structures of vacuoles of parasites. In addition, the refractive index is translated into quantitative information about Hb content of individual Pf-RBCs. During the intraerythrocytic stages of *P. falciparum*, we show the decrease of both the total amount and the concentration of Hb in the cytoplasm of Pf-RBCs. Thermally driven membrane fluctuations in Pf-RBCs are strongly correlated with the material properties of cell mem-

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$$k_e(x, y) = k_B T / \langle \Delta h(x, y)^2 \rangle,$$

Fig. 2. Host RBC Hb concentrations decreases as *P. falciparum* matures. (A) Refractive index of healthy RBC and *Pf*-RBCs at the indicated stages. (B) Mean corpuscular Hb concentration (MCHC) of healthy and *Pf*-RBCs at the indicated stages. (C) Cytoplasm volume of *Pf*-RBCs at the indicated stages. (D) Hb content in healthy and *Pf*-RBCs at the indicated stages. Each point in A represents average refractive index for one cell. Graphs in B–D show the median (central horizontal line), standard deviations (box), and minimum and maximum values (vertical lines). For each condition, 15 samples were tested.

where k_B is the Boltzmann constant, T is the absolute temperature, and $\langle \Delta h(x, y)^2 \rangle$ the mean-squared displacement. Representative k_e maps of RBCs at the indicated stages of parasite development are shown in Fig. 3 E–H. The map of instantaneous displacement of cell membrane fluctuation, $\Delta h(x, y, t)$, was obtained by subtracting time-averaged cell shape from each thickness map in the series. A histogram showing membrane displacements for all parasite stages is shown in Fig. 4A.

RBC deformability is sensitive to membrane stiffness. Our DPM experiments provide quantitative information from which in-plane shear modulus of RBC membrane with attached spectrin cytoskeleton could be determined. The in-plane shear modulus G can be obtained by using the Fourier-transformed Hamiltonian (strain energy) and equipartition theorem (20):

$$G \approx \frac{k_B T \ln(A/a)}{3\pi \langle \Delta h_1^2 \rangle},$$

where A is the projected diameter of RBC ($\approx 8 \mu\text{m}$), and a is the minimum spatial wavelength measured by DPM ($\approx 0.5 \mu\text{m}$). The tangential component of displacement in membrane fluctuations, Δh_t , was decoupled from axial membrane fluctuation Δh^2 by using the angle α between the direction of Δh_t and the normal direction of membrane as illustrated in Fig. 4B Inset. The angle α was extracted from cell topographical information measured by DPM. Δh_t^2 was calculated and averaged along the circumference of cell, from which in-plane shear modulus G was calculated. We determined that $G = 5.5 \pm 0.8 \mu\text{N/m}$ for healthy RBCs (Fig. 4B), which compares well with independent modulus measurements, extracted for healthy RBCs from micropipette aspiration and optical tweezers (14, 21, 22). The modulus for ring ($G = 15.3 \pm 5.4 \mu\text{N/m}$), trophozoite ($G = 28.9 \pm 8.2 \mu\text{N/m}$), and schizont ($G = 71.0 \pm 20.2 \mu\text{N/m}$) stages is in good quantitative agreement with that inferred from large-deformation stretching with optical tweezers of *Pf*-RBCs over all stages of parasite maturation (22).

Fig. 3. Topographic images and effective elastic constant maps of *Pf*-RBCs. (A and E) Healthy RBC. (B and F) Ring stage. (C and G) Trophozoite stage. (D and H) Schizont stage. The topographic images in A–D are the instant thickness map of *Pf*-RBCs. The effective elastic constant maps were calculated from the root-mean-squared displacement of the thermal membrane fluctuations in the *Pf*-RBC membranes. Black arrows indicate the location of *P. falciparum*, and the gray arrows indicate the location of hemozoin. (Bright-field and fluorescence micrographs provide information on locations of parasite and hemozoin; see SI Text). (Scale bar, $1.5 \mu\text{m}$.)

Fig. 4. Membrane fluctuations and in-plane shear modulus at different intraerythrocytic stages of *Pf*-RBCs. (A) Histograms of cell-thickness fluctuation of *Pf*-RBCs. (Histogram of the schizont stage is scaled down by a factor of 1.5.) (B) In-plane shear modulus of the RBC membrane versus developmental stage of *Pf*-RBCs. The in-plane shear modulus was calculated from the in-plane membrane displacement, Δh_r , over the rims of RBCs. Also shown for comparison are the estimated from optical tweezers (22). (Inset) Illustration of RBC and membrane fluctuations: Δh , thickness fluctuations measured by DPM; Δh_r , in-plane membrane displacement; Δh_n , out-of-plane membrane displacement, and α , the angle between Δh and Δh_r . The measurements were performed at the room temperature (23°C) and for each group 20 samples were tested.

Effects of Temperature on Deformability of *Pf*-RBC During All Intraerythrocytic Stages. The effect of febrile temperature exposure on deformability of *Pf*-RBCs was evaluated at each parasite stage. Measurements were made at physiological temperature (37°C), febrile temperature (41°C), and physiological temperature after 45 min exposure at 41°C (one fever cycle) by using DPM. The results of these tests are summarized in Fig. 5 (membrane fluctuation) and Fig. 6 (in-plane shear modulus). The measured membrane in-plane shear modulus values reflect the overall cell deformability. The in-plane shear modulus for healthy RBCs are 6.2 ± 1.4 , 4.9 ± 1.1 , and 5.9 ± 1.0 $\mu\text{N/m}$, respectively, for 37°C, 41°C, and 37°C after one fever cycle. For ring stage, 14.5 ± 3.8 , 20.4 ± 7.2 , and 13.5 ± 4.2 $\mu\text{N/m}$; for trophozoite stage, 35.0 ± 9.0 , 56.6 ± 14.9 , and 46.7 ± 13.4 $\mu\text{N/m}$; and for schizont stage, 71.8 ± 21.0 , 95.0 ± 25.5 , and 99.6 ± 21.6 $\mu\text{N/m}$, respectively. Previous work consistently reports a shear modulus of 4–8 $\mu\text{N/m}$ for healthy RBCs at physiological and febrile temperature (14). A higher shear modulus indicates a loss in

deformability. As a control, healthy RBCs were evaluated at all three temperature conditions (Figs. 5A and 6A).

Results from testing ring stage display a marked decrease in deformability at febrile temperature compared with physiological temperature (Figs. 5*B* and 6*B*). On returning to physiological temperature, the deformability of ring stage returned to the level observed before the fever episode. Similar to ring stage, measured shear modulus values for trophozoite and schizont stage increase at febrile temperature compared with physiological temperature. However, measurements at physiological temperature after one fever cycle indicate permanent changes to deformability as a result of fever conditions. In trophozoites, a partial recovery of deformability was observed after returning to physiological temperature (Fig. 6*C*). Only half of the loss in deformability associated with a fever episode was recovered at the trophozoite stage. For schizont stage, essentially all loss of deformability as a result of febrile temperature exposure was permanent and was not recovered after fever conditions end (Fig. 6*D*).

Fig. 5. Membrane fluctuations of *PF*-RBCs at different temperatures. Membrane fluctuation at different parasitic development stages at 37°C, 41°C, and 37°C after one fever cycle for healthy RBCs (A), ring stage (B), trophozoite stage (C), and schizont stage of *PF*-RBCs (D). The values for fluctuations are FWHM of the membrane fluctuation histograms. Graphs show the median (central horizontal line), standard deviations (box), and minimum and maximum values (vertical lines). For each group 15 samples were tested.

Fig. 6. In-plane shear modulus of *Pf*-RBCs at different temperatures. In-plane shear modulus at different parasitic development stages at 37°C, 41°C, and 37°C after one fever cycle for healthy RBCs (A), ring stage (B), trophozoite stage (C), and schizont stage (D). Symbols indicate individual *Pf*-RBCs. For each group 15 samples were tested.

Discussion

With *P. falciparum* parasitic development into the schizont stage, the normal discocyte shape is lost. Parasite modifications to the internal and membrane structure of invaded RBCs are implicated in the observed morphological changes (Fig. 3 *A–D*). Membrane stiffness also increases progressively with parasite development. In particular, the spatially averaged effective stiffness, $\langle k_e(x, y) \rangle$, at the schizont stage is up to an order of magnitude higher than that for healthy RBCs (Fig. 3 *E–H*). These results indicate that parasite development stage directly correlates with the amplitude of membrane fluctuations, and that the distribution of membrane fluctuations becomes much sharper with parasite development.

Interestingly, there is significant increase in membrane fluctuations [53% in the full-width half maximum (FWHM) value of the fluctuation displacement histogram] from physiological to febrile temperature ($P < 0.01$), which is only a 7.5% increase in absolute temperature. Evidently, such enhancement in the fluctuation cannot be explained simply by equilibrium thermodynamics alone, that is, by the increase in the Boltzmann factor $k_B T$. The substantial increase in membrane fluctuations indicates that RBC phospholipid membrane and spectrin network undergo structural changes that alter elastic properties, which is reflected in changes to membrane in-plane shear modulus, G (Fig. 4). One possible explanation is that a transitional structural change in spectrin molecular architecture occurs between physiological and febrile temperature, causing altered and reorganized cytoskeleton network. Both α - and β -spectrin molecules have a significant structural transition near 40°C (23). It has been shown that membrane shear modulus of healthy RBCs decreases by $\approx 20\%$ when the temperature increases from 23 to 41°C (21). From physiological to febrile temperature, membrane fluctuations decrease significantly ($P < 0.01$) for all infected test conditions (Fig. 3), with trophozoite and schizont stages showing the strongest temperature dependence. Based on comparison with results from healthy RBC and associated changes of in-plane shear modulus (Fig. 4*B*), we postulate that intraerythrocytic parasite development causes an opposite effect. Parasite-exported proteins that target RBC membrane can alter thermally induced spectrin-folding transitions involved in stabilizing erythrocyte cytoskeleton (24). Expression of *P. falciparum* genes is known to be affected by exposure to 41°C (25). Under febrile conditions, increased levels of parasite-exported proteins could contribute to large changes in membrane fluctuations observed by DPM between physiological and febrile temperatures. One example is the ring-infected erythrocyte surface antigen (RESA). When RESA binds to spectrin, normal balance between spectrin dimers and tetramers in the cytoskeletal network is skewed toward tetramer formation, which results in increased membrane stability (26). As a result, RESA enhances RBC resistance to mechanical and thermal damage. Moreover, elevated thermal stability conferred by RESA plays a protective role for *Pf*-RBCs against damage at febrile temperature (27). The possible specific role of other membrane proteins and histidine-rich proteins in influencing thermal and mechanical stability of RBCs over the temperature range of interest is presently not fully understood. The development of adhesion properties at the trophozoite and schizont stages can also contribute to significant decrease in fluctuation observed by DPM at these stages, especially at febrile temperature. For the same febrile temperature, our results indicate that fluctuation amplitude progressively decreases as a parasite matures from the ring to the schizont stage.

The findings presented in Figs. 5 and 6 reveal that effects of fever episodes cause irreversible changes to deformability of *Pf*-RBCs. These experiments mimic *in vivo* conditions that *Pf*-RBCs experience and provide information that cannot be extracted from mechanical experiments performed at a constant temperature. Plastic behavior caused by a febrile episode is not observed at the ring stage. Decreased deformability observed under febrile condi-

tions is recovered on returning to physiological temperature. The decrease in deformability of *Pf*-RBCs may be attributed to exported parasite proteins that interact with the host RBC membrane. The ability of ring stage to regain deformability could play an important role in their ability to avoid splenic clearance when circulating. Trophozoite and schizont stages display significant plastic changes to deformability. Again, decreased deformability for mature stages may be also attributed largely to parasite-exported proteins that associate with the membrane. Parasite protein exportation is likely accelerated by thermal energy. In turn, febrile episodes may be accelerating parasite protein exportation to the membrane that results in advancing the loss of deformability. Both reduced deformability and the development of adhesion properties contribute to sequestration of trophozoite and schizont stage. The temperature-related permanent loss of deformability found in our experiments is greater than anticipated and can be viewed as an additional driving force to impede microcirculation and promote sequestration. The plastic behavior of trophozoite and schizont stages in response to fever could also have implications when treatments are used to suppress fever episodes. Additional studies would be needed to explore this possibility.

Finally, the optical experimental techniques (TPM and DPM) used to measure the refractive index maps and the membrane fluctuations in this study offer unique advantages over other more commonly used techniques, such as micropipette aspiration, optical tweezers, laminar shear flow, and magnetic twisting cytometry. TPM quantitatively and noninvasively measures the three-dimensional maps of refractive index, which provides a measure of Hb content of single *Pf*-RBCs. Indeed, TPM does not require any special sample preparation because the refractive index is an intrinsic optical property. DPM is able to measure nanometer level membrane fluctuations without any direct, and potentially invasive, contact with a cell. The optical layout of DPM is such that the sample temperature can be regulated easily without affecting of the rest of the apparatus, a common limitation of other techniques. In addition, deformability of living cells, measured by thermally driven membrane fluctuations, ensures mechanical properties in the linear regime. Also, measurements are made within seconds once a specific cell is identified. Thus, the technique provides the flexibility to experiment on a large number of samples under a variety of well controlled test conditions in a reasonable time span.

Conclusions

We have presented three-dimensional maps of refractive index of *Pf*-RBCs during all intererythrocytic stages, from which we determine the Hb concentration of *Pf*-RBCs. We have also presented systematic measurements of nanoscale fluctuations associated with RBCs parasitized at all stages by *P. falciparum* at physiological and febrile temperatures. Our approach to studying *Pf*-RBCs uniquely combines optical interferometry, biophysics, and cell nanomechanics. A method has been presented to extract elastic properties from membrane fluctuation results, and this technique is validated by quantitative comparisons of elastic moduli of RBCs over all parasite maturation stages with prior, independent experimental data obtained with laser tweezers (14, 22). Compared with other techniques for assessing RBC mechanical properties, such as electric field deformation, micropipette aspiration, optical tweezers, and magnetic bead excitation, the method presented here has distinct advantages of being spatially resolved and noncontact. We envision that this methodology could offer potentially powerful means to link cell membrane fluctuations with pathological conditions that lead to human disease states by providing quantitative information that could not be extracted through other experimental techniques.

These results point to major new avenues for exploiting membrane fluctuations as quantitative indicators of the onset and progression of pathological states that could lead to diseases. In addition, the information provided by diffraction phase microscopy can guide theory and computational simulations that address a